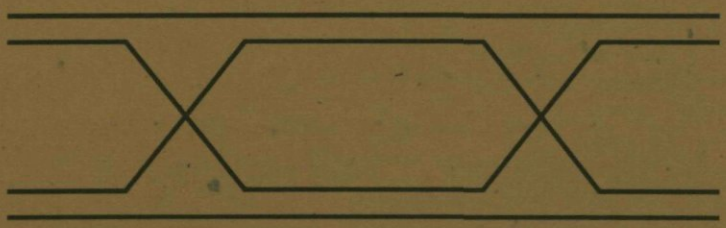
ON HOMOLOGOUS RECOMBINATION IN *ASPERGILLUS NIDULANS*



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On homologous recombination in *Aspergillus nidulans*

Proefschrift
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Stellingen

Stellingname door de promovendus over onderwerpen buiten diens vakgebied dient, gelijk het rolletje pepermunt in de kerk, uitsluitend ter overbrugging van een voor leken uiterst saaie plechtigheid

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Ouderschap houdt een mens meer op de tenen dan wetenschap

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BSE is een gekke mensenziekte

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Genetisch modificatie van voedingsgewassen is een vruchteloze weg, behalve voor biotechnologen die dan ook over het algemeen het tegendeel beweren

~

Routinematig kloneren van mensen zal leiden tot een ongekende bloei van de farmaceutische industrie

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Het zou verboden moeten zijn de zondagsrust met klokgelui te verstoren

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Een te respecteren religie kent geen zendingsdrang

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Tabak heeft de Zwarte Piet

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Het geringe gewicht dat wordt toegekend aan de stellingen bij proefschriften blijkt zowel uit de traditie deze losbladig te publiceren, alsook uit de lage frequentie waarmee zij bij door de opponenten bij de promotie betrokken worden; dit is alleszins terecht

Stellingen behorende bij het proefschrift '*On homologous recombination in Aspergillus nidulans*' van Hans Thijs

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CONTENTS

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SUMMARY

This thesis deals with the molecular mechanisms that underlie homologous recombination in eukaryotes. Most molecular genetic studies on homologous recombination have been performed in budding yeast (*Saccharomyces cerevisiae*). However, it remains to be investigated to what extent the results obtained in this unicellular organism can be extrapolated to other eukaryotes. We therefore initiated a series of molecular genetic studies on homologous recombination in *Aspergillus nidulans*. This filamentous fungus is amenable to most molecular techniques and is phylogenetically closer to higher eukaryotes than *S. cerevisiae*; *A. nidulans* may thus provide a powerful eukaryotic model system. We have examined meiotic and mitotic recombination events in the *nii*A-*nia*D genomic region of *A. nidulans*. The *nii*A and *nia*D genes encode nitrite and nitrate reductase respectively, and are both required for the utilization of nitrate as a nitrogen source.

The *nii*A-*nia*D region was chosen after a preliminary genetic analysis of meiotic recombination, which suggested the presence of an initiation site for meiotic recombination nearby, possibly in the intergenic promoter region (chapter 2). Subsequently, we designed an assay to analyze the products of recombination in more detail. As parental strains for a series of two-point crosses, we constructed *nii*A and *nia*D mutant strains that differed by nonsense or missense mutations in the *nii*A and/or *nia*D gene and by a series of 11 silent point mutations. We analyzed recombinants selected on nitrate for the presence or absence of individual mutations to determine the position and endpoints of conversion tracts. We found evidence for an initiation site for meiotic recombination in the promoter region located between the *nii*A and the *nia*D gene. Such initiation sites have also been found in the promoter regions of several genes of *S. cerevisiae*. Conversion tracts with an average length of 1.5 kb extended from this site into the *nii*A gene, the *nia*D gene or both. Meiotic conversion tracts were continuous in almost all recombinants. Co-conversion of markers declined steeply with distance from the proposed initiation site. In crosses where one of the parents harbored an ectopic copy of the *nii*A-*nia*D region instead of the allelic copy, conversion tracts were similar in size and position to those observed in allelic crosses. Ectopic recombinants were detected at a significantly lower frequency. The majority of the allelic recombinants but not of the ectopic recombinants harbored a crossover within the *nii*A-*nia*D region (chapter 3).

Some initial molecular genetic studies on meiotic recombination in a higher plant (maize) have recently been published. In chapter 4, we draw attention to an important similarity between meiotic recombination in fungi and maize, namely a 5' to 3' polarity of gene conversion in the loci analyzed. It is thus possible that maize, like *A. nidulans* and *S. cerevisiae*, has specific initiation sites for meiotic recombination at the 5' ends of its genes.

We also examined mitotic recombination in a diploid *A. nidulans* strain with the same assay system (chapter 5). The observed patterns of mutations in the haploid derivatives of the mitotic recombinants suggest that the initiation site for meiotic recombination that we found in the *nii*A-*nia*D locus, does not function in mitotic recombination; furthermore, the patterns suggested that long heteroduplex tracts (> 8.4

kb) are formed during mitotic recombination, and that conversion occurs in patches within these tracts. Mitotic recombination differs in these respects from meiotic recombination in *A. nidulans*.

SAMENVATTING

Dit proefschift behandelt de moleculaire mechanismen van homologe recombinatie in eukaryoten. De meeste moleculair-genetische studies naar homologe recombinatie zijn tot nu toe uitgevoerd met bakkersgist (*Saccharomyces cerevisiae*). Nog onbekend is in hoeverre de resultaten van de experimenten met deze eencellige geëxtrapoleerd kunnen worden naar andere eukaryoten. Wij hebben ter vergelijking een serie experimenten uitgevoerd met *Aspergillus nidulans*, een filamenteuze schimmel die zich goed leent voor moleculaire analyse en bovendien fylogenetisch dichter bij hogere eukaryoten staat dan gist; *A. nidulans* kan dus wellicht een zeer bruikbaar model-organisme zijn. We hebben zowel meiotische als mitotische recombinatie geanalyseerd in het *nii*A-*nia*D gebied van *A. nidulans*. De *nii*A en *nia*D genen coderen voor respectievelijk nitriet- en nitraatreductase; beide zijn noodzakelijk voor het aanwenden van nitraat als stikstofbron.

Het *nii*A-*nia*D gebied werd gekozen na een genetische analyse, waaruit bleek dat initiatie van meiotische recombinatie vermoedelijk in het intergene promoter gebied tussen de *nii*A en *nia*D genen plaatsvindt (hoofdstuk 2). Op basis van deze informatie hebben we een aantal *A. nidulans* stammen gemaakt om additionele details van het recombinatie-proces te kunnen bestuderen. In elke gebruikte combinatie verschilden deze ouderstammen, afgezien van de nonsense of missense mutaties in het *nii*A gen of het *nia*D gen (noodzakelijk voor de selectie van recombinanten), door de gerichte introductie van 11 stille puntmutaties in één van beide ouders (markers). Het systeem werd zo ontworpen dat de aan- of afwezigheid van elk van deze markers in recombinanten bepaald kon worden. De patronen van mutaties die in de recombinanten werden aangetroffen, bevestigden de preferentiële initiatie van recombinatie in het intergene promoter gebied. Zulke 'hotspots' voor recombinatie zijn eerder aangetoond in promoter gebieden in gist. Conversie-trajecten, meestal ononderbroken, met een gemiddelde lengte van 1.5 kb liepen vanaf het intergene promoter gebied tot in het *nii*A gen, het *nia*D gen, of beide. De co-conversie van markers nam scherp af met de afstand tot het intergene promoter gebied. Recombinanten uit kruisingen waarbij één van de ouders een ectopische kopie i.p.v. de oorspronkelijke kopie van het *nii*A-*nia*D gebied bezat, vertoonden conversie-trajecten vergelijkbaar met die in allelische recombinanten, maar ectopische recombinanten werden met een significant lagere frequentie gevonden. In de meerderheid van de allelische maar niet van de ectopische recombinanten werd een geassocieerde overkruising aangetroffen (hoofdstuk 3).

Recentelijk zijn enkele moleculair-genetische studies over meiotische recombinatie in mais gepubliceerd. In hoofdstuk 4 wordt de aandacht gevraagd voor een belangrijke overeenkomst tussen meiotische recombinatie in schimmels en mais, namelijk een sterke

polariteit van conversie. Het lijkt waarschijnlijk dat ook mais, net als *A. nidulans* en bakkersgist, beschikt over recombinatie 'hotspots' in promoter gebieden.

We hebben ook mitotische recombinatie (in een diploide *A. nidulans* stam) onderzocht, gebruik makend van hetzelfde systeem (hoofdstuk 5). De patronen van mutaties in mitotische recombinanten suggereren dat de meiotische 'hotspot' in het intergene promoter gebied van de *nii*A and *nia*D genen, niet functioneel is gedurende de vegetative levenscyclus. Bovendien lijken mitotische heteroduplex trajecten zeer lang (> 8.4 kb) te zijn, waarbinnen meerdere conversies kunnen optreden. In deze opzichten lijken mitotische en meiotische recombinatie in *A. nidulans* te verschillen.

1. GENERAL INTRODUCTION

A PH.D. PROJECT

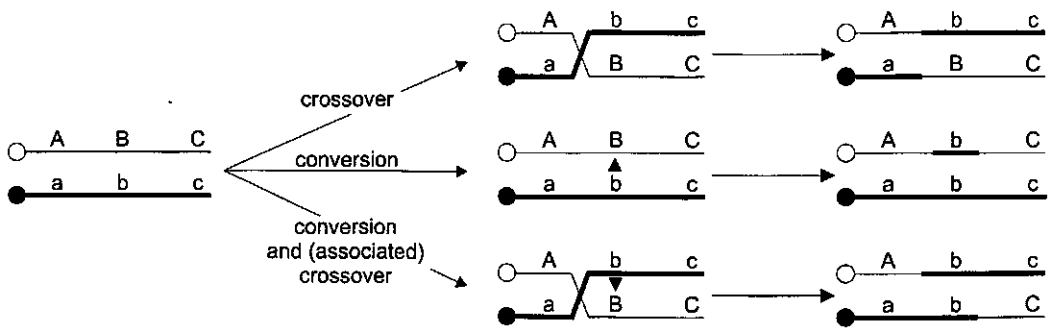
In the spring of 1992, I was invited to contribute to the project "Meiotic and mitotic recombination and gene conversion in relation to transcription in *Aspergillus nidulans*" in the form of this thesis. The project proposal was written by Dr. T. Goosen, Dr. K. Swart, Dr. H. W. J. van den Broek and Prof. Dr. C. Heyting, all of whom are staff members of the Laboratory of Genetics of the Wageningen Agricultural University. The project concerned the study of homologous recombination in filamentous fungi at the molecular level; *Aspergillus nidulans* was chosen to exemplify filamentous fungi. This ascomycete was instrumental in the emergence of the fundamental concept of specific initiation sites for meiotic recombination (reviewed in Whitehouse 1982), but has received little attention in recent years with respect to the mechanisms that underlie recombination. As these things go, the choice for *A. nidulans* was partly pragmatic (few other filamentous fungi are as accessible to genome modification), partly laboratory tradition. The route of the project was to introduce specific mutations into one of the loci of *A. nidulans* as molecular markers and to monitor these in recombinants. This approach has been very informative in other organisms, particularly the yeast *Saccharomyces cerevisiae*; the accumulated data obtained in this organism are therefore the major point of reference throughout this thesis.

The project proposal named the analysis of the relationship between initiation of recombination and gene transcription as the primary objective. Our attempts to analyze this relationship did not provide any indication that gene transcription influences (the initiation of) recombination in *A. nidulans* and consequently the focus of this thesis has shifted towards the mechanism of recombination itself.

HOMOLOGOUS RECOMBINATION

Recombination involving extensive sequence homology between the interacting DNA molecules is generally referred to as homologous recombination, to distinguish it from other types of recombination (e.g., random integration of a transforming DNA fragment, site-specific recombination, illegitimate recombination and transposition), which fall beyond the scope of this introduction. Here, the focus is on homologous recombination between eukaryotic chromosomes; the term recombination will be used to refer to these events. Current models of the molecular mechanisms of recombination are based largely on data obtained in the yeast *S. cerevisiae*. The question whether recombination in this unicellular organism is prototypic for eukaryotic recombination can only be answered by comparative studies in other eukaryotes. Filamentous fungi are phylogenetically much closer to higher eukaryotes than *S. cerevisiae* (Smith 1989), and may thus provide a potent model system.

Figure 1. Conversions and crossovers



Two interacting (non-sister chromatids of) homologous chromosomes (thin and thick lines) are depicted; circles represent the position of the centromeres, A/a, B/b and C/c are heterozygous markers. If a crossover occurs (in this drawing between markers A/a and B/b) the markers distal to the location of the crossover are reciprocally exchanged. In the case of a conversion of marker B the effect is local, but a conversion can be associated with a crossover.

Recombination is observed as the transfer of genetic information between two stretches of DNA, either non-reciprocal (a conversion event) or reciprocal (a crossover event) as illustrated in Figure 1. Conversions and crossovers may occur in a single event and are therefore generally considered mechanistically related (Whitehouse 1982; Petes *et al.* 1991). Recombination during the vegetative cell cycle (mitotic recombination) occurs primarily in the context of DNA repair and replication; the relation of recombination to DNA repair is illustrated by the elevated levels of recombination after treatment of cells with DNA-damaging agents and by the concerted effects of some mutations on DNA repair and recombination (Petes *et al.* 1991; Zhao and Kafer 1994). The high frequency of recombination during meiosis is the consequence of a programmed introduction of numerous DNA lesions by the cell, at least in *S. cerevisiae* (see below).

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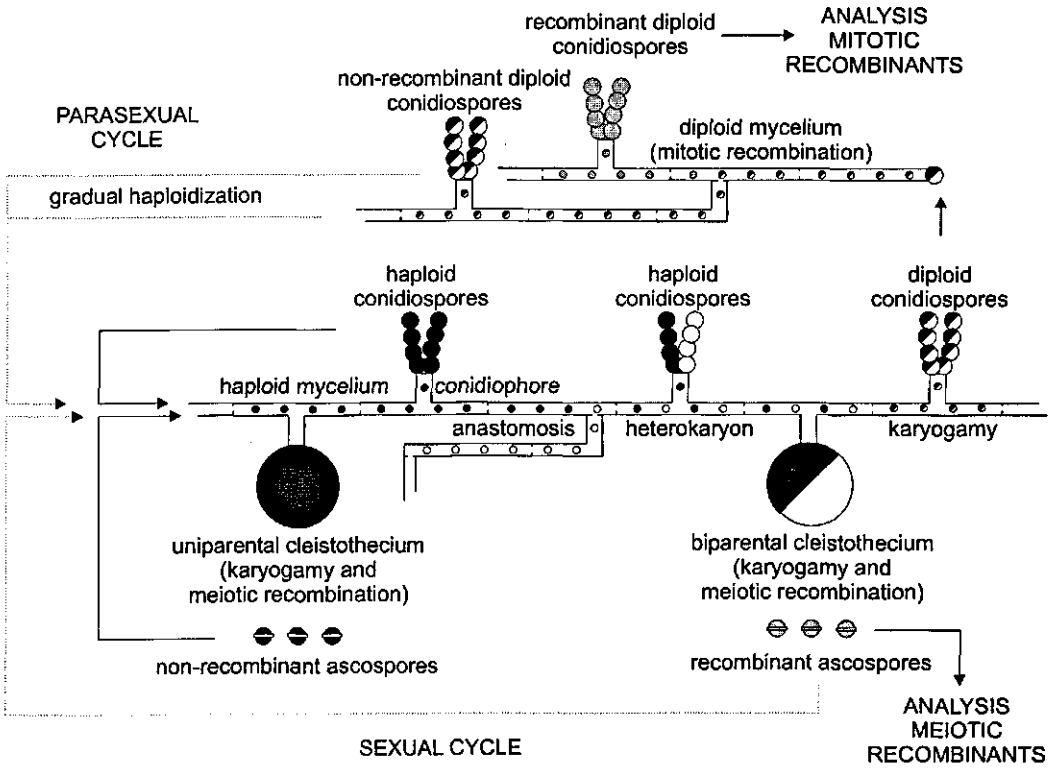
Figure 2 shows the generative (sexual) and vegetative (parasexual) life cycles of the homothallic ascomycete *A. nidulans*. On solid media, mycelial colonies grow from the vegetative conidiospores or the sexual ascospores. Within the resulting multinuclear mycelial layer, specialized cells (conidiophores) develop that carry chains of conidiospores. Anastomosis occurs readily between hyphae of the same strain or between hyphae of different (compatible) strains; the latter event gives rise to heterokaryotic mycelium. Karyogamy of two nuclei in such a heterokaryon may produce a diploid mycelium and diploid conidiospores. Mitotic recombination can be observed in a heterozygous diploid strain.

Meiosis in *A. nidulans* occurs in a specialized structure (cleistothecium). Within a cleistothecium, karyogamy occurs in a programmed fashion. The resulting diploid nuclei immediately undergo meiosis, so that four haploid nuclei are produced; after DNA replication and a mitotic division, the resulting eight haploid cells differentiate into ascospores. In *A. nidulans*, the eight ascospores that originate from a single meiosis remain together in a separate ascus. If the cleistothecium stems from a heterokaryon, i.e., from two genetically different (haploid) strains, meiotic recombinants can be identified among the ascospores.

Figure 2. The life cycles of *A. nidulans*



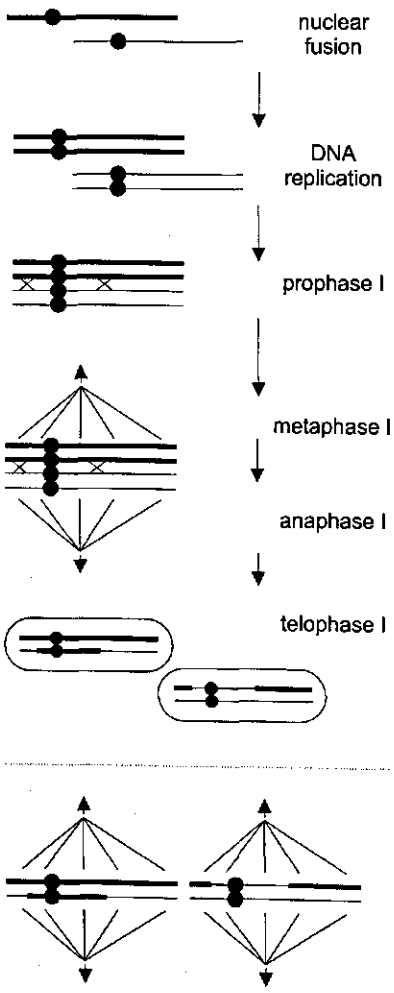
Small circles represent nuclei, the color (black and white) indicates parental origin; larger circles represent conidiospores and ascospores (with a bar). Mycelium may grow from conidiospores or ascospores, anastomosis between hyphae of two different strains leads to a heterokaryon. Meiotic recombinants can be selected among the ascospores contained in a cleistothecium of mixed parental origin. Mitotic recombinants can be selected from diploid mycelium.

MEIOTIC RECOMBINATION

The process of meiosis

During meiosis, a diploid cell undergoes a single round of DNA replication, followed by two rounds of chromosome segregation (meiosis I and meiosis II), so that four haploid cells are produced from one diploid cell (see Figure 3 for details).

Figure 3. The process of meiosis.



Schematic drawing of the stages of meiosis. For clarity, only one set of homologous chromosomes (homologs) is shown (thick and thin lines). Circles indicate centromeres.
Fusion of two haploid nuclei produces one diploid nucleus. Prior to melotic prophase I, DNA replication of each homolog produces two sister-chromatids. Subsequently, the cell enters meiosis. **Prophase** I consumes most of the time required for meiosis. The homologs are brought into proximity during a search for homology and align. Non-sister chromatids of homologs recombine so that corresponding segments are exchanged (crossover). This results in a physical connection of the homologs, which in some stages of meiosis and in favorable organisms are visible as chiasmata. Two crossovers are indicated (X). During **metaphase** I, the homologs, connected by chiasmata, line up on the spindle. During **anaphase I**, the chromosomes segregate to opposite poles.
At the end of meiosis I (**telophase I**), two daughter nuclei have formed, each with the haploid number of chromosomes.

During subsequent stages (prophase II, metaphase II, anaphase II and telophase II), the sister-chromatids are separated, so that four (recombinant) haploid cells are formed. In *A. nidulans*, an additional cell division precedes sexual spore formation.

In meiosis I, the homologous chromosomes align and usually synapse to form a synaptonemal complex (SC), a tripartite proteinaceous structure (Von Wettstein *et al.* 1984; Heyting 1996) observed in most organisms but not in *A. nidulans* (Egel-Mitani *et al.* 1982). After disappearance of the SC (if any), the homologous chromosomes segregate to opposite poles. Recombination occurs during meiotic prophase I, i.e., after bulk DNA replication and before the transition to metaphase I, when the pairs of homologs orient themselves in the spindle in preparation of chromosome disjunction. In Ascomycetes, sexual spore formation may directly follow meiosis as in the yeast *S. cerevisiae*, or may be delayed until after a post-meiotic division, so that 8 ascospores per meiosis are formed, as in *A. nidulans*.

Detection of recombinants

In fungi that produce eight sexual spores per meiosis, a meiotic cell heterozygous for a genetic marker M (denoted as M/m) will usually produce 4 M-spores and 4 m-spores, a normal or Mendelian segregation (denoted 4M:4m). This is not influenced by crossovers since the genetic information is merely swapped between chromatids. Deviations from this normal pattern of segregation, jointly referred to as conversion events, are indicative of a recombination event at the M locus. Usually, two major types of aberrant segregation patterns are observed.

I) The first major type of aberrant segregation is 6M:2m or 2M:6m and is referred to as whole chromatid conversion (WCC), since the genetic information present on one chromatid (both strands) is converted into that originally residing on the homologous chromosome.

II) The second major type of aberrant segregation, 5M:3m or 3M:5m, is the result of a transfer of information from a single strand of one chromatid to a single DNA strand of another at the corresponding locus of a non-sister chromatid of the homolog. This type of segregation is referred to as post-meiotic segregation (PMS). This term reflects the segregation of the two strands of the affected chromatid in the first mitotic division after meiosis. A double PMS (Ab(errant)4M:4m) is indicative of the symmetric information transfer of single DNA strands, in contrast to the asymmetric transfer which may result in 5M:3m or 3M:5m segregations (see

III)Figure 4). The frequency of PMS is marker-specific, defining WCC (low frequency of PMS) and PMS (high frequency of PMS) markers (reviewed in Petes *et al.* 1991).

The systematic analysis of aberrant segregations is only feasible in organisms in which the products of a single meiosis are kept together, as in ascomycetes, and if the frequency of aberrant segregation is high. Analysis of multiple markers in a single locus may provide valuable insight into the extent of the region involved in a recombination event.

If recombination frequencies are low, recombinant progeny has to be identified by selection. This selection is usually accomplished by the use of two-point crosses, in which recombination between two non-identical markers in one locus can result in a selectable (or detectable) phenotype different from either parent. Additional markers flanking the locus are used for the distinction between reciprocal and non-reciprocal recombination events. This method allows the analysis of the genetic composition of only one of the two chromatids involved; consequently, non-reciprocal (conversion) events can only be inferred. Also, the array of recombinant types that can be recovered by this method is limited by the selective conditions.

A third method to investigate the segregation patterns resulting from recombination events is the physical analysis of recombination intermediates by microscopy or, if meiosis is synchronous in the majority of the cells, by "real-time" analysis of components of the recombination system; some laboratory strains of *S. cerevisiae* allow such studies.

A model for meiotic recombination in *S. cerevisiae*

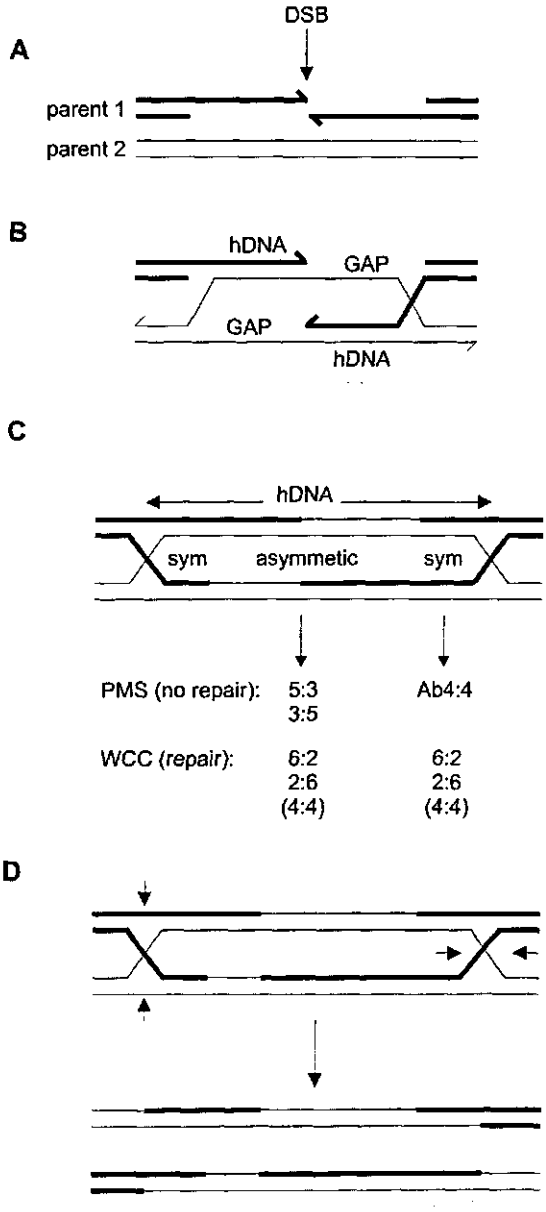
The model described in this section is a version of the double-strand-break repair model proposed by Szostak *et al.* (1983), modified as proposed by Sun *et al.* (1991) and Schwacha and Kleckner (1995) and is illustrated in Figure 4.

Recombinational interactions between homologs are initiated at specific chromosomal locations by the introduction of a double strand break (DSB) in one of the chromatids during prophase I; DSBs have been detected in the promoter region of the *ARG4* gene (Sun *et al.* 1989), in an artificial *HIS4-LEU2* construct (Cao *et al.* 1990), upstream of the *THR4* gene (Goldway *et al.* 1993) and in the promoter regions of the *HIS4* and *CYS3* genes (Fan *et al.* 1995; de Massy *et al.* 1995). On a larger scale, numerous sites of meiosis-specific DSBs were found in whole chromosomes and in various chromosomal regions; many of these sites are located upstream of open reading frames (Zenvirth *et al.* 1992; Game 1992; Wu and Lichten 1994).

It is possible that interhomolog interactions precede the formation of DSBs (Xu and Kleckner 1995; Rocco and Nicolas 1996). The alignment of the homologous chromosomes during meiotic prophase I requires a search for homology which may precede DSB formation, but the precise mechanism and timing of this search is unknown (Kleckner *et al.* 1991; Roeder 1995). The observation of recombination between homologous sequences located at non-allelic positions (ectopic recombination) supports the view that a genome-wide search for DNA homology precedes chromosome alignment. Ectopic conversion occurs at appreciable levels in *S. cerevisiae*, though at frequencies 3- to 17-fold lower than for allelic positions (reviewed in Petes *et al.* 1991).

DSBs occur within a region, rather than at a specific site; they consist of nicks on opposite strands, giving short 5' overhanging termini or blunt ends (Xu and Kleckner 1995; de Massy *et al.* 1995; Liu *et al.* 1995). The *SPO11* gene product is required for the formation of DSBs; possibly, this protein catalyzes the primary cleavage reaction in a topoisomerase-like and sequence non-specific fashion (Xu and Kleckner 1995; de Massy *et al.* 1995; Liu *et al.* 1995; Keeney *et al.* 1997). The DSBs are rapidly processed by a 5'-3' exonuclease activity to give 3' single-stranded tails of several hundreds of nucleotides; no evidence for substantial resection of the 3' ends has been obtained, implying that no or very little genetic information present on the broken chromatid is lost in the process (Sun *et al.* 1991, Bishop *et al.* 1992; Xu and Kleckner 1995; de Massy *et al.* 1995; Liu *et al.* 1995). In a non-null mutant of the *RAD50* gene (*rad50S*), the processing of DSBs is blocked and non-resected breaks associated with the *SPO11* gene product accumulate (Alani *et al.* 1990; Keeney *et al.* 1997).

Figure 4. A model for meiotic recombination in *S. cerevisiae*



A. Recombination is initiated at specific chromosomal locations by the introduction of a double-strand break (DSB) in one of the chromatids during prophase I. DSBs occur within a region, rather than at a specific site; the primary reaction is a topoisomerase-like and sequence non-specific cleavage, which results in short 5' overhanging termini or blunt ends.

B. DSBs are rapidly processed by a 5'-3' exonuclease activity to give 3' single-stranded tails of several hundreds of nucleotides. Invasion of the other chromatid by the 3' tails results in single stranded gaps (GAP) and asymmetric heteroduplex regions (hDNA).

C. Processed DSBs are converted into branched joint molecules (JMs) which consist of four parental (non-crossover) DNA strands, joined by a double Holliday junction. No interrupted strands were observed, which suggests a rapid repair of the single stranded (and possibly small double-stranded) gaps, which originate from the resection of the broken ends. The region encompassed by the two Holliday junctions contains two duplexes of hybrid parental origin. If the heteroduplex region is enlarged by movement of the Holliday junctions (branch migration), symmetric heteroduplex regions (sym) flank the asymmetric regions. Segregation patterns for PMS and WCC events are denoted; those that will go unnoticed are between brackets.

D. *In vitro*, the JMs are resolved into recombinant and non-recombinant products by Holliday junction resolving enzymes, which supports the view that reciprocal and non-reciprocal recombination events share a common intermediate. The example shows resolution into a crossover, arrows indicate the cut strands.

Recently, evidence has been obtained for a transition of processed DSBs into branched joint molecules (JMs) at the *HIS4-LEU2*, *ARG4*, *THR4* and *TRP1* loci (Schwacha and Kleckner 1994, 1995). The transition probably involves the *RAD51* and *DMC1* gene products; like the bacterial RecA enzyme, Rad51 and Dmc1 form nucleoprotein filaments on ssDNA and promote strand exchange *in vitro* (Modrich 1991; Shinohara *et al.* 1992; Bishop *et al.* 1992). *RAD51* and *DMC1* are required for connecting the homologs (Rockmill *et al.* 1995). JMs consist of four parental (non-crossover) DNA strands linked by two Holliday junctions (Schwacha and Kleckner 1994, 1995). No interrupted strands were observed, suggesting a rapid repair of the single stranded (and possibly small double stranded) gaps, which originate from the resection of the broken ends. In meiosis, JMs form preferentially between non-sister chromatids (Schwacha and Kleckner 1994). The region encompassed by the junctions contains two duplexes of hybrid parental origin (Schwacha and Kleckner 1995). Invasion of the homolog by the 3' single-stranded tails of the broken chromatid generates mismatches if heterologies are present in the transferred single-stranded DNA segments. The heteroduplex thus generated is confined to one of the homologs (asymmetrical). Schwacha and Kleckner (1995) have reported the absence of mismatches in JMs at the *HIS4-LEU2* locus, which suggests that, like the single-stranded gaps, mismatches are readily repaired.

In vitro, Holliday junction resolving enzymes can resolve JMs into crossover and non-crossover products. This suggests that reciprocal and non-reciprocal recombination events share a common intermediate (Schwacha and Kleckner 1995; Storlazzi *et al.* 1995). *In vivo*, recombinant (crossover) molecules appear at the end of prophase I, prior to or concurrent with transition into metaphase I (Padmore *et al.* 1991). Interestingly, heteroduplex DNA containing mismatches has been detected at this stage but not at earlier time points; heteroduplex DNA with mismatches detected at the end of prophase I may be the result of branch migration of the Holliday junctions and will consequently be symmetrical (Goyon and Lichten 1993; Nag and Petes 1993; Schwacha and Kleckner 1994; Storlazzi *et al.* 1995).

According to the model described above, WCC (6:2 segregation) and PMS (5:3 or Ab4:4 segregation) are the consequence of repair or the absence of repair, respectively, of mismatches contained in a heteroduplex (reviewed in Nicolas and Petes 1994). Repair of a mismatch may lead to either conversion (6:2 segregation) or restoration (4:4 segregation), depending on which strand of the heteroduplex is used as the template. Absence of mismatch repair leads to PMS (5:3 segregation) if the heteroduplex is asymmetrical and to a double PMS (Ab4:4 segregation) if the heteroduplex is symmetrical.

Polarity of recombination

The frequency of aberrant segregation of a marker is dependent on the position of the marker within a locus. Characteristically, a 5' to 3' gradient in the frequency of WCC is observed in a series of one-point crosses in *S. cerevisiae* (reviewed in Nicolas and Petes 1994). A notable exception is the *HIS2* gene, where highest WCC frequencies are found for 3' markers (Malone *et al.* 1992). Sun *et al.* (1991) noted that the frequency gradient observed in the *ARG4* gene correlates well with the size distribution of the 3' tails of the broken chromatid. The frequency of both WCC and PMS has been investigated in the *HIS4* gene; while a clear 5' to 3' gradient was found for WCC markers, for PMS markers this was not the case (Detloff *et al.* 1992). The gradients of WCC in the *ARG4* and *HIS4* genes appear to be biphasic: a steep curve close to the initiation site and a less steep curve further

away; the gradient of PMS in the *HIS4* gene shows the same steep curve close to the initiation site, but a constant frequency further away (Nicolas *et al.* 1989; Detloff *et al.* 1992). This suggests an influence of mismatch repair systems (that do not efficiently detect PMS markers) on the extension of heteroduplex regions by movement of the Holliday junctions (Alani *et al.* 1994).

In a two-point cross system, meiotic polarity of WCC is observed as a preferential conversion of one of the alleles. This has been observed in a range of organisms and appears to be a general phenomenon (Whitehouse 1982).

Meiotic recombination in *A. nidulans*

Tetrad analysis in *A. nidulans* is extremely laborious and this method has been reported only once (Strickland 1958a); among 1642 hybrid asci examined for a number of loci, Strickland found 6 asci that displayed a non-Mendelian segregation. For individual loci, the frequency of aberrant segregation was found to range from 1/120 to 0/1142. In *S. cerevisiae*, frequencies range from 0.5% to 30% for individual alleles (Petes *et al.* 1991). Consequently, meiotic recombination in this organism is usually analyzed by the selection of recombinants among the ascospores produced in two-point cross systems. Meiosis is not synchronous in *A. nidulans* cleistothecia, precluding "real-time" analysis of recombination.

The *ad*E (Pritchard 1955, 1960), *ad*F (Calef 1957), *paba*A (Siddiqi 1962), *lys*F (Pees 1965, 1967) and *brl*A (Clutterbuck *et al.* 1992) loci have been examined genetically; additional molecular data are available only for the *brl*A locus. In each case, the frequency of conversion of an allele was found to be dependent on its genetic location (polarity). The *ad*E, *ad*F, *paba*A and *brl*A loci show a high frequency and a low frequency end, while for the *lys*F locus alleles located on both sides of the locus show a high frequency of conversion when compared to internal alleles. For the *brl*A locus, polarity was determined to be 5' to 3'.

In summary, the available data on meiotic recombination in *A. nidulans* do not contradict the model described above: polarity of conversion is a feature of all loci examined, and is 5' to 3' for the *brl*A gene; conversion is often accompanied by a crossover.

MITOTIC RECOMBINATION

Detailed molecular analysis of mitotic recombination in eukaryotes has so far been largely restricted to the yeast *S. cerevisiae*, despite the importance of this process for DNA repair, mammalian antigen and immunoglobulin production, and its possible role in the development of cancer (Roeder and Stewart 1988). Like meiotic recombination, mitotic recombination includes non-reciprocal exchanges (conversions) and reciprocal exchanges (crossovers), presumably through a heteroduplex intermediate (reviewed in Petes *et al.* 1991). In *S. cerevisiae*, mitotic recombination during both the G1 phase and the G2 phase has been observed (Fabre 1978; Esposito 1978; Roman and Fabre 1983; Fabre *et al.* 1984).

In *A. nidulans* and in mammalian cells, the G2 phase is substantially longer than in *S. cerevisiae* (Bainbridge 1971; Bergen and Morris 1983). Therefore, mitotic post-replicational recombination may be more frequent in *A. nidulans* than in *S. cerevisiae* (Osman *et al.* 1993). The frequent recovery of parental chromosomes from recombinant diploids has prompted the assumption that all mitotic recombination occurs after DNA replication in *A. nidulans* (Whitehouse 1982), although this has not been systematically investigated. All

studies on mitotic recombination in *A. nidulans* have been restricted to genetic analysis; as in *S. cerevisiae*, polarity of mitotic conversion is low or absent in *A. nidulans* (reviewed in Whitehouse 1982, and in Petes *et al.* 1991).

OUTLINE OF THIS STUDY

Selection of a locus

The aim of the experiments presented in chapter 2 was to establish whether the *nii*A-*nia*D locus of *A. nidulans* (Figure 5) could be used to analyze some of the molecular aspects of recombination events in a filamentous fungus. The major prerequisites for such a model system were I) a strong polarity of conversion, which would indicate a recombination initiation site nearby, and II) a general frequency of recombination that would allow the collection of a sufficient number of recombinants.

Figure 5. The *crn*A-*nii*A-*nia*D gene cluster of *A. nidulans*

The locus is drawn to scale. CEN, centromere; P, promoter region. Arrows indicate the direction of transcription. The *fac*C and *brl*A loci, used as flanking markers in crosses, are located 26 map units centromere-proximal and 10 map units centromere-distal to the *nii*A-*nia*D locus, respectively. The *crn*A gene product is involved in nitrogen uptake (Unkles *et al.* 1991).

At the start of the project, we had essentially two options. One of the loci in the *A. nidulans* genome that had previously been shown to display polarity of meiotic conversion could be cloned and sequenced. This would guarantee suitability of the locus beforehand, but would require a considerable investment of time and money. The alternative strategy would be based on the assumption that promoters of gene transcription are preferred initiation sites of meiotic recombination and that consequently meiotic polarity of conversion is a property of many if not all genes, as predicted by most data on meiotic recombination in *S. cerevisiae* and other fungi. Although it inserted an amount of risk into the project, we opted for this second approach and selected the *nii*A-*nia*D region, a locus which had already been cloned and sequenced.

The *nii*A-*nia*D region

The *nii*A gene (encoding nitrite reductase) and the *nia*D gene (encoding nitrate reductase) are located on the right arm of chromosome VIII of *A. nidulans* (Johnstone *et al.* 1990). The products of these genes are essential for the utilization of nitrate as a nitrogen source. Nitrate reductase catalyses the conversion of nitrate to nitrite, which is converted to ammonium by nitrite reductase (Cove 1979). The *nii*A and *nia*D genes have several assets that were useful for our purposes:

I) Recombinants among the progeny of two-point crosses can be easily selected on medium with nitrate as the only nitrogen source. This is essential as intragenic meiotic recombination frequencies *in A. nidulans* are generally too low (less then 0.5%) to allow analysis of unselected progeny (Whitehouse 1982). Also, mitotic recombinants derived from a diploid strain can be identified with the use of a simple plate-assay based on the same selection.

II) The whole region of the genome containing the *nii*A and *nia*D genes (depicted in Figure 5) has been cloned and sequenced; it contains a bi-directional promoter region that directs transcription of the adjacent *nii*A and *nia*D genes (Johnstone *et al.* 1990). A third gene, *crn*A, involved in nitrogen uptake, is located centromere-proximal to *nii*A and is transcribed from a separate promoter immediately downstream of *nii*A (Unkles *et al.* 1991).

III) Selection on nitrate can be used in both intergenic and intragenic crosses involving *nii*A and/or *nia*D alleles to identify recombinants. We would therefore be able to analyze both intergenic and intragenic recombination in a single genomic region.

IV) Transcription of the *nii*A and *nia*D genes is subject to nitrate induction and ammonium repression (Cove 1979; Punt *et al.* 1991). This would allow us to (indirectly) analyze the influence of transcription on recombination by varying the nitrogen sources during crosses.

V) A large number of *nii*A and *nia*D alleles were already available (Tomsett and Cove 1979). This would facilitate the necessary preliminary check for polarity of meiotic conversion in this region. Moreover, a number of *nia*D alleles had been partially sequenced to identify the position and type of the mutations. These alleles and the sequence information have been kindly made available to us (Peter Strike, personal communication). The use of these alleles would greatly increase the value of an analysis of meiotic recombination in the *nia*D gene, since it would allow a correlation of recombination frequencies and physical position and type of the mutations involved.

A recombination assay

The principle conclusion drawn from the genetic analysis of recombination in the *nii*A-*nia*D locus (chapter 2) is that conversion is highly polarized within the *nia*D gene. Also, the range of recombination frequencies in the *nii*A-*nia*D locus (3 to 384 per 10^5 viable ascospores) allowed the collection of sufficient numbers of recombinants. Thus the *nii*A-*nia*D locus appeared to be suitable for our purposes. This served as a basis for the design of a recombination assay, which consisted of a set of strains harboring neutral mutations in the *nii*A-*nia*D region, which could be detected by restriction enzyme analysis. A similar assay system was previously used for the analysis of recombination in *S. cerevisiae* (Nicolas *et al.* 1989) and *Schizosaccharomyces pombe* (Grimm *et al.* 1994).

We have examined meiotic recombinants obtained from allelic intergenic (*nii*A-*nia*D) crosses and from, both allelic and ectopic, intragenic (*nia*D/*nia*D) crosses; mitotic intragenic recombinants were obtained from a heterozygous (*nia*D/*nia*D) diploid strain. In chapter 3, the analysis of meiotic recombinants with the use of this system is described. Chapter 5 describes the analysis of spontaneous mitotic recombinants. Details on the construction of the necessary strains are given in Figure 16 (page 69). The system was designed to generate answers to the following questions:

I) Are recombination events that lead to functional restoration of the *nii*A-*nia*D locus in two-point crosses initiated in the intergenic promoter region?

II) Does induction of transcription during crosses quantitatively or qualitatively influence the recombination process?

III) What is the extent of the genomic region involved in individual meiotic recombination events? What happens in this region during recombination?

IV) Does the type and position of the introduced heterologies influence the outcome of the recombination event?

V) How does meiotic recombination compare with mitotic recombination in the above respects?

VI) Is meiotic recombination in the *nii*A-*nia*D region influenced by the chromosomal context (allelic *vs.* ectopic) of this region?

2. THE *nii*A-*nia*D LOCUS OF *A. NIDULANS* AS A MODEL SYSTEM

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SUMMARY

We have examined polarity of meiotic conversion in the *nii*A-*nia*D gene cluster of *A. nidulans* in two-point crosses. The type and position of the mutations represented by the *nia*D alleles and the correlation between the relative frequency of conversion and the physical position of these mutations were determined. We show that polarity of meiotic conversion is 5' to 3' (transcribed strand) within the *nia*D gene. Additional crosses involving a *nii*A allele and a *nia*D allele show a low polarity of conversion, which suggests that the recombination events leading to restoration of the *nia*D gene are initiated upstream of the coding region of the *nia*D gene but within the *nii*A-*nia*D gene cluster, possibly within the intergenic promoter region.

INTRODUCTION

Genetic recombination embodies the conflicting concepts of stability and flexibility of the genome as the primary carrier of genetic information. In vegetative cells, interchromosomal recombination is suppressed to a low level, ensuring faithful transmission of the genetic material to the daughter cells and repair of DNA damage. In contrast, cells undergoing meiosis display strongly elevated levels of recombination, which results in a wide array of gametic genotypes. Recombination events are observed in the progeny as an exchange of chromosomal markers (crossing over) or the non-reciprocal transfer of information (conversion) between the interacting non-sister chromatids (see Figure 1, page 8).

In several organisms, including *Drosophila melanogaster*, filamentous fungi and yeast, polarity of meiotic conversion has been demonstrated in most of the loci analyzed (reviewed by Whitehouse 1982). Within these loci, the frequency of conversion of a marker allele is primarily dependent on the location of the marker within the locus: markers on one side of the locus show a higher frequency of conversion than markers on the other side. Originally proposed by Holliday (1964), the most widely accepted interpretation of polarity of conversion is that there is a specific site at the high frequency side of a locus where recombination events between the interacting non-sister chromatids can be initiated during meiotic prophase I. Starting from this initiation site, a tract of heteroduplex of variable length is formed between strands of the non-sister chromatids. Subsequent mismatch repair may lead to conversion or restoration of a marker located within the heteroduplex tract. Markers located close to the initiation site will show a higher frequency of conversion than more distant markers as the probability that a marker is included in the heteroduplex tract (and subject to mismatch repair) declines with distance from the point of initiation (Holliday 1964). A model for meiotic recombination in *S. cerevisiae* is presented in Figure 4 (page 13).

Support for this interpretation was provided by the molecular analysis of meiotic conversion in the *ARG4* and *DED81-DED82* genes of yeast, where a bipolar initiation site of meiotic recombination is located within the *ARG*4 promoter region (Nicolas *et al.* 1989, Schultes and Szostak 1990). Like the *ARG*4 locus, the *HIS4* locus of yeast (White *et al.* 1991) and the *brl*A locus of *A. nidulans* (Clutterbuck *et al.* 1992) show 5' to 3' polarity of meiotic conversion, suggesting that promoter regions may serve as initiation sites. The binding of transcription factors but not transcription *per se* has been implicated in the initiation of recombination at the *HIS4* locus (White *et al.* 1993). In contrast, the polarity of meiotic conversion is 3' to 5' at the *HIS2* locus of yeast (Malone *et al.* 1992).

The main aim of the work presented here was to establish whether the *nii*A-*nia*D gene cluster of *A. nidulans* is a useful model system to investigate the initiation of meiotic recombination events in relation to gene transcription at the molecular level in filamentous fungi. Prerequisite for such a model system is a strong polarity of conversion in one or both of these genes. We have examined the properties of meiotic conversion in the *nii*A-*nia*D gene cluster (Figure 5, page 16) using a series of alleles in two-point crosses. The *nii*A gene (encoding nitrite reductase) and the *nia*D gene (encoding nitrate reductase) are located on the right arm of chromosome VIII of *A. nidulans* and are divergently transcribed from an intergenic promoter region (Johnstone *et al.* 1990). Both genes are subject to nitrate induction and ammonium repression at the transcription level (Punt *et al.* 1991). We have

determined the physical position of the mutations in the nine *nia*D alleles used in this study, and the correlation between the location of a mutation and its relative frequency of conversion. We show that polarity of meiotic conversion is 5' to 3' within the *nia*D gene. Additional data suggest that the initiation site of conversion events resulting in restoration of the *nia*D gene is located upstream of the coding region of the *nia*D gene but within the *nii*A-*nia*D gene cluster, and polarity in the *nii*A gene may not be strictly 5' to 3'.

RESULTS

Crosses

To distinguish between reciprocal and non-reciprocal recombination events leading to functional restoration of the *nii*A-*nia*D gene cluster, five tester strains carrying *fac*C301 and *brl*A42 as outside markers were constructed by crossing the appropriate strains (Table 5, page 64). Analysis of unselected progeny from these crosses confirmed that the *fac*C marker is located 26 map units proximal to *nii*A, and the *brl*A marker is 10 map units distal to *nia*D (Clutterbuck 1993). The tester strains carried either the *nia*D1017 (WG440 and WG441), the *nia*D1078 (WG453 and WG454), or the *nii*A4 (WG466) allele. Subsequently, *nii*A and *nia*D mutant strains were crossed with these tester strains. Recombinant progeny colonies showing wild type growth on nitrate as sole nitrogen source were isolated and tested for all markers (Table 1).

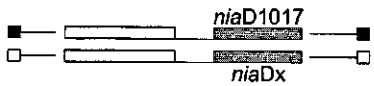
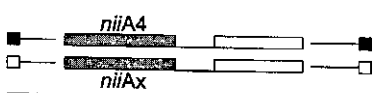
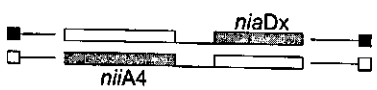
The frequency of recombination at the *nii*A-*nia*D gene cluster varied a 100-fold between crosses, but was also found to vary widely between cleistothecia of one cross; in Table 1 average frequencies are given. Highest frequencies were detected when the tester strains carrying the *nia*D1017 or *nia*D1078 alleles were crossed to strains carrying centromere proximal *nia*D alleles and in intergenic *nii*A-*nia*D crosses.

In each cross, approximately half (55% on average) of the nitrate-utilizing progeny carried a non-parental set of outside markers and only one of the two possible non-parental sets of the outside markers (R1 and R2 in Table 1) was predominantly found for each combination of alleles. If we assume that this bias is the consequence of crossovers between the mutant sites, the genetic order of the alleles used in this study is: *nii*A18, *nii*A4, *nii*A10, (*nia*D17, *nia*D21, *nia*D35, *nia*D169), *nia*D1017, (*nia*D15, *nia*D20, *nia*D124), *nia*D1078 (centromere proximal to distal, brackets indicating unresolved internal order).

Location of the *nia*D mutation

To allow for a correlation between the physical position and relative conversion frequency of the markers, the location and nature of the mutations in the *nia*D1017 and *nia*D1078 alleles were determined by partially sequencing PCR products; for the other *nia*D alleles this information was already available (Table 2). Identified genetically as point mutations (Tomsett and Cove 1979), three of the alleles were found to harbor a small deletion (*nia*D1017 (1 bp), *nia*D20 (13 bp) and *nia*D124 (9 bp)). The location of the mutations match the relative order derived from crossover frequencies perfectly with the exception of *nia*D1017 and *nia*D21, which show a reverse physical order.

Table 1 .Meiotic recombination in the *nii*A-*nia*D gene cluster.

Parental genotypes[a]				flanking marker combinations[c]			
				P1	P2	R1	R2
Type of cross	allele x	frequency[b]	# scored	■—■	□—□	■—□	□—■
A. Intragenic (*nia*D/*nia*D)							
	*nia*D35	384	125	5	**36**	**53**	6
	*nia*D17	98	217	8	**41**	**45**	6
	*nia*D169	15	95	11	**46**	**37**	6
	*nia*D21	4	168	17	**28**	**47**	8
	*nia*D15	3	88	**28**	16	16	**40**
	*nia*D20	11	63	**24**	11	7	**48**
	*nia*D124	14	134	**37**	12	16	**35**
	*nia*D1078	4	70	**43**	8	6	**43**
	*nia*D35	126	146	3	**40**	**53**	4
	*nia*D17	112	143	8	**49**	**41**	2
	*nia*D169	45	57	7	**32**	**51**	10
	*nia*D21	25	93	9	**40**	**44**	7
	*nia*D15	15	53	11	**33**	**46**	10
	*nia*D20	3	98	8	**42**	**41**	9
	*nia*D124	7	60	7	**35**	**50**	8
B. Intragenic (*nii*A/*nii*A)							
	*nii*A18	24	142	9	**36**	**47**	8
	*nii*A10	30	63	16	**27**	9	**48**
C. Intergenic (*nii*A/*nia*D)							
	*nia*D1017	413	85	14	**26**	**53**	7
	*nia*D1078	342	86	17	**25**	**52**	6

[a] The constitutions of the parental strains are drawn. Functional genes are white, non-functional genes are gray. A black square represents the presence of the mutant allele of the corresponding outside marker (*facC* on the left, *brlA* on the right), a white square the wild type allele.

[b] the frequency of recombination is denoted per 10^5 viable ascospores.

[c] The four combinations of outside markers (parental: P1, P2; recombinant: R1, R2) are schematically drawn and given as the percentage of the recombinants analyzed per cross (# scored): P1, *facC brlA*; P2, *facC*$^+$ *brlA*$^+$; R1, *facC brlA*$^+$; R2, *facC*$^+$ *brlA*. The most frequent P and R combinations for each cross are bold-faced.

A possible explanation for the reverse genetic order of *nia*D1017 and *nia*D21 could be that the amino acid change resulting from the point mutation in *nia*D21 (Gly to Asp) is not the cause of the *nia*D phenotype of the strain carrying this allele, and a second mutation is located 5' to the equivalent position of the *nia*D1017 mutation. To investigate this possibility, strains carrying the *nia*D1017 and *nia*D21 alleles were transformed with two DNA fragments (*Hin*cII fragment A (bp 1460-1898) and *Hin*cII fragment B (bp 1899-2408). The positions of the *Hin*cII sites and fragments are depicted in Figure 6B. The strain carrying *nia*D1017 could be rescued with fragment A and the strain carrying *nia*D21 with fragment B (data not shown). This is in agreement with the positions of the *nia*D1017 (bp 1715) and *nia*D21 (bp 1918) mutations as determined by sequencing. Although these experiments do not exclude the possibility of secondary mutations, they do demonstrate that the *nia*D21 allele does not carry a second mutation 5' to *nia*D1017.

Conversion

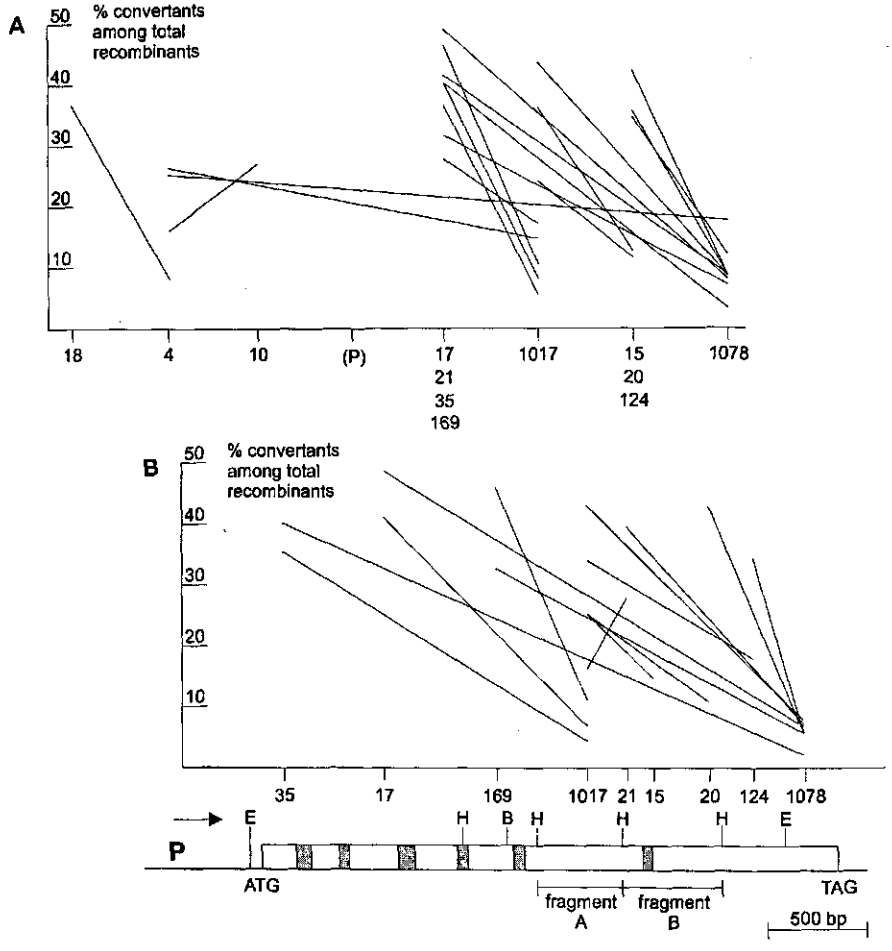
If nitrate-utilizing progeny colonies harboring one of the two parental sets of outside markers (P1 and P2 in Table 1) are interpreted as conversion events, our data for crosses involving two *nia*D alleles show an absolute correlation between the relative map positions of the markers as determined from the crossover frequencies and the polarity of conversion: in all crosses, the proximal marker is preferentially converted (Figure 6A). Given the location of the mutant sites of the *nia*D alleles used in this study, this shows that, in general, polarity is 5' to 3' in the *nia*D gene (Figure 6B). Although not extensive, the data generated with *nii*A alleles suggest a different situation for the *nii*A locus. A uniform polarity of conversion is not apparent as extreme marker alleles (*nii*A18 and *nii*A10) are preferentially converted relative to *nii*A4 (Figure 6A). Polarity is low when crosses involve a *nii*A and a *nia*D allele.

Table 2. Position and nature of mutations in *nia*D alleles.

allele	position[a]	mutation	effect
*nia*D35	133	G to A transition	amino acid substitution: Glu to Lys
*nia*D17	655	G to A transition	aminoacid substitution: Gly to Arg
*nia*D169	1251	G to A transition	aminoacid substitution: Gly to Arg
*nia*D1017	1751	1 bp deletion	frameshift
*nia*D21	1918	G to A transition	aminoacid substitution: Gly to Asp
*nia*D15	2058	G to A transition	intron-exon boundary disrupted
*nia*D20	2342-2354	13 bp deletion	frameshift
*nia*D124	2569-2577	9 bp deletion	deletion of Glu-Arg-Phe
*nia*D1078	2842	T to A transversion	amio acid substitution: Asn to Ile

[a] The position of the mutations is given relative to the *nia*D ATG codon (Johnstone *et al.* 1990).

Figure 6. Polarity of conversion in the *nii*A-*nia*D gene cluster of *A. nidulans*.



Panel A. The x-axis shows the genetic position of the *nii*A and *nia*D alleles as deduced from the recombination analysis (Table 1), P represents the intergenic promoter region. One line represents the result from a cross between strains carrying the alleles corresponding to the end points of the line. The percentage conversions of the parent alleles is given on the y-axis.

Panel B. The x-axis shows the physical location of the *nia*D mutations as determined by sequencing (Table 2). At the bottom the *nia*D gene is drawn to scale, P represents the promoter region, the arrow shows the direction of transcription and shaded boxes represent introns; B = *Bgl*II, E = *Eco*RI, H = *Hin*cII; *Hin*cII fragments A and B have been used in transformation experiments (see text).

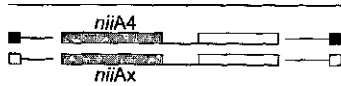
Correction for secondary crossovers

Due to the considerable genetic distance between the *nii*A-*nia*D locus and the outside markers, a fraction of the recombination events leading to restoration of the *nii*A-*nia*D locus will be incorrectly classified as a crossover or a conversion as a consequence of crossovers between the *nii*A-*nia*D locus and the outside markers. In an attempt to correct our data set for these misinterpreted events, we have made the following assumptions. I) The recombination event leading to restoration of the *nii*A-*nia*D locus is either a conversion to wild type of the left hand allele (COL), conversion to wild type of the right hand allele (COR) or a crossover between the mutant sites (CRO). Since we have confirmed by determining the physical location of the mutant sites that genetic mapping, based on crossover frequencies, provides a good (but not a perfect) order of the *nia*D alleles, CRO must account for a considerable number of recombination events. Since we are unaware of the precise mechanism of recombination in filamentous fungi, we have assumed that the COL, COR and CRO events are independent. However, if the initiation point of the recombination events is not located between the mutant sites, recombination tracts must cover the first mutation to result in a CRO. This may render CRO and COL/COR events interdependent. II) The recombination event leading to restoration of the *nii*A-*nia*D locus does not alter the genetic distances between the *fac*C, *nii*A-*nia*D and *brl*A loci. This may be a reasonable assumption, as chiasma interference has been reported absent in *A. nidulans* (Strickland 1958b). Furthermore, re-analysis of the data previously obtained for recombination at the *ad*E locus (Pritchard 1955) point in this direction. In this case, two outside markers (*paba*A and *y*A) on the left side of the *ad*E locus were included in the crosses. The *paba*A marker is located 15 map units to the left of *ad*E, the *y*A locus is adjacent to the *ad*E locus (0.3 map units). Among progeny colonies, selected for recombination at the *ad*E locus, the genetic distance between the *paba*A and *y*A loci varied from 15 to 21 map units, depending on the *ad*E allele used. This suggests that the genetic distance between two loci is not severely affected by a recombination event nearby.

The line of reasoning for the correction of our data is the following. True conversion of the right hand allele (COR) may be misinterpreted as the consequence of an accompanying crossover between the *fac*C and *nii*A loci (COR/XL), a crossover between the *nia*D and *brl*A loci (COR/XR) or both (COR/XL/XR). Other recombination events (COL and CRO) may be misinterpreted as a COR as a consequence of secondary crossovers (COL/XL/XR and CRO/XR). The fraction of the progeny colonies that is expected to have undergone a crossover between *fac*C and *nii*A (XL) is, based on the genetic distance between the two loci, 0.26. Similarly, XR is 0.10. Therefore, in a cross where the right hand allele carries the *fac*C and *brl*A markers, the P1 category (Table 1) equals COR - 0.26xCOR - 0.10xCOR - 0.026xCOR + 0.026xCOL + 0.10xCRO. The P2, R1 and R2 categories (Table 1) can, in a similar fashion, be dissected. The equations and the resolution of these equations are given in Table 3.

The corrected data set (Table 3) shows several remarkable features. I) The polarity of conversion in the *nia*D gene is more pronounced than in the raw data set: conversion of the 3' allele is dramatically lower than in the raw data set. II) Polarity is low when the mutant sites are close together and in intergenic crosses. III) In all crosses a high percentage of the nitrate-utilizing progeny colonies is classified as the consequence of a crossover between the mutant sites.

Table 3. Correction of the data for secondary recombination events.

parental genotypes[a]		recombination event[b]		
cross	allele x	COL	COR	CRO
	*nia*D35	28	-3	75
	*nia*D17	44	2	54
	*nia*D169	61	7	32
	*nia*D15	26	22	52
	*nia*D20	15	15	70
	*nia*D124	46	17	37
	*nia*D1078	49	2	49
	*nia*D35	35	-8	73
	*nia*D17	63	-2	39
	*nia*D169	24	4	72
	*nia*D15	29	10	61
	*nia*D20	49	5	46
	*nia*D124	29	2	69
	*nii*A18	33	5	62
	*nii*A10	-8	32	76
	*nia*D1017	8	11	81
	*nia*D1078	6	14	80

Legend
COL, percentage conversion of the left allele after correction;
COR, percentage conversion of the right allele after correction;
CRO, percentage crossovers between mutant sites after correction;
XL, predicted fraction of recombination events at the *nii*A-*nia*D locus accompanied by a crossover between the *fac*C and *nii*A loci (0.26);
XR, predicted fraction of recombination events at the *nii*A-*nia*D locus accompanied by a crossover between the *brl*A and *nia*D loci (0.10);
P1, P2, R1, R2 as in Table 1.

Correction procedure: sources of the outside marker combinations (P1, P2, R1, R2 in Table 1) if the right hand allele carries the *fac*C and *brl*A outside markers and assuming that (I) conversion events (COL and COR) and crossovers between the mutant sites (CRO) are independent and (II) the genetic distance between the *nii*A-*nia*D locus and the outside markers is not affected by the restoration event at the *nii*A-*nia*D locus (see text):

P1 = [COR] - [COR/XL] - [COR/XR] - [COR/XL/XR] + [COL/XL/XR] + [CRO/XR]
P2 = [COL] - [COL/XL] - [COL/XR] - [COL/XL/XR] + [COR/XL/XR] + [CRO+XL]
R1 = [CRO] - [CRO/XL] - [CRO/XR] - [CRO/XL/XR] + [COR/XR] + [COL/XL]
R2 = [COL/XR] + [COR/XL] + [CRO/XL/XR]

Equations for the reverse situation (the left hand allele is accompanied by the *fac*C and *brl*A markers) are identical, but the P1/P2 and the R1/R2 categories have to be interchanged. The equations can be used to extract correction formulae for COL, COR and CRO:

COL = 1.837x[P2] - 0.286x[P1] - 0.765x[R1] + 0.787x[R2]
COR = 1.265x[P1] - 0.127x[P2] - 0.192x[R1] + 0.946x[R2]
CRO = 2.102x[R1] - 0.551x[P1] - 0.962x[P2] + 0.587x[R2]

The data for the cross involving the *nia*D1017 and *nia*D21 could not be corrected due to conflicting data on the genetic and the physical position of the mutant sites (see text).

DISCUSSION

Frequency of recombination

Polarity of meiotic conversion has been detected in a large number of loci in both lower and higher eukaryotes and appears to be a general phenomenon. In *A. nidulans*, polarity of conversion in two-point crosses has been reported for the *ad*E, *paba*A, *lys*F and *brl*A loci (Pritchard 1955; Siddiqi 1962; Pees 1967; Clutterbuck *et al.* 1992). In each case, the frequency of conversion of an allele was found to be dependent on its genetic location. The *ad*E, *paba*A and *brl*A loci show a high frequency and a low frequency end, while for the *lys*F locus alleles located on both sides of the locus show a high frequency of conversion when compared to internal alleles. In these studies, progeny colonies harboring a restored locus were recovered at frequencies of up to 510 per 10^5 viable ascospores (*paba*A locus). In crosses with different *nia*D alleles, we found comparable frequencies, ranging from 3 up to 384 per 10^5 viable ascospores. It is noteworthy that the frequency of recombination in the *nia*D gene appears to be dependent on the location of the mutant sites, as crosses involving 5' alleles show higher frequencies than 3' alleles.

Crossovers

The percentage crossovers (non-parental set of outside markers) among total recombinants reported for the *A. nidulans* loci is 79% (*ad*E), 61% (*paba*A), 62% (*lys*F) and 55.4% (*brl*A). As a rule, one of the two possible crossover configurations of outside markers is predominantly found. In our crosses, 55% of the recombinants had a non-parental set of outside markers. We have determined the physical location of the mutant sites in the *nia*D alleles and have shown that the genetic order of the alleles, based on crossover frequencies, is in good agreement with the physical location of the mutant sites. This strongly suggests that a large fraction of the nitrate-utilizing progeny have arisen through recombination intermediates that are resolved as a crossover between the mutant sites. A similar suggestion has been made by Whitehouse (1982).

Polarity of conversion

In most genes examined at the molecular level the direction of meiotic polarity of conversion is 5' to 3'. Generally explained as the consequence of fixed or preferred initiation sites for recombination, 5' to 3' polarity of conversion implicates the promoter regions of these genes in the initiation of recombination. Here we present evidence for 5' to 3' polarity of meiotic conversion in the *nia*D gene of *A. nidulans.* Our observations are in agreement with models on recombination, which propose specific sites where recombination is initiated, subsequent heteroduplex formation, and resolution of the intermediate (Radding 1982; Szostak *et al.* 1983). The inclusion of one or both of the mutant sites in heteroduplex tracts must be highly dependent on the distance between the point of initiation and the mutant sites to account for the observed differences in recombination frequency between crosses involving 5' or 3' *nia*D alleles, and the strong polarity of conversion.

The low polarity in crosses involving a *nii*A and a *nia*D allele suggests that the gene cluster contains an initiation site for recombination events, possibly within the intergenic promoter region, as reported for the *ARG*4 and *HIS*4 loci of yeast (Nicolas *et al.* 1989, White *et al.* 1991). The bi-directional polarity in the *nii*A gene, although not thoroughly documented in this study, can be interpreted as the consequence of initiation points on both sides of the *nii*A gene. The location of this putative centromere-proximal initiation site may be the

promoter region of the *crn*A gene (Unkles *et al.* 1991), located immediately downstream of the *nii*A gene (see Figure 5, page 16). This bi-directional type of polarity is also found for other loci, including the *lys*F locus of *A. nidulans* (Pees 1967).

We have attempted to correct our data set for crossovers between the *nii*A-*nia*D gene cluster and the outside markers. Correction of the data does not alter our principle conclusion that polarity of meiotic conversion in the *nia*D gene is 5' to 3'.

Conflicting results

As neither the *nia*D1017 nor the *nia*D21 allele show apparent aberrant behavior in other crosses and we have confirmed the physical order of the two mutations by transformation experiments, the interpretation of the outcome of the cross involving these alleles remains difficult. The equations used for correction of our data could not be applied to the *nia*D1017/*nia*D21 cross as the consequence of conflicting genetic and molecular data on the location of the mutant sites. In current models on recombination that entail the formation of a heteroduplex as an intermediate in the recombination process (Radding 1982, Szostak *et al.* 1983), the frequency of conversion of a marker depends, apart from the distance of the mutant site from the point of initiation, on the efficiency of the repair of a specific mismatch. This could account for a reversal of the polarity of conversion for a specific pair of marker alleles. However, this does not clarify the reversal of the genetic positions found for the *nia*D1017/*nia*D21 pair of alleles. Possibly, the close proximity of these mutations (203 bp) is of crucial influence on the repair of the mismatches included in a heteroduplex and/or the resolution of recombination intermediates.

3. ANALYSIS OF MEIOTIC RECOMBINANTS

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The data presented in this chapter have been submitted for publication as: Meiotic recombination in the *nii*A-*nia*D gene cluster of *Aspergillus nidulans* initiates in the intergenic promoter region (submitted to Genetics).

SUMMARY

We have examined meiotic recombination events in the *nii*A-*nia*D region of the filamentous fungus *A. nidulans*. The *nii*A and *nia*D genes are required for the utilization of nitrate and are divergently transcribed from an intergenic promoter region. The *nii*A and *nia*D mutant strains that were used as parents in two-point crosses differed by a nonsense or missense mutation in the *nii*A or *nia*D gene and by a series of 11 silent point mutations. We analyzed recombinants selected on nitrate for the presence or absence of individual markers to determine the position and endpoints of conversion tracts. We found evidence for an initiation site for meiotic recombination in the intergenic promoter region. Conversion tracts with an average length of 1.5 kb extended from this site into the *nii*A gene, the *nia*D gene or both. Conversion tracts were continuous in almost all recombinants. Co-conversion of markers declined steeply with distance from the proposed initiation site. In crosses where one of the parents harbored an ectopic copy of the *nii*A-*nia*D region instead of the allelic copy, conversion tracts were similar in size and position to those observed in allelic crosses. Ectopic recombinants were detected at a significantly lower frequency. The majority of the allelic recombinants but not of the ectopic recombinants harbored a crossover within the *nii*A-*nia*D region.

INTRODUCTION

The role of transcriptional promoters as preferred initiation sites for meiotic recombination (hotspots) is well-documented for the yeast *S. cerevisiae* (see
Figure 4 (page 13) for details and references); for other eukaryotes this is not the case. However, preferential conversion of one of the two mutant sites in two-point crosses has been observed in a range of organisms; in a series of one-point crosses, a gradient in the frequency of recombination is usually observed (Whitehouse 1982; Petes *et al.* 1991). This polarity of conversion is generally, though not universally (Detloff *et al.* 1992), interpreted as the consequence of fixed initiation sites and the formation of heteroduplex tracts of variable length extending from this site (Hastings and Whitehouse 1960; Holliday 1964; Meselson and Radding 1975; Szostak *et al.* 1983). In *A. nidulans*, polarity of conversion has been observed for a number of loci (reviewed in Whitehouse 1982), including the *brl*A locus (Clutterbuck *et al.* 1992) and the *nii*A-*nia*D region (chapter 2). Previous genetic analysis suggested that the intergenic promoter region of the *nii*A-*nia*D locus contains an initiation site for meiotic recombination (chapter 2). The primary objective of the work presented here was to confirm this by molecular analysis of a series of unselected markers in recombinants, an approach pioneered in *S. cerevisiae* by Nicolas *et al.* (1989).

In *S. cerevisiae*, initiation sites for meiotic recombination co-localize with meiosis-specific double-strand break (DSB) sites (
Figure 4, page 13). So far, no meiosis-specific DSBs have been reported in other organisms (Baehler *et al.* 1991). Recently, evidence has been obtained for a transition of DSBs into branched joint molecules (JMs), in which the chromatids are connected by a double Holliday junction; the region encompassed by the two individual Holliday junctions contains two heteroduplexes (Schwacha and Kleckner 1994, 1995).

The heteroduplex regions in recombination intermediates in the *DED81-ARG4* region and the *URA3* gene of *S. cerevisiae* and the *ade6* gene of *S. pombe* have been deduced from detailed examination of (additional) unselected markers in the region; this approach allows the analysis of co-conversion of markers and an estimation of the size of the region involved. (Nicolas *et al.* 1989; Schultes and Szostak 1990; Judd and Petes 1988; Grimm *et al.* 1994). General observations are that co-conversion tracts are usually not interrupted by non-converted markers and that co-conversion of markers on both sides of the initiation site is frequently observed (reviewed in Nicolas and Petes 1994; Grimm *et al.* 1994). The lengths of individual conversion tracts have been measured as the distance between markers known to have been co-converted (minimum tract length), the distance between the markers adjacent to a conversion tract (maximum tract length), or the average of the minimum and maximum lengths per tract (average tract length). For *S. cerevisiae*, mean minimum tract lengths vary between 0.4 kb and 1.6 kb, while mean average tract lengths vary between 1.5 kb and 3.7 kb, depending on the locus under observation (Petes *et al.* 1991). For the *ade*6 gene of *S. pombe*, the mean minimal tract length is 0.7 kb (Grimm *et al.* 1994).

The purpose of the experiments presented in this chapter was to assess in which respects the observations on meiotic conversion tracts in the yeasts *S. cerevisiae* and *S. pombe* could be extended to other eukaryotes. We chose to analyze this in the filamentous fungus *A. nidulans*, which we have adopted to study recombination. *A.*

nidulans is a homothallic ascomycete with specialized structures for meiosis; asci with eight sexual spores are produced in cleistothecia (Elliot 1958). We have analyzed the transmission through meiosis of a series of silent mutations, introduced into the *nii*A-*nia*D region, in a two-point cross system. The *nii*A and *nia*D genes encode nitrite and nitrate reductase respectively and are divergently transcribed from a promoter region located between the two genes (Figure 5, page 16). All strains used in crosses were unable to grow on nitrate as a consequence of a nonsense or a missense mutation (hereafter collectively referred to as stop mutation) in the *nii*A or the *nia*D gene; recombinants were selected on nitrate as the sole nitrogen source. On the basis of the position of the conversion tracts observed, we concluded that an initiation site for meiotic recombination is located in this intergenic promoter region; the initiation site remained functional when moved to an ectopic location in the genome. The initiation site functioned bi-directionally, as co-conversion of silent mutations on both sides of the initiation site were frequently observed.

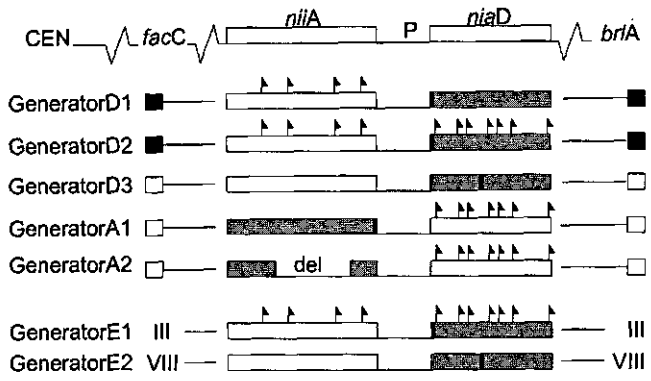
RESULTS

Experimental system

We have developed a system for the molecular analysis of recombinants in the *nii*A-*nia*D region by introducing a series of silent point mutations in a set of *A. nidulans* strains (Figure 7 and, for details on construction, Figure 16 on page 69). This approach was pioneered by Nicolas *et al.* (1989) for the analysis of *ARG4* locus of *S. cerevisiae*. All strains carried a stop (nonsense or missense) mutation in the *nii*A gene or the *nia*D gene to allow the selection of recombinants on nitrate as the sole nitrogen source in two-point crosses. The presence or absence of individual silent mutations (heterozygous in all crosses) was determined by PCR or Southern blotting analysis. Previous experiments (chapter 2) indicated the presence of an initiation site for meiotic recombination in the intergenic promoter of the *nii*A-*nia*D region, and our objective was to confirm this by the analysis of both intragenic (*nia*D/*nia*D) and intergenic (*nii*A-*nia*D) crosses. Also, we examined the influence of local non-homology on recombination and we compared allelic with ectopic recombination in the *nii*A-*nia*D region. For ectopic recombination, we used a strain which carried the *nii*A-*nia*D region on chromosome III instead of chromosome VIII; a strain carrying a 2 kb deletion in the *nii*A gene was used to examine the influence of local non-homology on recombination.

The nitrogen source during crosses was glutamate, a combination of glutamate and nitrate or urea. Expression of the *nii*A and *nia*D gene products is influenced by the nitrogen source(s) present: glutamate is non-inducing and non-repressing, the addition of nitrate induces expression (Cove 1979; Tomsett and Cove 1979; Punt *et al.* 1991). In previous experiments (chapter 2), urea was used as the nitrogen sources during crosses. Although urea, like glutamate, does not induce or repress expression of the *nii*A and *nia*D gene products (Cove 1979), the binding of transcription factors may depend on the specific nitrogen source and may influence the frequency of recombination (White *et al.* 1991; White *et al.* 1993). We therefore performed some crosses both on glutamate (with and without the addition of nitrate) and on urea to allow comparison of the data with those obtained previously.

Figure 7. Relevant details of the *A. nidulans* strains used in crosses



The top drawing shows chromosome VIII, including the centromere (CEN), coding regions of the *nii*A and *nia*D genes (rectangles), the intergenic promoter region (P), and the flanking markers *fac*C and *brl*A. Functional genes are white, non-functional genes are gray. A black square represents the presence of the mutant allele of the corresponding outside marker, a white square the wild type allele; for strains GeneratorE1 and GeneratorE2, the chromosome number is given. Flags indicate the position of the introduced silent point mutations, black rectangles the stop mutations. Construction of the strains is diagrammed in Figure 16 (page 69), except for GeneratorE2, which was selected among the progeny of a cross between WG443 and WG466 (Table 5, page 64).

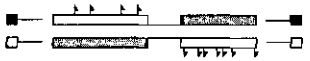
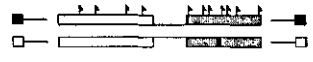
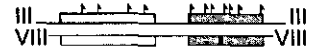
Frequency of recombination

For each cross, the frequency of recombination was estimated for at least three independent batches of ascospores (Table 4). The frequency of allelic recombination was in the range observed previously (chapter 2) and similar ($P = 0.96$) for crosses on different nitrogen sources (crosses I and III). Table 4 shows that the absence of homology outside the *nii*A-*nia*D region (ectopic recombination, cross IV) reduced the frequency of recombination (crosses III *vs.* IV, $P < 0.01$). However, the frequency of ectopic recombination was underestimated slightly, because of inviability of a specific class of recombinants (discussed below). Also, a 2 kb deletion in strain GeneratorA2 significantly ($P < 0.05$) elevated the recombination frequency (crosses I and II).

Genetic analysis

The recombination frequencies between the *nii*A-*nia*D region and the proximal flanking marker *fac*C and the distal flanking marker *brl*A are 26% and 10% respectively (Clutterbuck 1993). The percentage of the recombinant progeny colonies carrying each of the 4 possible combinations of the flanking markers (P1, P2, R1 and R2 in Table 4) was determined for all allelic recombinants isolated (crosses I, II and III). No heterogeneity was detected among the distributions of the flanking marker combinations between crosses performed on different nitrogen sources (Table 4).

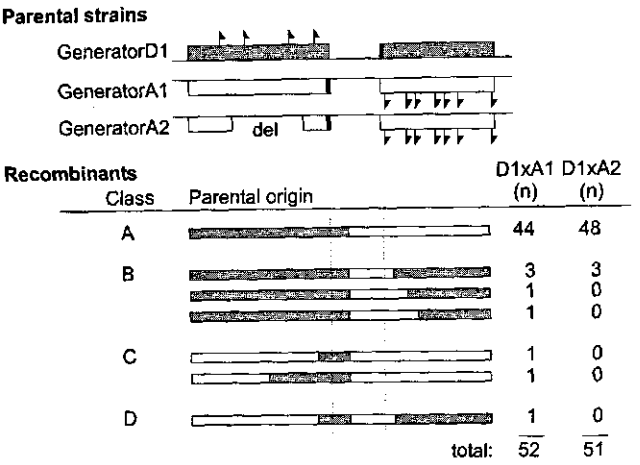
Table 4. Genetic analysis of recombinants.

Parental genotypes[a]	nitrogen source	frequency[b]	# scored (100%)	Flanking marker combinations[c] P1	P2	R1	R2	heterogenity[d]
I) GeneratorD1 x GeneratorA1	glutamate	142 ± 77 (4)	119	11	41	43	5	
	glutamate/nitrate	161 ± 20 (4)	161	14	36	45	5	n.s. (p = 0.42)
	urea	187 ± 73 (3)	188	12	30	50	8	
	total:	158 ± 55 (11)	468					
II) GeneratorD1 x GeneratorA2	glutamate	357 ± 152 (4)	184	9	34	50	7	n.s. (p = 0.74)
	glutamate/nitrate	350 ± 33 (3)	206	9	38	48	5	
	total:	354 ± 110 (7)	390					
III) GeneratorD2 x GeneratorD3	glutamate	81 ± 23 (3)	298	37	9	8	46	
	glutamate/nitrate	82 ± 17 (3)	282	35	6	7	51	n.s (p = 0.20)
	urea	95 ± 10 (3)	226	31	13	6	50	
	total:	87 ± 14 (9)	806					
IV) GeneratorE1 x GeneratorE2	glutamate	1.5 ± 0.8 (3)	168					
								

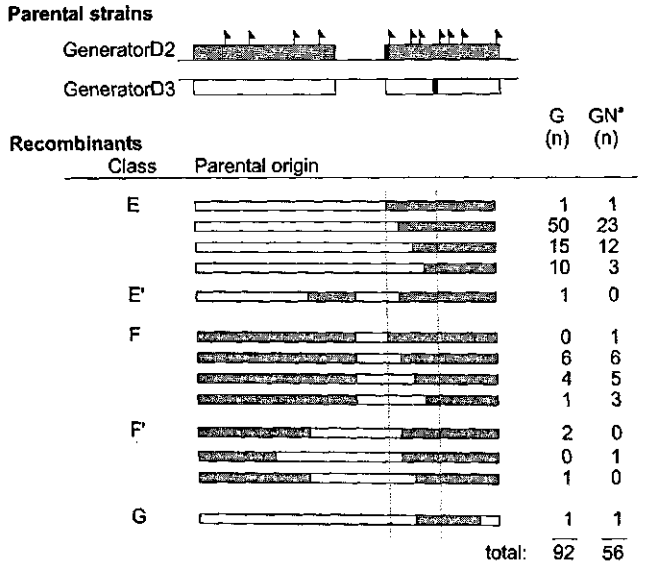
[a] The constitutions of the parental strains are given in Figure 7.
[b] the frequency of recombination is denoted per 10^5 viable ascospores with the standard deviation; the number of observations is given between brackets.
[c] The four combinations of outside markers (parental: P1, P2; recombinant: R1, R2) are given as the percentage of the recombinants analyzed per cross (# scored): P1, *facC brlA*; P2, *facC*$^+$ *brlA*$^+$; R1, *facC brlA*$^+$; R2, *facC*$^+$ *brlA*.
[d] The distributions of the flanking marker combinations (expressed in numbers) of crosses with the same parental genotype on different media were tested for heterogeneity in a 2x4 or 3x4 contingency table by a chi square test; n.s., no significant heterogeneity.

Figure 8. Molecular analysis of allelic recombinants

A. Intergenic recombinants



B. Intragenic recombinants



The configurations of the *nii*A and *nia*D genes of the parental strains are drawn at the top: the parental origin of the recombinants is color-coded (white and gray). The number of recombinants with a specific marker combination is denoted for each cross (n). Dotted lines mark the position of the stop mutations in the parental strains.

The relative frequencies of the flanking marker combinations were in full agreement with our previous results (chapter 2) as I) the major and minor crossover classes (R1 or R2) were as expected on the basis of positions of the stop mutations in the parental strains; and II) in intragenic crosses, the P1 class consistently exceeded the P2 class, which is in agreement with a 5' to 3' polarity of conversion in the *nia*D gene. The nitrogen source used nor the presence of the deletion strongly affected the distribution of the flanking marker combinations (Table 4).

Molecular analysis of allelic intergenic recombinants

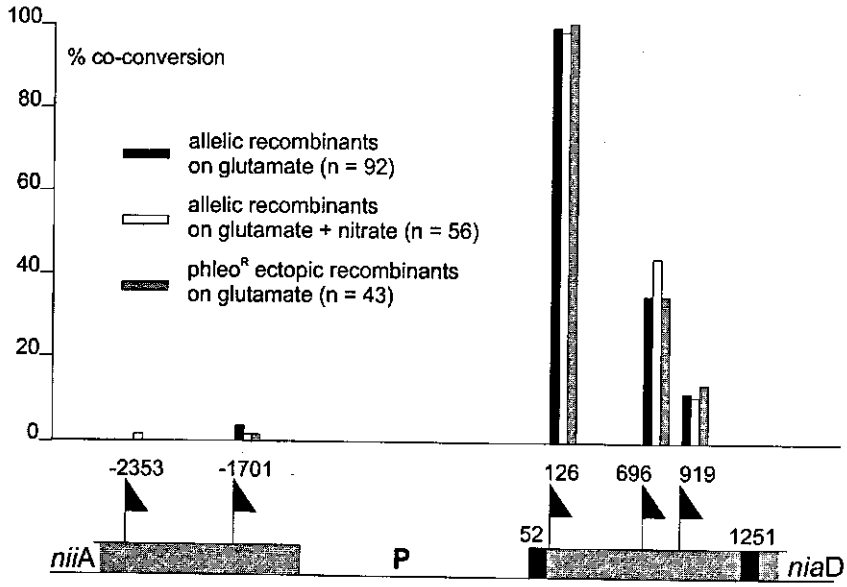
The presence or absence of individual silent mutations in the *nii*A-*nia*D region was determined for about 50 recombinants of each of the intergenic crosses on glutamate (Figure 8A). Most of the recombinants showed a pattern of silent mutations consistent with a crossover between the stop mutations (class A), irrespective of the presence or absence of the 2 kb deletion in the *nii*A gene in one of the parents. This supports the idea that the initiation site (or region) of the recombination events detected in our assay is located in the intergenic promoter region of the *nii*A and *nia*D genes. Minor classes of recombinants showed patterns consistent with conversion events, with conversions tracts extending into the *nia*D gene (class B), the *nii*A gene (class C) or both (class D). Conversion of silent mutations in the *nii*A gene was somewhat rarer than those in the *nia*D gene; in the cross involving the deletion in the *nii*A gene, recombinants showing conversion of *nii*A silent mutations were not observed.

Molecular analysis of allelic intragenic recombinants

Similarly, allelic intragenic recombinants derived from crosses on glutamate and on glutamate plus nitrate were analyzed (Figure 8B). The highest number of recombinants fell into class E. We have interpreted the event leading to recombinants of this class as conversions which include the 5' stop mutation, accompanied by a crossover; class F recombinants were interpreted as similar to those in class E but without a crossover (see Discussion). Conversion tracts that include silent mutations in the *nii*A gene were found at a low frequency (class E' and F'). Recombinants in class G showed a pattern suggestive of a conversion that included the 3' stop mutation, without an associated crossover.

Conversion of a stop mutation was usually accompanied by co-conversion of adjacent silent mutations and these co-conversion tracts were never interrupted by a non-converted site. Conversion of the 5' stop mutation (classes E, E', F and F') never included silent mutations 3' of the second stop mutation. The frequency of co-conversion of individual silent mutations with the 5' stop mutation displayed a steep bi-directional gradient (Figure 9). This suggests the presence of a recombination initiation site in the proximity of the 5' stop mutation.

Figure 9. Co-conversion



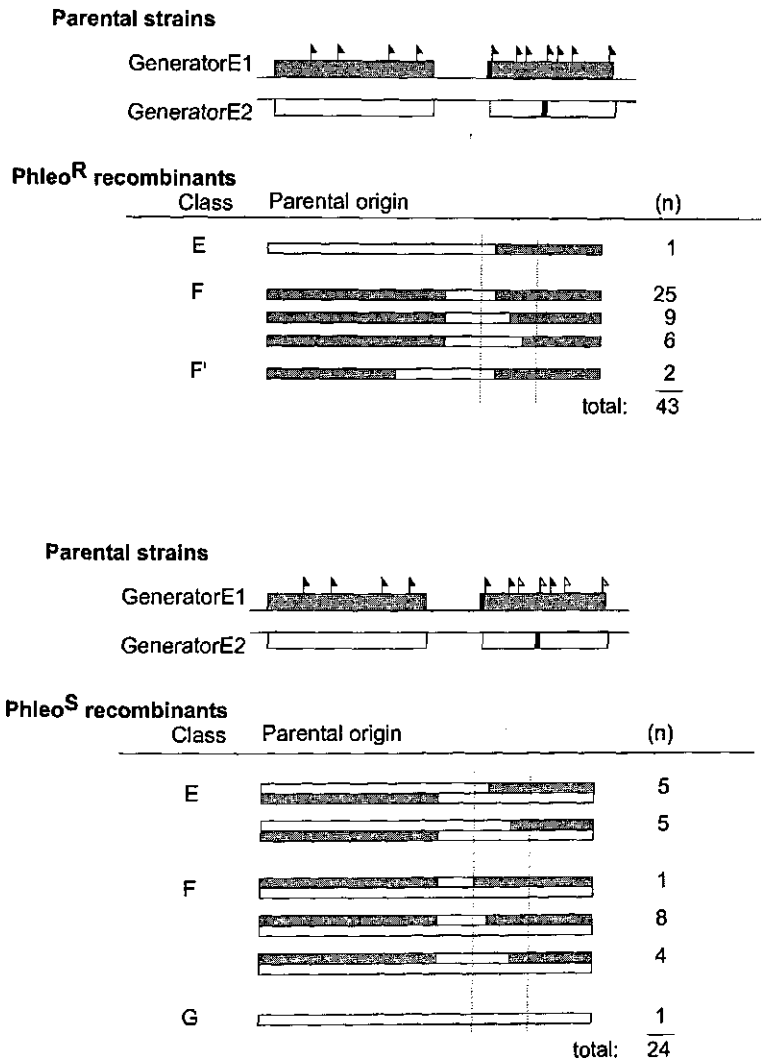
The percentage co-conversion of individual markers with the 5' stop mutation in intragenic crosses is given (see Figure 8). At the bottom the position (in base pairs) of the relevant silent and stop mutations (flags and black rectangles, respectively) is denoted relative to the ATG codon of the *nia*D gene. P indicates the position of the promoter region.

Molecular analysis of ectopic intragenic recombinants

We have examined ectopic meiotic recombination between the copy of the *nii*A-*nia*D region at its original position on chromosome VIII (strain GeneratorE2) and an ectopic copy at the *arg*B locus on chromosome III (strain GeneratorE1). Chromosome III and VIII constitutions of the parental strains are depicted at the top of Figure 10. Recombination is followed by (assumed random) segregation of chromosomes; consequently, recombinant progeny colonies may carry any combination of chromosomes III and VIII that includes at least one functional copy of the *nii*A-*nia*D region. A crossover associated with the recombination event will lead to a reciprocal translocation. In this case, the progeny colony must harbor both interacting copies of the *nii*A-*nia*D region to retain both chromosome arms.

Chromosome VIII of GeneratorE1 could be identified genetically in recombinants by the phleomycin resistance cassette (prc). Of the 168 recombinants isolated, 39% was resistant to phleomycin (phleo^R). The likely constitution of these recombinants is the, now functional, copy of the *nii*A and *nia*D genes on chromosome III and the phleomycin resistance cassette on chromosome VIII, both derived from GeneratorE1 (Figure 11A).

Figure 10. Molecular analysis of ectopic recombinants



The configurations of the *nii*A and *nia*D genes of the parental strains are drawn at the top: the parental origin of the recombinants is color-coded (white and gray). The number of recombinants with a specific marker combination is denoted for each cross (n). Dotted lines mark the position of the stop mutations in the parental strains. Markers not analyzed are indicated with white flags (in the case of ectopic intragenic phleo^S recombinants).

Molecular analysis of 43 of these recombinants showed that all harbored a single copy of the *nii*A-*nia*D region and all but one of the recombination events were the result of conversion of the 5' stop mutation with co-conversion of up to 3 adjacent silent mutations and without an associated crossover (Figure 10). The exception (class E) showed an apparent co-conversion of the entire *nii*A gene. Alternatively, this pattern can be interpreted as the result of an associated crossover, analogous to allelic class E recombinants (Figure 8A and B); to account for the phleomycin resistance of the recombinant, a second crossover at the 3' end of the *nii*A gene or a complex genomic rearrangement has to be assumed. Ectopic recombination displays a gradient of co-conversion with the 5' stop mutation similar to allelic intragenic recombination (Figure 9).

The analysis of the phleomycin sensitive (phleo^S) recombinants (Figure 10) was somewhat obstructed by the possibility of two copies of the *nii*A-*nia*D region in a single colony. Although theoretically feasible, the standard method of PCR-analysis failed to reveal interpretable restriction fragment patterns in those phleo^S recombinants that contained two copies of the *nii*A-*nia*D region. This was partly circumvented by the use of Southern analysis. To ascertain that the gradient of conversion was not limited to the specific (phleo^R) class of recombinants analyzed, 24 phleo^S recombinants were subjected to Southern analysis to analyze the *Nsp*V polymorphisms in the *nia*D gene by which the parental strains differed. One of the recombinants showed a single pattern, identical to the parental GeneratorE2 strain (Figure 10, class G). The *nii*A gene of this recombinant was fully analyzed by the standard method and showed patterns identical to those of GeneratorE1, which suggested that this was either a revertant or the result of a conversion of the 3' stop mutation. The other 23 recombinants showed double patterns, consistent with conversion of the 5' stop mutation, with or without co-conversion of other silent mutations (Figure 8C, classes E and F).

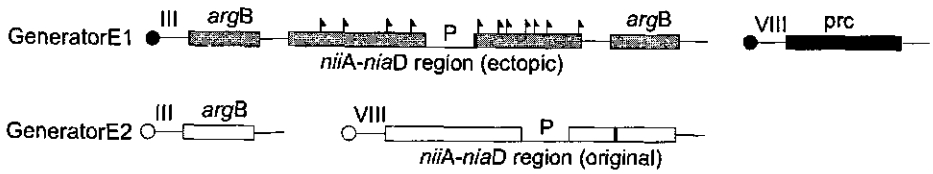
Legend to Figure 11 ⇒

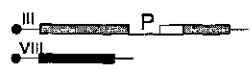
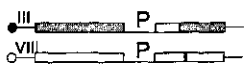
Panel A. At the top, chromosomes III and VIII of the parental strains are drawn. The copy of the *nii*A-*nia*D region is at an ectopic position on chromosome III in GeneratorE1 and at the original position on chromosome VIII in GeneratorE2. Circles represent the centromeres, roman numerals indicate the chromosome numbers. The position of *arg*B (duplicated in GeneratorE1) and the phleomycin resistance cassette is shown (prc, black rectangle). Recombinants were selected on nitrate, tested for phleomycin resistance and analyzed molecularly (Procedure and observations). Schematic diagrams of the deduced chromosome constitutions show only the relevant details (centromeres are color-coded). An interpretation of the recombination event is given (the origin of the chromosomes is abbreviated: III-E1, chromosome III of Generator E1 etc.). The estimated percentage of the recombinants with a specific constitution is denoted (%): the total number of recombinants was corrected for the loss of 43 crossover recombinants (due to segregation of a crossover and a non-crossover chromosome, see text) to 211 and the percentages of recombinants in a specific class were based on this number.

Panel B. The distinction of crossovers and non-crossovers was made by Southern analysis of *Stu*I digested genomic DNA. The approximate lengths of the fragments detected is indicated.

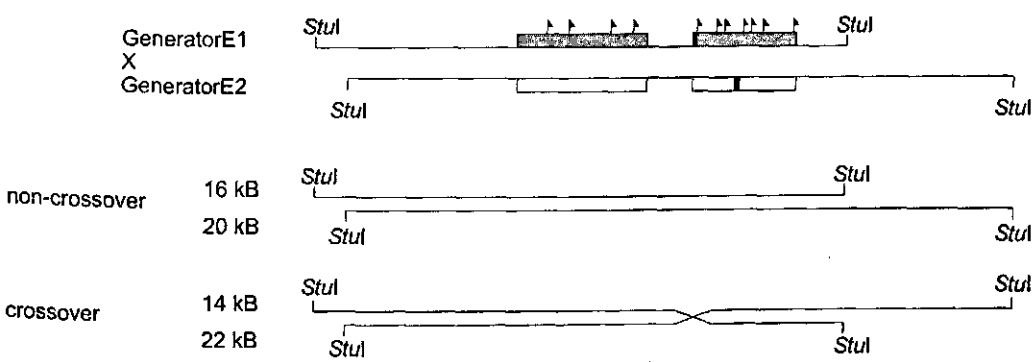
Figure 11. Ectopic recombination

A. Analysis of ectopic recombinants



Procedure and Observations	Deduced chromosome III and VIII constitution (schematic)	Interpretation	%
Selection of recombinants on nitrate (n = 168)			
Phleomycin resistent (66 of 168 recombinants)		Conversion III-E1 segregation with VIII-E1	31%
Phleomycin sensitive (102 of 168 recombinants)			
1 copy of the *nii*A-*nia*D region (1 of 24 recombinants)		Conversion VIII-E2 segregation with III-E2 (or revertant)	2%
2 copies of the *nii*A-*nia*D region (23 of 24 recombinants)			
Crossover (10 of 23 recombinants)		Conversion III-E1 with associated crossover	41%
Non-crossover (13 of 23 recombinants)		Conversion III-E1 segregation with VIII-E2	26%

B. Detection of crossovers



Crossovers were detected by Southern analysis (Figure 11B) in 10 of the 23 recombinants with double patterns (Figure 10, class E). As a crossover leads to a reciprocal translocation, only those crossover recombinants that inherit both interacting chromosomes will be viable, whereas all non-crossover recombinants will be viable. Consequently, crossovers will be underrepresented in the set of recombinants analyzed. To estimate the percentage of all recombination events that were accompanied by a crossover, we have assumed that the number of crossover events that lead to a non-viable recombinant equals the number of crossover recombinants detected (random segregation of the chromosomes); the other types of events were corrected accordingly. The results are summarized in Figure 11A.

DISCUSSION

A recombination initiation site in *A. nidulans*

In this chapter, we describe a detailed molecular analysis of meiotic recombination events at the *nii*A-*nia*D region of *A. nidulans*. Our major observations on recombinants derived from allelic crosses were the following: I) in intergenic crosses, apparently uncomplicated crossovers confined to the promoter region were the prominent class of recombinants; when observed, conversion tracts extended from the promoter region, and II) in intragenic crosses, a bi-directional gradient of co-conversion is observed with the peak located in the proximity of the 5' stop mutation. These observations are in agreement with the presence of an initiation site of meiotic recombination in the promoter region of the *nii*A and *nia*D genes.

The intergenic promoter region of the *nii*A-*nia*D region is the first recombination hotspot that has been defined at the molecular level in *A. nidulans*. Our results thus extend the role of transcriptional promoters in the initiation of meiotic recombination from the yeasts *S. cerevisiae* and *S. pombe* (Sun *et al.* 1989; Cao *et al.* 1990; Zenvirth *et al.* 1992; Game 1992; Goldway *et al.* 1993; Wu and Lichten 1994; Grimm *et al.* 1994; Fan *et al.* 1995; de Massy *et al.* 1995) to a filamentous fungus. Polarity of meiotic conversion has been observed in a number of fungi (reviewed in Whitehouse 1982, and in Lichten and Goldman 1995), consistent with the presence of recombination hotspots in the regions flanking the loci examined.

Frequency of recombination

Recombinants among the progeny of allelic crosses were detected at frequencies within the range observed previously for the *nii*A-*nia*D region (chapter 2). Ectopic recombinants were detected at a significantly lower frequency than allelic recombinants (Table 4); taking into account the crossovers that lead to non-viable progeny, the reduction was 40-fold. This suggests that an homologous chromosomal context is required for normal levels of recombination, but that the homology required for recombination does not exceed the size of the *nii*A-*nia*D region (8.4 kb). Similar results were obtained in *S. cerevisiae*, where ectopic recombination frequencies are 3 to 17-fold lower than allelic frequencies (reviewed in Petes *et al.* 1991).

The frequency of recombination was, somewhat surprisingly, stimulated 2-fold by the presence of a 2 kb deletion in the *nii*A gene of one of the parents (Table 4). In several

fungi (but not in *S. cerevisiae*), the inclusion of a deletion in a heteroduplex tract leads to the preferential use of the longer strand as the template (reviewed in Nicolas and Petes 1984). This disparity, not investigated in *A. nidulans*, may lead to more nitrate-utilizing progeny colonies in the cross involving the 2 kb deletion. However, frequent inclusion of the deletion in a heteroduplex tract is an assumption not supported by our data as conversion tracts that cover a major part of the *nii*A gene were rare. Alternatively, sensing the presence of the deletion by the cell may limit the heteroduplex tract length and enhance reciprocal exchanges, as observed in *Ascobolus immersus* (Nicolas and Rossignol 1989). This would lead to more class A recombinants (Figure 8) as a consequence of the presence of the 2 kb deletion. As class A recombinants do not require repair of mismatches, this may lead to an elevated frequency of recombination.

Conversion tracts

The detection of aberrant marker segregations in *A. nidulans*, indicating non-reciprocal recombination, is laborious but has been reported once (Strickland 1958a). The analysis of both interacting copies of the *nii*A-*nia*D region as present in some of the ectopic recombinants (Figure 10) confirmed that *A. nidulans* is capable of meiotic non-reciprocal recombination. Both heteroduplex formation (and subsequent repair of mismatches in the heteroduplex tract) and gap repair (
Figure 4) may result in the patterns found in the recombinants.

Bi-directional conversion tracts, covering markers on both sides of the initiation site, are frequently detected in the *DED81-ARG4* region of *S. cerevisiae* (Schultes and Szostak 1990) and the *ade6* gene of *S. pombe* (Grimm *et al.* 1994). In our study, conversion tracts covering markers on both sides of the initiation site in the *nii*A-*nia*D region were detected among allelic and ectopic recombinants (Figure 8 and Figure 10, classes D, E' and F'). Of the allelic intragenic recombinants without an associated crossover (classes F and F'), an average of 13% contain a bi-directional conversion tract (class F'). Assuming a similar ratio among class E and E' recombinants, where conversion tracts extending into the *nii*A gene are obscured, this suggests that bi-directional conversion tracts are a common intermediate in recombination in *A. nidulans*. Our results are therefore consistent with a model for recombination as depicted in
Figure 4, in which two Holliday junctions are formed that may move in opposite directions from the point of initiation.

Co-conversion

In intragenic crosses, the majority of the recombinants showed conversion of the 5' stop mutation, while conversion of the 3' stop mutation was rare (Figure 8B). This preferential conversion of one of the two stop mutations has been observed for many loci and is usually interpreted as the consequence of conversion tracts of variable length extending from a fixed initiation site (reviewed in Orr-Weaver and Szostak 1985). In agreement with this model, conversion of the 5' stop mutation was accompanied by co-conversion of up to three silent mutations, showing a steep 5' to 3' gradient of co-conversion (Figure 9). Co-converted markers were not interrupted by a non-converted marker, with the possible exception the class E' recombinant, in which silent mutation A1 appears to be not converted. Interestingly, this is the only silent mutation that leads to a C·C or G·G mismatch in a heteroduplex. Such a mismatch is poorly repaired in other organisms

(reviewed in Petes *et al.* 1991). Alternatively, the pattern of silent mutations in the class E' recombinant may be explained in the context of the model depicted in
Figure 4 as gap repair of A1 and mismatch repair of all other silent mutations involved. Conversion tracts covering both the 5' and the 3' stop mutations were not detected. This suggests that only one of the chromatids is used as a template in a recombination event and that consequently conversion tracts covering both stop mutations remain undetected in a two-point cross selection system. The prevalence of uninterrupted conversion tracts supports this notion. Conversion tracts in intragenic ectopic recombinants resembled those observed in allelic recombinants with respect to length and position.

In intergenic crosses, most conversion tracts were confined to the intergenic promoter region; this was not influenced by the presence of a heterologous 2 kb deletion in the *nii*A gene. Minor classes of recombinants show patterns consistent with conversion of markers in the *nii*A gene or the *nia*D gene or, in one case, both (Figure 8A). The latter recombinant (class D) is exceptional as both stop mutations are included in the conversion tract. Analogous to the class E' recombinant discussed above, gap repair of the A0 and A1 mutations in the class D recombinant may account for the pattern observed.

Conversion tract length

The length of individual conversion tracts has been measured as the distance between the markers known to have been co-converted (minimum tract length), the distance between the markers adjacent to a conversion tract (maximum tract length), or by averaging the minimum and maximum lengths per tract (average tract length). For *S. cerevisiae*, mean minimum tract lengths vary between 0.4 kb and 1.6 kb, while mean average tract lengths vary between 1.5 kb and 3.7 kb, depending on the locus under observation (Petes *et al.* 1991). For the *ade*6 gene of *S. pombe*, the mean minimal tract length is 0.7 kb (Grimm *et al.* 1994).

We have used the data sets on intragenic crosses (Figure 8B and Figure 10) to calculate these values for the *nii*A-*nia*D region; the data on intergenic crosses were excluded as the majority of these recombinants do not include a marker. All conversion tracts were confined to the *nii*A-*nia*D region with a maximum tract length of 3.7 kb (with the possible exception of one ectopic recombinant (Figure 10, class E)). The mean minimum tract length was 0.4 kb and the mean average tract length was 1.5 kb for both allelic and ectopic crosses. Thus, the length of conversion tracts appear to be similar in *S. cerevisiae*, *S. pombe*, and *A. nidulans*. However, the mean tract lengths observed must be influenced by the positions of the stop mutations in a cross. In intragenic crosses involving stop mutations which were both located downstream of the 3' stop mutation in the current system, recombinants (with long conversion tracts) were detected at appreciable frequencies (chapter 2).

Associated crossovers

Approximately half of the recombinants analyzed genetically harbored a non-parental set of outside markers, in agreement with earlier results (chapter 2). However, in intergenic crosses, an average of 90% of the recombinants showed a pattern consistent with a crossover in the intergenic region and in intragenic crosses, 84% of the recombinants were the result of a conversion event associated with a crossover. The discrepancy between the genetic and molecular data is presumably the consequence of secondary crossovers in the genetic intervals between the *nii*A-*nia*D region and the flanking markers.

High frequencies of associated crossovers have been observed for many loci in *A. nidulans* and other fungi (reviewed in Whitehouse 1982).

Among ectopic recombinants the percentage crossovers was estimated at 41% of the recombinants (Figure 11), suggesting that resolution of a recombination intermediate as a crossover is to some extent dependent on a homologous chromosomal context. No reduction of associated crossovers is observed among ectopic recombinants in *S. cerevisiae* (Jinks-Roberson and Petes 1985; Lichten *et al.* 1987; Lichten and Haber 1989).

Crossover interference

We have analyzed whether the selected recombination events in the *nii*A-*nia*D region affected the recombination frequency between this region and the proximal flanking marker *fac*C (interference). This distance is 26% (Clutterbuck 1993) and was 24% in our strains (78 colonies tested). Of 251 non-ectopic recombinants fully analyzed, 209 (83%) contained an exchange of the outmost markers (crossover) in the *nii*A-*nia*D region. Irrespective of the event (crossover or non-crossover), 36% of the recombinants contained a crossover between the *nii*A-*nia*D locus and the proximal *fac*C marker. This constitutes a 1.4-fold map expansion (or negative interference) as the consequence of the selection for recombinants in the *nii*A-*nia*D locus. In many organisms, including *S. cerevisiae*, positive interference (a crossover decreases the likelihood of a second crossover in an adjacent interval) is observed (Whitehouse 1982; Petes *et al.* 1991). In *A. nidulans* and *S. pombe*, interference is reported absent (Strickland 1958b; Snow 1979), but Calef (1957) reported increased map distances (up to 4-fold) for intervals adjacent to the *acr*F locus of *A. nidulans*, when selecting for intragenic recombinants. Interestingly, *A. nidulans* and *S. pombe* appear to be exceptional as no structures resembling SCs are observed (Egel-Mitani *et al.* 1982; Olson *et al.* 1978). SC formation appears to be required for proper disjunction and for crossover interference in *S. cerevisiae*; it is not a prerequisite for recombination (Sym and Roeder 1994). Thus, the observation of negative interference in *A. nidulans* supports a role of the SC in crossover interference.

4. A COMMENT ON MEIOTIC RECOMBINATION IN MAIZE

Hans Thijs
Christa Heyting

This chapter has been submitted as a letter to the editor of The Plant Cell in response to: Dooner HK, Martínez-Férez IM (1997) Recombination occurs uniformly within the *bronze* gene, a meiotic recombination hotspot in the maize genome. The Plant Cell 9, pp. 1633-1646

Meiotic recombination hotspots in plants are of considerable interest because their occurrence has important implications for the mechanism of meiotic recombination and the development of plant breeding strategies. We therefore enjoyed reading the detailed investigations of recombination events in the *bronze* locus of maize by Dooner and Martínez-Férez (1997). Although these authors conclude that recombination occurs uniformly within the *bronze* gene, we would like to draw attention to the clear evidence in their data for a 5' to 3' polarity of meiotic conversion in this locus.

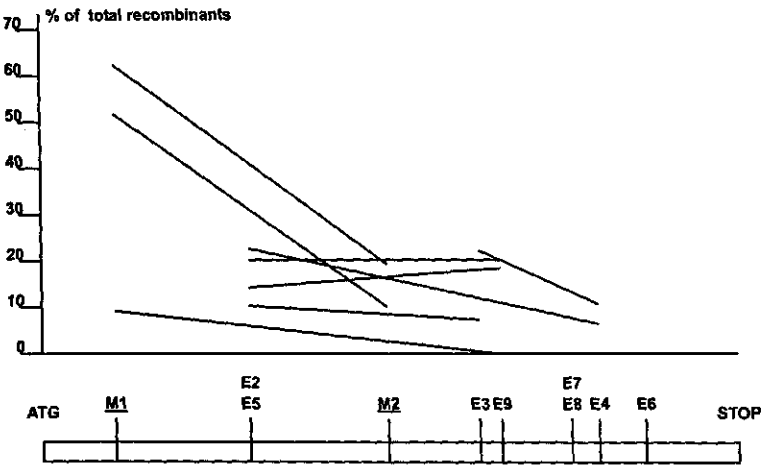
Dooner and Martínez-Férez interpret their data in the context of the double-strand DNA-break (DSB) model for the initiation of meiotic recombination, and for convenience we will also keep to this model in this letter, although as yet no meiotic DSBs have been demonstrated in other organisms than budding yeast (reviewed in Lichten and Goldman 1995). In a large number of intragenic recombinants (IGRs) in the *bronze* locus, Dooner and Martínez-Férez mapped the recombination junctions, which in the context of the DSB-model can be interpreted as sites where a Holiday junction was resolved, or the initiating DSB occurred (see their Figure 8). Because the authors did not observe a gradient in the frequency of recombination (recombination junctions) in the *bronze* locus, they concluded that there was no polarity of recombination in this locus, and thus no preferred sites for the initiation of meiotic recombination.

However, polarity is usually not detected as gradients in the frequency of recombination (or of recombination junctions), but as gradients of gene conversions. A sensitive method of detection of polarity is the comparison of the relative frequency of conversion of the two involved markers in heteroallelic two-point crosses (reviewed in Whitehouse 1982). The data collected by Dooner and Martínez-Férez allow such a

comparison, and we show the results in Figure 12. In all except two marker pairs, the most 5' marker is converted more frequently than the 3' marker. This represents clear evidence for polarity of gene conversion in the *bronze* locus, and suggests the existence of (a) preferred site(s) for the initiation of meiotic recombination 5' to the *bronze* gene. Both exceptional marker pairs include mutation E9. It is possible that a marker-specific effect, for instance the efficiency by which E9 is repaired in heteroduplexes, accounts for the apparent reversal of polarity of gene conversion in these two marker pairs.

The polarity of gene conversion is also evident if the co-conversion patterns are considered. With a single exception, all recombination junctions detected by Dooner and Martínez-Férez are located between the E and M mutations used in a cross, which is in agreement with conversion of the 5' E or M mutation, co-conversion of adjacent mutations and crossover resolution. Although a clear gradient of recombination junctions in the region between the mutations is indeed debatable, the polarity of co-conversion is not: mutations upstream to the 5' E or M mutation are (almost) invariably co-converted, mutations between the two E or M mutations sometimes are, and mutations downstream of the 3' E or M mutation are never co-converted. This is a strong indication for a 5' to 3' polarity of gene conversion in the *bronze* gene.

Figure 12. Polarity of meiotic conversion in the *bronze* locus



The x-axis shows the physical locations of the E and M mutations. One line represents the result from a cross between strains carrying the mutations corresponding to the end points of the line. The percentage conversions of the two mutations among the recombinants (the P1 and P2 categories) is given on the y-axis.

5. ANALYSIS OF MITOTIC RECOMBINANTS

Hans Thijs
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The data presented in this chapter will be submitted for publication as: Molecular analysis of spontaneous mitotic recombination in the *nii*A-*nia*D region of *Aspergillus nidulans* (to be submitted to Molecular and General Genetics)

SUMMARY

We have examined mitotic recombination at the molecular level in the *nii*A-*nia*D locus of *Aspergillus nidulans*, using a diploid strain which was heterozygous for a series of mutations in this region. The diploid was unable to grow on nitrate as a nitrogen source due to the presence of dissimilar, non-functional alleles of the *nia*D gene in the genomes of the two haploid strains from which it was derived; recombinants were selected on nitrate. Eleven unselected polymorphisms (silent point mutations), located in the coding regions of the *nii*A and the *nia*D genes, and flanking markers (*fac*C and *brl*A) were analyzed in haploid derivatives of recombinant diploids. The observed combinations of mutations in the recombinants suggest that the initiation site for meiotic recombination that we found in the *nii*A-*nia*D locus, does not function in mitotic recombination; furthermore, they suggested that long heteroduplex tracts (> 8.4 kb) are formed during mitotic recombination, and that conversion occurs in patches within these tracts. Mitotic recombination differs in these respects from meiotic recombination in *A. nidulans*.

INTRODUCTION

Detailed molecular analysis of mitotic recombination in eukaryotes has so far been largely restricted to the yeast *Saccharomyces cerevisiae*, despite the importance of this process for DNA repair, its possible role in the development of cancer and the production of antibodies (Roeder and Stewart, 1988). Filamentous fungi are phylogenetically much closer to higher eukaryotes than *S. cerevisiae* (Smith, 1989), and may thus provide a powerful model system. We previously monitored a series of silent point mutations in the *nii*A-*nia*D locus of the filamentous fungus *A. nidulans* in a two-point cross system (chapter 3). Here, we report on the analysis of spontaneous mitotic recombination using the same experimental system.

It is reasonable to assume that mitotic recombination, associated with the repair of DNA damage, can occur throughout the cell cycle; this in contrast to meiotic recombination which is strictly post-replicational (reviewed in Roeder and Stewart, 1988). Like meiotic recombination, mitotic recombination includes non-reciprocal exchanges (conversions) and reciprocal exchanges (crossovers), which presumably arise through a heteroduplex intermediate (reviewed in Orr-Weaver and Szostak, 1985). Crossing over in the G1 phase of the cell cycle (i.e., before DNA replication), affects the single chromatids of both homologous chromosomes, so that two recombinant chromosomes will segregate to each pole in the next mitosis. Crossing over in G2 affects only one of the two chromatids of each homolog, so during the next mitosis, the two crossover chromosomes may segregate to either opposite poles (x-segregation) or to the same pole (z-segregation); x-segregation leads to loss of heterozygosity of all markers distal to the crossover, z-segregation does not (Chua and Jinks-Robertson, 1991). In *S. cerevisiae*, mitotic recombination during both G1 and G2 has been observed (Fabre, 1978; Esposito, 1978; Roman and Fabre, 1983; Fabre *et al.* 1984). However, the G2 phase in *S. cerevisiae* is substantially shorter than in *A. nidulans* and in mammalian cells (Bainbridge, 1971; Bergen and Morris, 1983). Therefore, mitotic post-replicational recombination may be more frequent in *A. nidulans* than in *S. cerevisiae* (Osman *et al.* 1993).

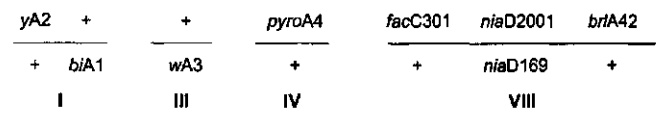
A. nidulans was one of the first experimental systems in which mitotic recombination was examined, with the use of heteroallelic diploids which harbored heterozygous flanking markers (Pontecorvo and Roper, 1953). Genetic analysis of mitotic recombination at the *ad*E and *paba*A loci has identified four major classes of recombinant diploids, interpreted as: I) conversion of the proximal allele, II) conversion of the distal allele, III) both crossover chromosomes, and IV) a crossover chromosome and a parental chromosome; minor classes were detected at appreciable frequencies (Pritchard, 1955; Pritchard, 1960; Putrament, 1964; Zhao and Kafer, 1992).

In two-point crosses, meiotic conversion of alleles at one side of a locus is typically much more frequent than conversion of alleles at the other side (reviewed in Whitehouse, 1982). This polarity of meiotic conversion is usually interpreted to indicate the presence of a preferred initiation site (hotspot) at the high frequency side of the locus; the promoter region of the *nii*A-*nia*D locus contains such a hotspot (chapter 2 and 3). Polarity of mitotic conversion is less pronounced or absent (reviewed in Whitehouse, 1982, and Petes *et al.* 1991), which may be the result of random initiation of recombination events and/or the

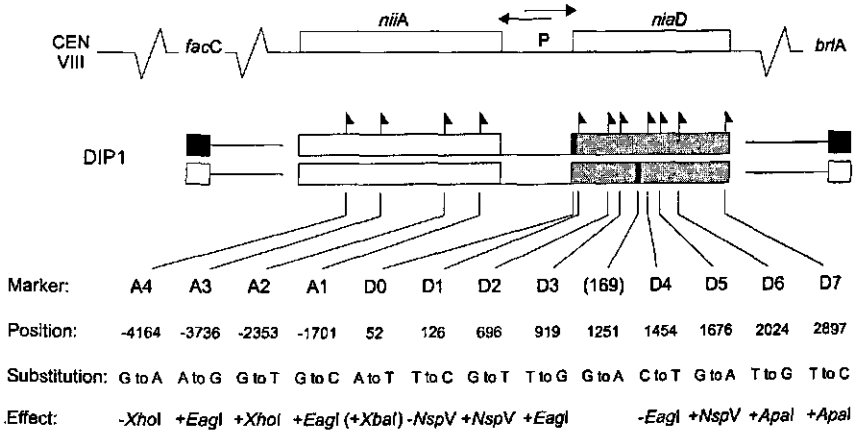
consequence of very long conversion tracts extending from preferred initiation sites (Petes *et al.* 1991).

Figure 13. Constitution of the diploid *A. nidulans* strain DIP1

A. genetic markers



B. Molecular markers



Panel A. The genetic markers of the diploid strain DIP1, obtained from a heterokaryon of the haploid strains GeneratorD2 (top line) and ANGW1126 (bottom line). The chromosome number is denoted in roman numerals. The genetic markers are described by Clutterbuck (1993). The *y*A2 and *w*A3 mutations are recessive and cause a yellow (*y*A2) or a white (*w*A3) conidiospore color; *w*A3 is epistatic over *y*A2. The diploid strain DIP1 produces, like wild-type strains, green conidiospores. The temperature-sensitive *brl*A42 allele (bristle conidiospores) is epistatic over both the *y*A and *w*A markers and causes a bristle conidiospore morphology at 37°C but not at 26°C. The *nia*D2001 allele contains a missense mutation and the *nia*D169 allele contains a nonsense mutation in the nitrate reductase gene.

Panel B. The *nii*A-*nia*D region of *A. nidulans* is drawn to scale. CEN, centromere; P, promoter region. Arrows indicate the direction of transcription. The *fac*C and *brl*A loci, used as flanking markers in crosses, are located 26 map units centromere-proximal and 10 map units centromere-distal to the *nii*A-*nia*D locus, respectively (Clutterbuck, 1986). Below, the constitutions of the two copies of the *nii*A-*nia*D region in DIP1 are drawn. Functional genes are white, non-functional genes are gray, a flag indicates a silent mutation, a black rectangle a stop mutation (i.e. a missense or a nonsense mutation), a gray square indicates the presence of an outside marker, a white square its absence. For each mutation, the position is given relative to the *nia*D ATG codon; the base-substitutions generated (coding strand) and gain or loss of restriction sites (+ or -) are indicated. Restriction sites not used in the analysis of recombinants are between brackets.

In this chapter, we describe the analysis of mitotic recombination in a diploid strain of *A. nidulans*, which harbored a missense and a nonsense mutation (hereafter collectively referred to as stop mutations) *in trans* in the *nia*D gene, a series of silent mutations in the *nii*A-*nia*D region in one of the chromosomes and heterozygous flanking markers. Recombinants were selected by their ability to utilize nitrate as a nitrogen source (i.e., the presence of a functional *nia*D gene). Genetic analysis of the recombinants revealed the same four major classes that have been found among recombinants in the *ad*E and *paba*A loci. However, the patterns of silent mutations showed that many recombination events were more complex than could be suspected from genetic analysis. Our results suggest that long heteroduplex tracts have been formed during mitotic recombination, that conversion occurred in short patches in these tracts, and that in some of the recombinants both DNA strands within a heteroduplex tract had served as a template for conversion. Mitotic recombination differs in these respects from meiotic recombination in *A. nidulans*. Also, the hotspot located in the intergenic promoter region of the *nii*A and *nia*D genes does not function in mitotic recombination.

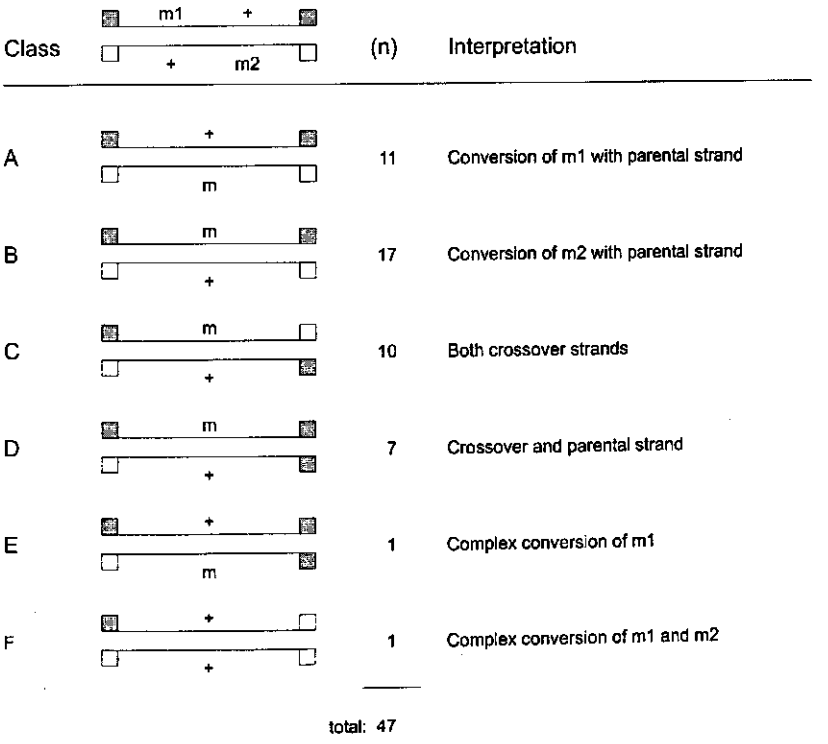
RESULTS

Genetic analysis

For the analysis of mitotic recombination, we isolated the green-spored diploid strain DIP1, derived from a white-spored (*w*A) and a yellow-spored (*y*A) haploid parental strain (Figure 13A). The recessive spore-color mutations of the parental strains facilitated the identification of haploid sectors after haploidization. The recessive temperature-sensitive *brl*A42 allele (bristle conidiospores), used as the centromere-distal flanking marker, is epistatic over both the *y*A and *w*A markers. A total of 93 diploid recombinants were isolated from selective plates inoculated with DIP1. After growth at 37°C, the majority of the recombinants was green, 22% produced bristle conidiospores (e.g., were homozygous *brl*A), and none were yellow or white; all recombinants were green when grown at 26°C, the permissive temperature of the *brl*A42 allele.

Haploid derivatives of 47 of the diploid recombinants were isolated and purified. The recombinants were classified according to functionality of the *nia*D gene and the presence or absence of the flanking markers *fac*C and *brl*A in the haploid derivatives (Figure 14). For most diploid recombinants, two chromosome VIII genotypes were detected among the haploid derivatives and these were taken to represent the two copies of chromosome VIII of the diploid recombinant. From eight recombinants, we obtained more than two genotypes. Repurification of these recombinants and subsequent haploidization led to the identification of two genotypes among the haploid derivatives in all cases. We have assumed that initial impurity of these recombinant colonies was the cause of the multiple genotypes observed among the haploid derivatives and have included these recombinants in the analysis.

Figure 14. Genetic analysis of mitotic recombinants



Classification of the recombinants was based on the status of the *nia*D gene and the presence or absence of the *fac*C and *brl*A outside markers in haploid derivatives. The constitution of the *nii*A-*nia*D region and outside markers on the two copies of chromosome VIII of the parental diploid are depicted at the top: gray squares represent the presence of the outside markers, white squares their absence, m1 and m2 represent the stop mutations (*nia*D2001 and *nia*D169, respectively). Below, the genetic constitutions of recombinant diploids are shown. Six classes (A to F) can be distinguished, each with a characteristic set of two chromosome VIII genotypes. Growth on nitrate of a haploid derivative is denoted with a +-sign (the selected chromosome of the recombinant); no growth on nitrate and thus the presence of either or both of the mutant sites is indicated with an m; (n), number of recombinants.

We observed four major and two minor classes of recombinants: major classes A and B had parental combinations of flanking markers on both copies of chromosome VIII, whereas major classes C and D had at least one chromosome VIII with a non-parental combination of flanking markers (Figure 14). We interpreted the recombinants in classes A and B as the result of conversions to wild type of the *nia*D2001 and the *nia*D169 allele, respectively. Recombinants in class C harbored reciprocal non-parental combinations of

flanking markers on both copies of chromosomes VIII; presumably, these chromosomes represent the two interacting copies of chromosome VIII, which segregated together (z-segregation). Recombinants in class D harbored a crossover chromosome with a functional *nia*D allele and a chromosome with a parental combination of flanking markers. Class D recombinants are homozygous for the distal flanking marker *brl*A and may be the result of a crossover in G2, followed by x-segregation. Alternatively, a long heteroduplex tract including both the *nia*D locus and the *brl*A locus (located 10 map units centromere-distal to the *nii*A-*nia*D locus), occurring in either G1 or G2, may have led to class D recombinants. Conversion of the *brl*A allele concurrent with, but not associated with, the recombination event at the *nii*A-*nia*D locus is unlikely as no yellow or white recombinants, which could have been generated in such a fashion, were observed. Furthermore, no homozygosity of the centromere-proximal flanking marker *fac*C, at a distance of 26 map units from the *nii*A-*nia*D locus, was observed. Minor classes E and F are each represented by a single recombinant, and possibly resulted from multiple interactions between the chromosome VIII homologs.

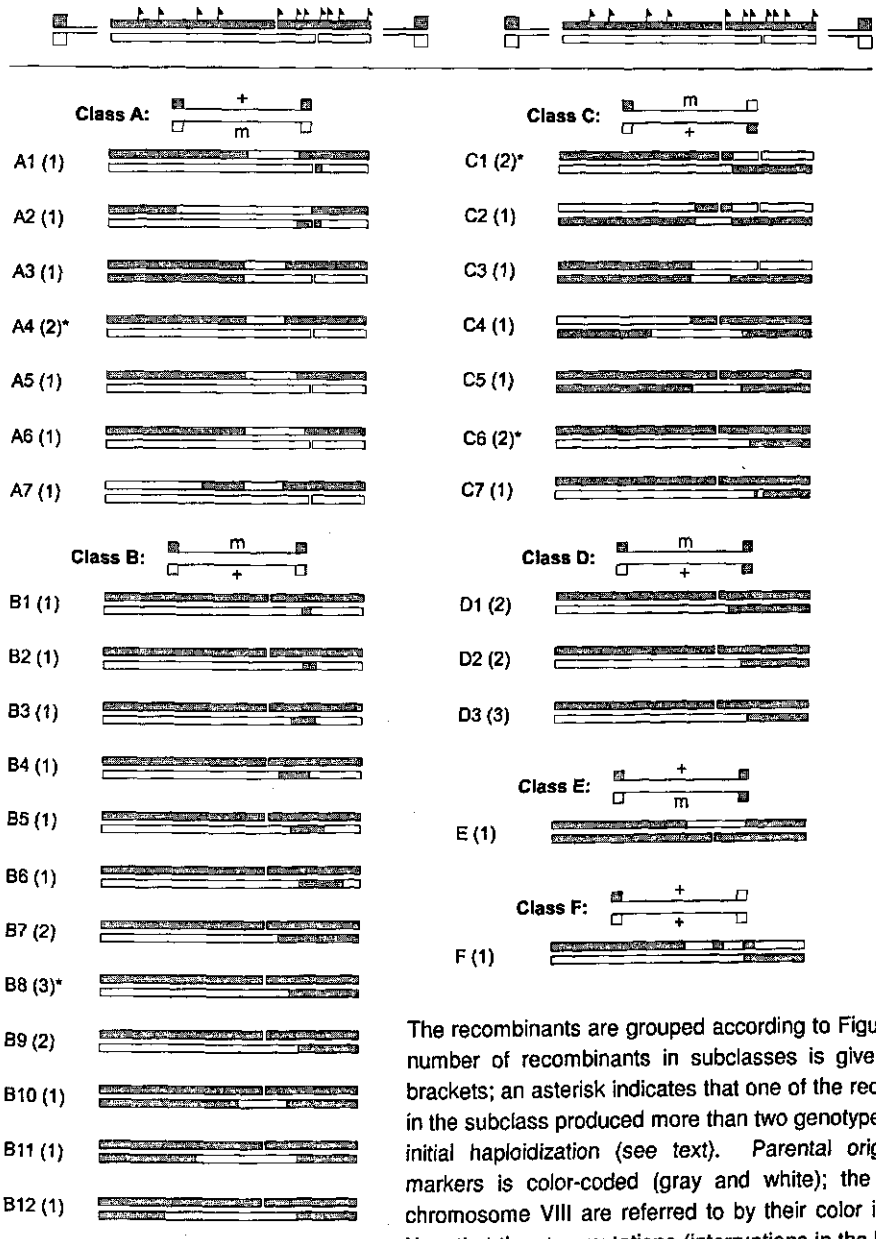
Polarity of meiotic conversion is 5' to 3' for the *nia*D gene, and we have postulated the presence of an initiation site for meiotic recombination (hotspot) upstream of the *nia*D gene to account for this; in a heteroallelic cross involving the *nia*D2001 and the *nia*D169 alleles, the 5' *nia*D2001 allele is preferentially (35% *vs.* 10% of the recombinants) converted (chapter 3). In contrast, the relative frequency of mitotic conversion the 3' *nia*D169 allele (Figure 14, class B) appears to exceed that of the *nia*D2001 allele (class A), although the observed ratio of class A and class B recombinants (11:17) does not differ significantly from a 1:1 ratio, expected if no preference for conversion of either allele exists (P = 0.35). This suggests that the meiotic hotspot located upstream of the *nia*D gene does not function in mitotic recombination.

Molecular analysis

Of 42 diploid recombinants belonging to each of the six classes, the silent mutations in the haploid derivatives were analyzed (Figure 15). In the following section, the patterns found among the recombinants in each of the genetic classes will be described; the parental chromosomes will be identified by the color code ("gray" and "white") used in Figure 15; the chromosome with the functional copy of the *nia*D gene is referred to as the selected chromosome; if a subclass contains a recombinant that initially produced more than two genotypes, this is indicated by an asterisk in Figure 15.

Class A recombinants were genetically interpreted as conversion to wild type of the *nia*D2001 allele ("gray" chromosome). In three class A recombinants (subclasses A1-A3), both copies of chromosome VIII showed non-parental patterns of mutations, which presumably resulted from a two-way exchange of genetic information in a single recombination event. Five class A recombinants (subclasses A4-A7) harbored a "white" chromosome indistinguishable from the parental copy. These recombinants may be the result of a one-way transfer of information, or a two-way transfer G2 event followed by segregation with the non-involved copy of the "white" chromosome (x-segregation). In all but one class A recombinants, the selected chromosome showed a single conversion patch (one or several adjacent mutations converted in the same direction), which included the 5' stop mutation and several (1 to 5) adjacent silent mutations. The exception (subclass A7) showed a pattern with (at least) two conversion patches on the selected chromosome; subclass A2 harbored more than one conversion patch in the unselected chromosome.

Figure 15. Molecular analysis of mitotic recombinants



The recombinants are grouped according to Figure 14. The number of recombinants in subclasses is given between brackets; an asterisk indicates that one of the recombinants in the subclass produced more than two genotypes after the initial haploidization (*see text*). Parental origin of the markers is color-coded (gray and white); the copies of chromosome VIII are referred to by their color in the text. Note that the stop mutations (interruptions in the bars) were not analyzed, but inferred from genetic analysis and adjacent markers.

Class B recombinants were interpreted as conversion to wild type of the *nia*D169 allele ("white" chromosome). In contrast to class A recombinants, class B recombinants provided no evidence for a two-way transfer of information: the "gray" chromosome was indistinguishable from the parental chromosome in all cases (Figure 15). In all class B recombinants, the selected chromosome showed a conversion patch of the 3' stop mutation and up to six adjacent silent mutations. Some recombinants (subclasses B10-12) have at least two conversion patches in the *nii*A-*nia*D region.

The non-parental and complementary flanking markers in class C recombinants suggested that both crossover chromosomes have segregated together. In recombinants in subclasses C1-C4, the *nii*A-*nia*D regions of both copies of chromosome VIII harbored non-parental patterns of mutations, which is in agreement with this interpretation. In contrast, recombinants in subclasses C5-C7 harbored one copy of the *nii*A-*nia*D region indistinguishable from the "gray" parental copy. Patterns consistent with a crossover without any conversion of mutations (subclass C1) as well as patterns with conversion patches covering the whole *nii*A-*nia*D region (subclass C5), reminiscent of those observed among class B recombinants (subclasses B10-B12), were found. If our interpretation of class C recombinants is correct (two crossover chromosomes, see Figure 14), then most members of this class (C2-C5, C7) have more than one conversion patch in the *nii*A-*nia*D region.

The patterns among class D recombinants, homozygous for the distal but not the proximal flanking marker, were in agreement with the genetic interpretation as a segregation of the selected (crossover) chromosome with the non-involved copy of the "gray" chromosome. The ratio of class C (crossover, z-segregation) and class D (crossover, x-segregation) recombinants (10:7, see Figure 14) does not differ significantly from the 1:1 ratio expected if chromosome segregation is random ($P = 0.63$).

The class E recombinant showed a small conversion patch in the selected chromosome, and a "gray" chromosome with a non-parental set of flanking markers. Class F contained the only recombinant with two chromosomes with a functional *nia*D gene and the wild type *brl*A allele. Both the class E and class F recombinant were probably the result of multiple interactions.

Discussion

We have studied mitotic recombinants using a molecular detection system unprecedented in *A. nidulans*, as earlier studies were limited to the genetic analysis of a locus and flanking markers (Whitehouse, 1982; Zhao and Kafer, 1992). The diploid strain used for the generation of recombinants (DIP1) was unable to grow on nitrate as a nitrogen source due to the presence of dissimilar stop mutations in the two copies of the *nia*D gene; recombinants were selected on nitrate, i.e., the presence of at least one functional copy of the *nia*D gene. Genetic analysis of the *nia*D locus and the flanking markers in haploid derivatives of diploid recombinants (Figure 14) showed that recombination can be reciprocal (a crossover) or non-reciprocal (a conversion). Polarity of mitotic conversion was not observed. DIP1 was designed to monitor a series of silent point mutations in the *nii*A-*nia*D region, heterozygous in DIP1, in recombinants (Figure 13B)

The patterns of silent mutations in the recombinants (Figure 15) suggest that mitotic recombination occurs through the formation of long heteroduplex tracts with patches of

conversion within these tracts. Both one-way and two-way transfers of genetic information between the interacting chromosomes were observed. Therefore, most of the observations are compatible with recombination models that involve initiation by single- or double-strand breaks, followed by heteroduplex formation and subsequent repair of mismatches within the heteroduplex tracts (Holliday, 1964; Meselson and Radding, 1975; Szostak *et al.* 1983).

Mitotic conversion is patchy

The patterns of silent mutations observed in mitotic recombinants suggested that conversion tracts may frequently include regions flanking the *nii*A and *nia*D genes (Figure 15, classes A3, A7, B7-12, C3-7, D1-3, E and F). Also, whereas the majority of the mitotic recombinants showed a single conversion patch, a significant fraction contained multiple patches. In contrast, meiotic conversion tracts are confined to the *nii*A-*nia*D region and are rarely patchy (chapter 3). Similar differences between mitotic and meiotic recombination have been observed in *S. cerevisiae* (Judd and Petes, 1988). In those recombinants with non-parental patterns of silent mutations in both chromosomes (Figure 15, classes A1-4, C1-4 and F), the position of the conversion patches may be identical, overlap, or be at different locations in the two chromosomes. This indicates that no strict choice of template is implemented in mitotic recombination.

No preferred initiation site for mitotic crossovers

Recombinants were selected for an event between the two stop mutations present in DIP1. This event may conceivably be initiated between these sites and resolved as a crossover, or the endpoint of a conversion patch (which includes one of the stop mutations) may lie between these sites. The frequent discontinuity of mitotic conversion tracts prohibit a clear distinction between these alternatives. Judged by the non-parental flanking markers on the selected chromosome, classes C and D are the result of a crossover. These classes comprise one third of the recombinants analyzed genetically; at other *A. nidulans* loci, the percentage of the recombinants with a non-parental flanking marker combination on the selected chromosome varies widely (10-40%), depending on the specific pair of alleles used (reviewed in Whitehouse, 1982). Only in C1 recombinants, the crossover was probably initiated and resolved between the stop mutations. In the other cases, the initiation site for the recombination events that led to the crossover was located at a different location on chromosome VIII and a long heteroduplex tract has reached the *nia*D locus; patchy conversion must have led to a functional *nia*D copy. In summary, our data do not provide evidence for a specific initiation site for mitotic crossovers. In contrast, meiotic recombination, where 85% of the recombinants (identified by the same selection) contains an associated crossover, is initiated in the intergenic promoter region. This difference between mitotic and meiotic recombination is in agreement with the notion that mitotic recombination functions in the repair of accidental DNA damage (which may occur throughout the genome), whereas in meiosis recombination is initiated with carefully programmed and regulated steps, one of which is induction of DNA strand breaks at preferred sites, as documented for *S. cerevisiae* (Zenvirth *et al.* 1992; Game, 1992; Wu and Lichten, 1994).

Mitotic conversion is not polarized

Meiotic conversion in the *nia*D gene is highly polarized as the 5' allele is preferentially converted (chapter 2); this is also observed for the pair of alleles used in the current study

(chapter 3). The experiments presented in this chapter show that mitotic recombination was not polarized or weakly polarized in the direction opposite that of meiotic polarity. Mitotic recombination at the *ad*E and *paba*A loci of *A. nidulans* shows a weak polarity of conversion in the opposite direction of the strong polarity of meiotic recombination observed at both loci (reviewed in Whitehouse, 1982). For all three loci (*ad*E, *paba*A and *nia*D) it is the centromere-distal allele which is most often converted; whether this is co-incidental or a consequence of the mechanism of mitotic recombination cannot be decided. The absence of a strong polarity of mitotic conversion has also been observed in *S. cerevisiae* (reviewed in Petes *et al.* 1991).

G1 events *vs.* G2 events

After a crossover in G1, all descendants carrying the selected recombinant chromosome will also inherit the other involved (homologous) chromosome. If a crossover occurs in G2, only two of the four chromatids of homologous chromosomes interact: assuming random segregation, 50% of the descendants carrying the selected recombinant chromosome will then inherit the involved chromatid of the homolog (z-segregation; class C in Figure 14 and Figure 15), and 50% will inherit the non-involved chromatid (x-segregation, class D). From the observed fraction of crossover recombinants (class C+D) that belong to class C (10/17), we estimate ($P > 0.05$) that 44-100% of the crossover events occurred in G2. We cannot estimate the fraction of non-crossover events that occurred in G2, because we cannot distinguish a non-involved chromosome from a chromosome that was involved in a non-crossover event without undergoing conversion of one or more of the mutations analyzed. Among the class B recombinants, 100% ($n = 16$) of the unselected chromosomes (the "gray" chromosomes in Figure 15, class B) carried a parental set of mutations. This is significantly more ($P < 0.001$) than the percentage of non-involved chromosomes expected, even if all non-crossover events would have occurred in G2 (namely 50%). At least a fraction of the unselected chromosomes in class B should therefore be "involved" chromosomes, if both interacting chromosomes/chromatids are recovered from the events detected in class B. Non-crossover events in which both chromosomes/chromatids were recovered were detected among class A recombinants (see the "white" chromosomes of subclasses A1-3 in Figure 15). We do not know why two-way exchanges of information were detected in class A (conversion of *nia*D2001 to wild type) but not in class B (conversion of *nia*D169 to wild type) recombinants. Possibly, the mismatches in heteroduplex tracts generated by the stop mutation in the *nia*D2001 allele (T·T or A·A) and the stop mutation in the *nia*D169 allele (A·C or T·G) are repaired by different mechanisms. This would further complicate the consideration of polarity of mitotic conversion.

6. CONCLUSIONS

OVERVIEW

The experiments presented in this thesis concern the analysis of homologous recombination at the molecular level in the filamentous fungus *Aspergillus nidulans*. The aim of the experiments presented in chapter 2 was to establish whether the *nii*A-*nia*D locus would be suitable for such an analysis. The affirmative results prompted the design of a set of strains harboring neutral mutations in the *nii*A-*nia*D region, which could be detected by restriction enzyme analysis. With the use of this assay, we examined meiotic recombinants obtained from allelic intergenic (*nii*A-*nia*D) crosses and from, both allelic and ectopic, intragenic (*nia*D/*nia*D) crosses (chapter 3). Also, we have analyzed spontaneous mitotic recombinants obtained from a heterozygous (*nia*D/*nia*D) diploid strain (chapter 5). The assay system was designed to generate answers to a number of questions concerning the molecular mechanisms that underlie recombination in *A. nidulans* (see chapter 1); here, we will review the conclusions we have reached. Also, the question whether *A. nidulans* is a suitable organism to study the molecular mechanisms of recombination will be addressed.

ARE RECOMBINATION EVENTS INITIATED IN THE *NII*A-*NIA*D PROMOTER REGION?

A novel meiosis-specific recombination hotspot

We have demonstrated the presence of an initiation site for meiotic recombination (hotspot) in the *nii*A-*nia*D locus. The strong 5' to 3' polarity of meiotic conversion in the *nia*D gene, observed in a series of two-point crosses, indicated such a site upstream from the *nia*D coding region (chapter 2). Detailed analysis in recombinants generated with our assay system confirmed this and indicated that the hotspot is located in the intergenic promoter region of the *nii*A and *nia*D genes (chapter 3). This is the first recombination hotspot that has been defined at the molecular level in *A. nidulans*. Our results thus extend the role of transcriptional promoters in the initiation of meiotic recombination from the yeasts *S. cerevisiae* and *S. pombe* (see chapter 1 for details) to a filamentous fungus. Our data on mitotic recombination do not support the existence of a hotspot for mitotic recombination in the *nii*A-*nia*D region (chapter 5); the recombination initiation site in therefore meiosis-specific.

Why are promoters of transcription recombination hotspots?

The question why meiotic recombination hotspots are often located in promoters of transcription remains unresolved. Studies in *S. cerevisiae* have shown that an active hotspot is preferentially replaced by an inactive counterpart (Nicolas *et al.* 1989) during meiotic recombination; consequently, active hotspots will disappear unless an evolutionary force counteracts this. This topic has been addressed by Boulton and co-workers (1997) by the use of computer simulations; they have tried but failed to identify evolutionary benefits for the preferential use of promoters of transcription as meiotic recombination hotspots.

Cooke (1997) has suggested that transcription, DNA repair and recombination may be spatially and mechanistically linked processes (located in transcription 'factories') and, as a consequence, (initiation of) recombination occurs preferentially in transcription units.

In this view, (initiation of) transcription of a gene is a prerequisite for recombination to occur.

DOES TRANSCRIPTION INFLUENCE RECOMBINATION IN *A. NIDULANS*?

We have tried to induce transcription of the *nii*A and *nia*D genes during crosses by the addition of nitrate to the media to see whether the level of transcription is of influence on meiotic recombination. Our attempts to analyze this relationship did not provide any indication that gene transcription influences (the initiation of) meiotic recombination in *A. nidulans* (chapter 3).

In easy retrospect, the experimental setup was flawed in several ways. Although the level of transcription of the *nii*A and *nia*D genes in vegetative tissue is strongly influenced by the nitrogen source(s) present in the medium (Cove 1979; Punt *et al.* 1991), we do not know if this effect applies to meiotic cells; a method to specifically measure the level of transcription in meiotic cells is currently unavailable in *A. nidulans*. Also, the non-inducing nitrogen sources used in our studies (urea and glutamate) do not repress transcription; the use of ammonium, which does repress transcription (Cove, 1979), as a nitrogen source might have increased the difference in the level of transcription between treatments. And finally, transcription of the *nia*D gene is not only influenced by the nitrogen source(s), but also by the *nia*D gene product itself; some *nia*D mutants express the gene product constitutively (Garde *et al.* 1995). We do not know the effect on (vegetative) transcription of the *nia*D mutations used in our studies.

WHAT HAPPENS DURING MEIOTIC RECOMBINATION IN *ASPERGILLUS NIDULANS*?

Bi-directional conversion tracts

Initiated in the intergenic promoter region, conversion tracts extend into the coding region of the *nii*A gene and/or the *nia*D gene; conversion tracts can be bi-directional, like those observed in the *DED81-ARG4* region of *S. cerevisiae* (Schultes and Szostak 1990) and the *ade6* gene of *S. pombe* (Grimm *et al.* 1994). Our results are therefore consistent with a model for recombination as depicted in
Figure 4 (page 13), in which a movable Holliday junction is formed on both sides of the point of initiation. Also, the average length of the conversion tracts and their generally uninterrupted nature are similar for *A. nidulans*, *S. cerevisiae*, and *S. pombe* (chapter 3).

In *A. nidulans*, meiotic recombination is usually reciprocal

Approximately 85% of the allelic meiotic recombinants harbored a crossover in the *nii*A-*nia*D region (chapter 3). This percentage of recombination intermediates that is resolved as a crossover is substantially higher then in *S. cerevisiae*, where an average of about 50% has been reported (Petes *et al.* 1991). A recent report on meiotic recombination in maize (Dooner and Martínez-Férez, 1997) shows that, like in *A. nidulans*, non-reciprocal resolution of recombination intermediates is rare in maize (see also chapter 4).

In our preliminary genetic analysis of recombination in the *nii*A-*nia*D region, the percentage of the recombinants with a non-parental set of flanking markers was 55%; for other *A. nidulans loci*, this percentage varies between 55% and 79% and is generally lower if the distance between the selected locus and the flanking markers is larger (see Whitehouse 1982). The discrepancy with respect to the number of crossovers between our genetic

(55%) and molecular (85%) data is probably due to secondary recombination events in the regions between the *nii*A-*nia*D locus and the flanking markers.

Crossover interference

In many organisms, including *S. cerevisiae*, positive crossover interference (i.e., a crossover decreases the probability of a second crossover nearby) is observed (see Sym and Roeder 1994). In *A. nidulans* however, positive crossover interference is reported absent (Strickland 1958b). For the interval between the *nii*A-*nia*D locus and the flanking marker *fac*C, we observed negative crossover interference, because the interval expanded 1.4-fold as a result of the selection of recombination events (usually crossovers) at the *nii*A-*nia*D locus (chapter 3). Calef (1957) accounted for the negative crossover interference observed in *A. nidulans* by assuming that pairing is not complete along the whole length of every pair of homologous chromosomes; selection for crossovers at one point would also select the products of those cells in meiosis in which the probability of pairing around the selected point is greater than average. This hypothesis of localized pairing is interesting, given the fact that most eukaryotes assemble synaptonemal complexes (SCs), but *A. nidulans* does not (Egel-Mitani *et al.* 1982). SC formation in *S. cerevisiae* is required for synapsis of the homologous chromosomes and for positive crossover interference (Sym and Roeder 1994). Possibly, *A. nidulans* has lost the ability to synaps its chromosomes during evolution and, as a consequence, crossover interference is absent in this organism.

However, SC formation is also required for a proper distibution of crossovers (at least one per bivalent) to ensure chromosome disjunction in *S. cerevisiae.* Sym and Roeder (1994) have hypothesized that multiple factors contribute to a low frequency of non-disjunction in the absence of SCs (as in *A. nidulans*): first, a high number of crossovers per meiosis (82 in *A. nidulans*, 90 in *S. cerevisiae*); second, a low number of chromosomes (8 in *A. nidulans*, 16 in *S. cerevisiae*); third, an approximately equal size of the chromosomes (2900-5000 kb in *A. nidulans*, 220-1900 in *S. cerevisiae*). A high frequency of crossover resolution of recombination intermediates may be yet another factor that is essential for a low frequency of non-disjunction in *A. nidulans*.

HOW DO HETEROLOGIES INFLUENCE MEIOTIC RECOMBINATION EVENTS?

Markers that distinguish the parental strains will produce mismatches when they are included in a heteroduplex tract. The way the meiotic cell handles such mismatches is of crucial influence on the types of recombinants observed. In *S. cerevisiae* and *S. pombe*, G·G and/or C·C mismatches are poorly repaired (PMS markers), while all other single base pair mismatches and most insertions and deletions (in *S. cerevisiae*) are repaired with high efficiency (reviewed in Petes *et al.* 1991). Also, the number and density of markers may influence meiotic recombination in *S. cerevisiae* (Borts and Haber 1987). In contrast, the presence of five markers (point mutations) at the *ade6* locus of *S. pombe* does not influence the frequency of recombination or the distribution of flanking markers (Grimm *et al.* 1994).

The parental strains used in our studies on meiotic recombination differed by the two mutations in the *nii*A and/or *nia*D genes used for the selection of recombinants (chapter 2), or by an additional series of 11 silent point mutations (chapter 3). We introduced different single base pair alterations to generate the silent point mutations (Table 6, page 67); only one of the alterations produced a C·C and/or G·G mismatch when included in a

heteroduplex tract (marker A1). We did not observe clear marker effects in *A. nidulans*, although in one recombinant we observed a pattern that may reflect the absence of repair of marker A1 (Class E' in Figure 8, page **Error! Bookmark not defined.**). This result is not unexpected as in *S. cerevisiae* the presence of a well-repaired (WCC) marker enhances repair of a nearby PMS marker (reviewed in Petes *et al.* 1991); in our experiments, most mismatches will presumably be well-repaired.

We have not found evidence for an influence of 13 *vs.* 2 mutations on the frequency of meiotic recombination or the distribution of flanking marker combinations (see Table 1, page 22 and Table 4, page 33), except that the presence of a 2 kb deletion in one of the parental strains elevated the frequency of recombination approximately two-fold. Interestingly, among the strains used in the preliminary genetic analysis of the *nii*A-*nia*D locus (chapter 2), two harbored a small deletion. In crosses where these deletions were the 3' mutations, recombinants were detected at frequencies higher than in comparable crosses in which the 3' mutation was a point mutation (Table 1, page 22). Speculating on this, we propose that in *A. nidulans* deletions are preferably excluded from heteroduplex tracts and that their presence enhances resolution into reciprocal exchanges. In our current assay system this elevates the observed recombination frequency.

HOW DOES ECTOPIC RECOMBINATION COMPARE WITH ALLELIC RECOMBINATION?

The use of a strain which harbored a copy of the *nii*A-*nia*D region at the *arg*B locus instead of the original location allowed a comparison of allelic and ectopic meiotic recombination. Compared to allelic recombination, the frequency of ectopic recombination was significantly reduced (40-fold) and the percentage of recombinants with a crossover in the region was two-fold lower (chapter 3). While the low frequency of ectopic recombination may simply reflect the low probability of an encounter between the two copies of the *nii*A-*nia*D region as a consequence of the absence of homologous interactions outside this region, the reduction of the frequency of reciprocal exchanges is intriguing. Apparently, *A. nidulans* is capable of sensing an ectopic interaction, and enhancing its resolution as a non-crossover.

HOW DOES MITOTIC RECOMBINATION COMPARE WITH MEIOTIC RECOMBINATION?

The analysis of mitotic recombination revealed several striking differences in comparison with meiotic recombination. First, no evidence for a specific initiation site for mitotic recombination was obtained; mitotic conversion showed no polarity. This is in agreement with the notion that mitotic recombination functions in the repair of accidental DNA damage (which may presumably occur throughout the genome), whereas in meiosis, recombination is initiated by carefully programmed and regulated steps. Second, our results suggest that during mitotic recombination long heteroduplex tracts are formed and conversion occurs in patches within these tracts. Mitotic recombination differs in these respects from meiotic recombination in *A. nidulans*.

ASPERGILLUS NIDULANS AS A RESEARCH TOOL

The use of *A. nidulans* for the analysis of homologous recombination has several limitations. First, tetrad analysis is extremely laborious in *A. nidulans*; this limits the analysis of the

products of meiotic recombination to one of the interacting DNA molecules (with some exceptions), and therefore precludes the distinction of WCC and PMS events. Also, assumptions on the reciprocal or non-reciprocal nature of the interaction are necessary. Secondly, *A. nidulans* is multicellular and its meiotic cells are asynchronous. This severely impairs the molecular analysis of meiotic recombination. Finally, the cytology of *A. nidulans* is not very well developed. An organism that combines accessibility to genome modification, the possibility of tetrad analysis and a good cytology is, at least currently, hard to find; consequently, the organism should fit the question. The central theme of the project proposal that prefaced the experiments described in this thesis was the relationship of meiotic recombination and gene transcription. In retrospect, this is an issue that called for a reseach organism that allows the analysis of gene transcription in meiotic cells.

However, the data presented in this thesis provide solid information on some of the details of the process of homologous recombination in *A. nidulans*. The accessibility of *A. nidulans* to genome modification has allowed us to collect these data with relative ease. Comparison with data obtained in other fungi suggests that the general mechanism by which meiotic recombinants arise is not dependent on the specific fungus under observation; our data support models for meiotic recombination that have been proposed on the basis of information obtained in *S. cerevisiae* and other fungi. Polarity of meiotic conversion, a key feature of meiotic recombination in fungi, has recently been observed in maize; this suggests that models based on fungal data may also be applicable to higher plants.

A. nidulans may prove to be a valuable tool with respect to mitotic recombination. Our data on mitotic recombination provide a detailed description of recombinants in a wild type background and a good starting point for the analysis of mutants impaired in recombination. In view of the importance of mitotic recombination in DNA repair, the development of cancer and the production of antibodies, we suggest that future research on homologous recombination in *A. nidulans* focuses on the mechanisms of mitotic recombination.

MATERIALS AND METHODS

A. NIDULANS STRAINS AND MEDIA

Strains

The *A. nidulans* strains (Table 5) were all descendants of the Glasgow stocks (Clutterbuck 1986). The strains used for the molecular analysis were nicknamed for clarity: strains used for transformation were named Receptors, strains used for the isolation of recombinants were named Generators. Suffices denote a *nia*D mutant (D),a *nii*A mutant (A), or a *nia*D mutant used in the analysis of interchromosomal recombination (E). DIP1 is a diploid strain, which was used for the generation of mitotic recombinants.

The genetic markers are all in general use (Clutterbuck 1993). To establish the presence or absence of auxotrophic markers in single-spore colonies, growth tests on appropriate media were performed. The *brl*A42 allele is temperature-dependent: at 37°C the conidiophores show a bristle morphology, while at 26°C the morphology is wild type (Clutterbuck 1992). Resistance to phleomycin was tested on plates containing 40 µg/ml phleomycin (Cayla, Toulouse, France).

Media recipes

Base medium (BM) was 1.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 1 mg/l $FeSO_4.7H_2O$, 1 mg/l $ZnSO_4.7H_2O$, 1 mg/l $MnCl_2.4H_2O$, 1 mg/l $CuSO_4.2H_2O$ (pH 5.8). Minimal medium (MM), sufficient for growth of strains without auxotrophic markers, was BM with 1% glucose and 10 mM $NaNO_3$. Complete medium (CM), sufficient for growth of all strains, was essentially as described by Pontecorvo *et al.* (1953). Supplemented medium (SM) was BM to which all components were added to enable growth of a particular strain. Selective medium (SN) was identical to SM, but contained 10 mM sodium nitrate as the sole nitrogen source. To solidify media, agar (Agar no. 2, Oxoid) was added as recommended by the supplier, except when testing or selecting for growth on various nitrogen sources. In these cases purified agar (Agar no. 1, Oxoid) was used.

Propagation and storage

For short-term storage and the production of vegetative spores, *A. nidulans* strains were grown in small bottles on solidified CM for 3 days at 37°C (4 days at 26°C if the strain carried the *brl*A42 marker). For the preparation of a spore suspension, glass beads and Tween/Saline were added and the bottle was vortexed vigorously. The suspension was filtered over glasswool prior to use. Bottles containing sporulating colonies remained viable for several months when stored at 4°C. For long-term storage, a spore suspension was obtained as above, collected and washed in Tween/Saline (2% Tween 80, 0.9% NaCl), resuspended in 1% peptone/30% glycerol and stored at -80°C. For the production of mycelium for the isolation of protoplasts or genomic DNA, liquid SM was inoculated with 10^6-10^8 vegetative spores per ml and incubated at 200 rpm in a waterbath at 37°C for 16 hours. The mycelium was harvested by filtration.

Table 5. *A. nidulans* strains.

Stockname	Nickname[a]	Genotype[b] Chromosome I-VII	Chromosome VIII	Source[c]
ANGW1108	Receptor1	*biA*1	*niiA-niaD*△R::prc	
ANGW1109	Receptor2	*biA*1, *niiA*$^+$	*niiA-niaD*△R::prc	
ANGW1111	GeneratorD1	*biA*1	*facC*301, *niaD*2001, *brlA*42	
ANGW1115	GeneratorA2	*yA*2, *pyroA*4	*niiA*2002	
ANGW1119	GeneratorA1	*yA*2, *pyroA*4	*niiA*2001	
ANGW1122	GeneratorD2	*yA*2, *pyroA*4	*facC*301, *niaD*2001, *brlA*42	
ANGW1123	Receptor3	*biA*1, *argB*2	*niiA-niaD*△R::prc	
ANGW1124	GeneratorE1	*biA*1, *niaD*2001 (*argB*2)	*niiA-niaD*△R::prc	
ANGW1125	GeneratorE2	*biA*1, *wA*3	*niaD*169	
ANGW1128	DIP1	+/*yA*2, *biA*1/+, *wA*3/+, +/*pyroA*4	+/*facC*301, *niaD*169/*niaD*2001, +/*brlA*42	
WG096		*yA*2, *pabaA*1	wild type	1
WG288		*biA*1, *amdI*18, *amdA*7	*niiA*4	1
WG427		*biA*1	*niaD*15	1
WG428		*biA*1	*niaD*17	1
WG430		*yA*2, *pyroA*4	*niaD*1017	2
WG432		*biA*1	*facC*301	1
WG440		*yA*2, *pyroA*4	*facC*301, *niaD*169, *brlA*42	
WG441		*biA*1	*facC*301, *niaD*1017, *brlA*42	
WG442		*biA*1	*niaD*35	2
WG443	GeneratorD3	*biA*1	*niaD*169	2
WG444		*biA*1	*niaD*20	2
WG445		*yA*2, *puA*2, *lysB*5, *suA*1-*lysB*5	*niaD*1078	2
WG446		*pantoB*5, *fwA*1	*niaD*124	2
WG447		*biA*1	*niaD*21	2
WG453		*yA*2, *pyroA*4	*facC*301, *niaD*1078, *brlA*42	
WG454		*pyroA*4	*facC*301, *niaD*1078, *brlA*42	
WG455		*yA*2, *pyroA*4	*niaD*1078	
WG466		*yA*2, *pyroA*4	*facC*301, *niiA*4, *brlA*42	
WG474		*yA*2, *metH*2, *argB*2	*fwA*1, *niiA-niaD*△509	1

[a] Some strains were nicknamed for clarity: Receptors were used for transformation with mutagenized constructs, Generators were used for the production of recombinants (suffixes denote a stop mutation in the *nii*A gene (A), in the *nia*D gene (D), or in the ectopic *nia*D gene (E)).

[b] Chromosome VIII constitutions are given separately; prc, phleomycin resistance cassette. The location of the *niiA*$^+$ allele in Receptor2 is unknown (see Figure 16).

[c] The source of a strain is given if it was not constructed in the course of this study: 1, Lab. Genetics, WAU, laboratory collection; 2, Dr. P. Strike and Dr. B. Tomsett, University of Liverpool.

A. NIDULANS STRAIN CONSTRUCTION AND ANALYSIS

Crosses to obtain new genotypes

Crosses to obtain new genotypes were performed essentially as described by Pontecorvo *et al.* (1953). In summary, vegetative spores of the parental strains were resuspended in 2 ml liquid CM and incubated at 37°C O/N. The mycelial layer was washed twice in Tween/Saline and small pieces were transferred to plates containing solid CM with 10 mM $NaNO_3$, 2% glucose, 10mM urea and 0.5% (w/v) active charcoal (to reduce cross-feeding of the strains) to produce a stable heterokaryon. After 2-3 weeks, plates were covered with heterokaryotic mycelium with ripe cleistothecia. Individual cleistothecia were isolated, cleaned and crushed in 1 ml Tween/Saline to release the ascospores. An aliquot of each was plated on CM and incubated at 37°C for 3 days. Crossed cleistothecia were identified using heterozygous spore color markers in each cross. Progeny colonies with the desired genotype were identified by plating an aliquot of ascospores on appropriately supplemented medium, isolation of single-spore colonies and testing these for all genetic markers on drop-out plates.

Isolation of genomic *A. nidulans* DNA

In the course of the experiments, two methods for the isolation of genomic *A. nidulans* DNA were employed. The first was used in the experiments described in chapter 2, the second in the experiments described in chapters 3 and 4.

I) Mycelium (1 g) was ground under liquid nitrogen, resuspended in 2.5 ml freshly made TPT-buffer (22 mM triisopropyl-naphtalene-sulphonic acid, 0.22 M 4-aminosalicylic acid, 0.25 M NaCl, 50 mM EGTA, 0.2 M Tris-HCl (pH 8.5)), mixed with 1.5 ml water-saturated phenol and incubated at 55°C for four minutes with repeated mixing. One ml chloroform/isoamylalcohol (24:1) was added and the mixture was incubated for two minutes. After separation from the organic phase by centrifugation, the genomic DNA was precipitated from the water phase and purified on a standard CsCl/ethidium bromide gradient (Sambrook *et al.* (1989)).

II) Mycelium (1-2 g wet weight) was ground under liquid nitrogen and resuspended in 10 ml extraction buffer (500 mM NaCl, 10 mM EDTA, 1% SDS, 10 mM Tris-HCl (pH 7.5)). The mixture was incubated at 65°C for 30 minutes and an equal volume of phenol/chloroform (1:1) was added. After careful vortexing and centrifugation, the waterphase was transferred to a fresh tube and the nucleic acids were precipitated with 0.4 volumes of isopropanol at room temperature. After centrifugation, the pellet was resuspended in 1 ml deionized water. RNA was removed by an RNase treatment (10 µg/ml RNase A, 30 minutes at 37°C). The solution was extracted with phenol/chloroform (1:1) and precipitated with 2 volumes of ethanol. After centrifugation, the pellet was resolved in deionized water.

E. coli strains, plasmids & media

E. coli DH5α (Gibco BRL) was used as receptor strain in routine transformation and propagation experiments. For electroporation-mediated transformation of *E. coli*, we followed the instructions of the supplier of the electroporation apparatus (Biorad, Hercules, CA, USA). The mutagenesis procedure (Altered sites *in vitro* mutagenesis system (Promega, Madison, WI, USA)) required the use of *E. coli mut*S strain BMH71-18. The following plasmids were used: pUC19 (Yannisch-Perron *et al.* 1985) and pHSS20 (Nickoloff

1991) as general cloning vectors; pAlter-1 (Promega) for *in vitro* mutagenesis; and pAN8-1 as the source of the phleomycin resistance cassette, which consists of the phleomycin resistance gene of *Streptoalloteichus hindustanus* bordered by the *A. nidulans gpd*A promoter and the *trp*C terminator (Mattern and Punt 1988). *E. coli* cells were grown in LB medium, when appropriate supplemented with ampicillin (125 μg/ml), kanamycin (50 μg/ml) or tetracycline (50 μg/ml); for blue/white screening, 20 μg/ml X-gal and 24 μg/ml IPTG were added. (Sambrook *et al.* 1989).

Standard DNA techniques

Standard procedures were followed for DNA manipulations (Sambrook *et al.* 1989). Restriction and modifying enzymes were from Gibco BRL (Breda, The Netherlands), except *Eag*I, which was purchased from New England Biolabs (Beverly, MA, USA). For Southern analysis (Southern 1975), DNA fragments separated on an agarose gel were alkali blotted onto a Hybond-N^+ membrane as recommended by the supplier (Amersham, Buckinghamshire, UK) and incubated at 65°C in modified Church buffer (7% SDS, 1 mM EDTA, 0.5 M $NaPO_4$ (pH 5.8)) with a random primed ^{32}P-labeled DNA probe; radiochemicals were purchased from Amersham. For the detection of fragments larger than 10 kb, the gel was soaked in 0.2 N HCl for 30 minutes prior to blotting. After hybridization, the membrane was washed under stringent conditions (0.2 x SSC, 0.5% SDS) at 65° for at least one hour; X-ray film (Konica, Tokyo, Japan) was exposed to the membrane to obtain an autoradiograph.

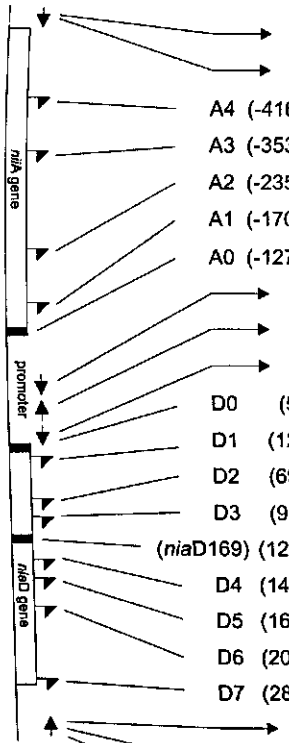
Sequencing of *nia*D1017 and *nia*D1078

Genomic DNA (100-500 ng) was used as a template in PCR amplification reactions with *Taq* polymerase (Boehringer) and 20 pmoles of each primer under standard reaction conditions as recommended by the supplier of the enzyme. The primers used for amplification were OH101 and OH102 (Table 6). The cycling conditions were the following: 30 cycles of DNA denaturation (1 minute, 95°C), annealing (30 seconds, 55°C), and extension (3 minutes, 72°C). The amplification products were purified from an agarose gel and used directly for sequencing. The *nia*D mutations were sequenced using primers OH101 (*nia*D1078) and *NIA*D-A1 (*nia*D1017) and the Circumvent sequencing kit (New England Biolabs) as recommended by the supplier. The experiments were repeated twice using newly isolated template.

Transformation of *A. nidulans*

A. nidulans protoplasts were transformed by a polyethylene glycol treatment essentially as described by van Heemst *et al.* (1997). In one exceptional case (a transformation of Receptor2 with pGW1115 (see Figure 16)), protoplasts were transformed by electroporation (Garde *et al.* 1995).

Table 6. Primers in the *nii*A-*nia*D region.



Mutation[a]		Effects[b]		Primer	Position[c]	Sequence[d]
			(+ *Kpn*I)	OHT2	-5145	TTAgGTAccacGCGTTCTATGGACTGTTACC (31)
				OHT2B	-5125	GGACTGTTACCGATTCGAG (19)
A4	(-4164)	G to A	-*Xho*I	NIIA-X2	-4175	CCCGGAAGGCTtTCGAGCCATCTCG (25)
A3	(-3536)	A to G	+*Eag*I	NIIA-E2	-3548	CAACTTGTCGGCcGTGATTTCACC (24)
A2	(-2353)	G to T	+*Xho*I	NIIA-X1	-2366	CCAGCTCGTTCGaGGTCGAATGCC (24)
A1	(-1701)	G to C	+*Eag*I	NIIA-E1	-1713	CCATGGGCCACGgCCGTATTATGCC (25)
A0	(-1277)	T to A	(+*Alu*I)	NIIA-	-1288	GACCGTCCAGCtACGGCATGATG (23)
				MBL516	-356	AGTGGATCGTAGGATCCAGCAT (22)
				MBL372	-321	GACAGCAGGAATATGCTGG (20 r)
				OH102	-17	TATCCCATACTCTCACAATG (20)
D0	(52)	A to T	(+*Xba*I)	OHT8	63	CTGGGAAGTCTaGAGAGTCTTG (22 r)
D1	(126)	T to C	-*Nsp*V	NIAD-N1	137	GGTTCTTTCGAgGGAGGAGGTAAAG (25 r)
D2	(696)	G to T	+*Nsp*V	NIAD-N2	708	CTTTGCTCTTCGaAGGGGCTTGGC (24 r)
D3	(919)	T to G	+*Eag*I	NIAD-E1	930	GCCCTCAGCGGcCGACCGTGGTCTG (25 r)
(*nia*D169)	(1251)	G to A				
D4	(1454)	C to T	-*Eag*I	NIAD-E2	1466	CATGTCGACGCGaCCGCCATAGAGC (25 r)
D5	(1676)	G to A	+*Nsp*V	NIAD-N3	1688	CAGTCGGATGtTCGAAGAGCAACC (24 r)
D6	(2024)	T to G	+*Apa*I	NIAD-A1	2038	CATAATAGAGGGCCcGGGTTGGAGAAC (27 r)
D7	(2897)	T to C	+*Apa*I	NIAD-A2	2909	CTCCATCGCCTCgGGCCCGCAAACC (25 r)
				OH101	2979	AACCCGTCTAGAAGTAATGA (20 r)
			(+*Bam*HI/*Mlu*I)	OHT1B	3230	GTCTACAGTGGCCGCCTG (18 r)
				OHT1	3243	AGAGGatccACgcGTCTACAGTGGCCGCCTG (31 r)

[a] The *nii*A-*nia*D region is drawn to scale, a flag indicates a silent mutation, a black rectangle indicates a stop (nonsense or missense) mutation, arrows indicate the position of primers used for cloning and PCR amplification. The position of the mutations is given relative to the *nia*D ATG codon; the position of the *nia*D169 allele is included for completeness.

[b] The base-substitutions generated (coding strand) and altered restriction sites (+ or -) are indicated. Restriction sites not used in the analysis of recombinants are between brackets.

[c] The position of the 5' base for the primers is given relative to the *nia*D ATG codon (Johnstone *et al.* 1990).

[d] The 5' to 3' sequence of the primers, length (bp) and orientation (r, reverse) is given; non-homologous nucleotides are in lower case.

In vitro site-directed mutagenesis and strain construction

Figure 16 shows our strategy for obtaining the *A. nidulans* strains harboring mutagenized copies of the *nii*A-*nia*D region which included the following steps: A) cloning of functional copies of the *nii*A and *nia*D genes, B) construction of receptor strains for transformation, C) *in vitro* site directed mutagenesis of the *nii*A and *nia*D genes, and D) transformation of receptor strains with the mutagenized plasmid constructs. The conditions for PCR amplification of the entire *nii*A and *nia*D genes prior to cloning are described below, except that for cloning of the *nii*A gene primers OHT2 and MBL312, and for cloning of the *nia*D gene primers OHT1 and MBL516 were used. Following restriction enzyme digestion (*Kpn*I/*Bam*HI (*nii*A), *Bam*HI (*nia*D), amplified DNA fragments were cloned separately into the polylinker site of pUC19. For mutagenesis, DNA fragments containing the *nii*A and *nia*D genes were recloned into the polylinker site of pAlter-1 and were subsequently mutagenized using the Altered Sites *in vitro* mutagenesis system (Promega) according to the manufacturers instructions. Up to seven mutations were introduced in a single reaction. Presence of the mutations was verified by restriction enzyme analysis. The mutagenic primers used are listed in Table 6. *A. nidulans* strains GeneratorD1, GeneratorD3 and GeneratorA1 (harboring the *nia*D2001 allele, the *nia*D169 allele and the *nii*A2001 allele respectively) were tested for reversion of the stop mutations by selfing the strains and selecting revertants among the progeny. No revertants were detected among 10^8 viable ascospores. The diploid strain DIP1 (Table 5) was selected among the conidiospores of a heterokaryon of the haploid strains ANGW1126 and GeneratorD2 by standard procedures essentially as described by Pontecorvo *et al.* (1953).

ISOLATION OF RECOMBINANTS

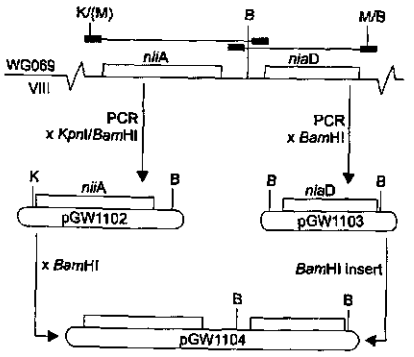
Crosses to obtain meiotic recombinants

The crosses described in chapter 2 were performed as described for crosses to obtain new genotypes. Samples of the ascospore suspensions were plated on SM plates, containing nitrate as the only nitrogen source to select for recombinant progeny colonies, harboring a restored *nii*A-*nia*D locus. After 2-3 days of incubation at 37°C, the recombinants were streaked on fresh plates and single-spore colonies were tested for all genetic markers. Diluted samples were plated on SM plates containing glutamate as the nitrogen source to determine the number of viable ascospores and the frequency of recombination.

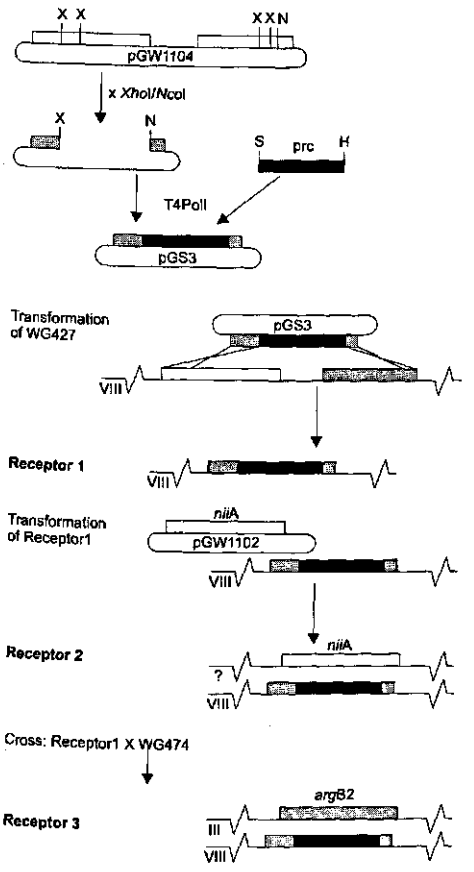
To circumvent problems associated with conventional plate-type crosses (trace contamination with nitrogen sources, laborious harvesting of ascospores), we developed a method to perform crosses with floating mycelial layers. This method was used in the experiments described in chapter 3. Several agar plugs with vigorously growing heterokaryon were transferred before the onset of cleistothecia formation to 50 ml liquid BM, supplemented with 2% glucose and a nitrogen source in a glass container. Nitrogen was supplied as 40 mM sodium glutamate, 40 mM sodium glutamate plus 10 mM sodium nitrate or 10 mM urea. The mycelium was allowed to grow and form cleistothecia by incubation at 37° for 2 weeks; subsequently, the mycelial mass was harvested and gently ground to release the ascospores.

Figure 16. Strain construction

A. Cloning of the *nii*A and *nia*D genes



B. Construction of the Receptor strains



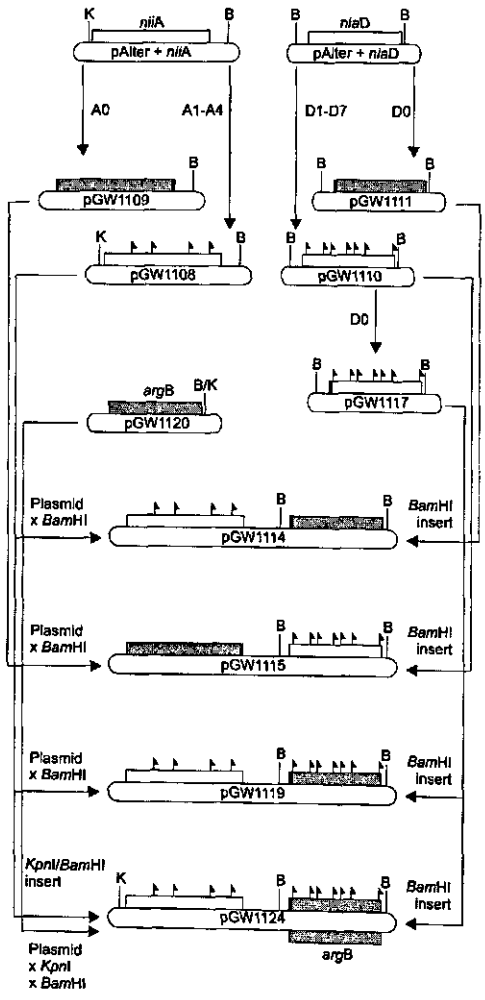
Functional *nii*A, *nia*D and *arg*B genes are white, non-functional genes are gray; a flag indicates a silent mutation, a black rectangle indicates a stop (nonsense or missense) mutation. When together, the *nii*A gene is to the left and the *nia*D gene is to the right. The phleomycin resistance cassette (prc) is black. Primers are black rectangles, roman numerals indicate chromosome numbers. The *facC* and *brlA* outside markers are depicted as squares (black: present, white: absent). Genotypes of all strains are listed in (Table 5). Relevant restriction sites are given for each construct: B, *Bam*HI; Bs, *Bst*EII; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; S, *Sst*I; X, *Xho*I.

A. Cloning of the *nii*A and *nia*D genes: Genomic DNA of *A. nidulans* strain WG096 was used as template in PCR reactions to amplify the *nii*A and *nia*D genes. The primers used (OHT2 and MBL372 for the *nii*A gene and OHT1 and MBL516 for the *nia*D gene, see Table 6) allowed cloning of the *nii*A gene as a 4.8 kb *Kpn*I-*Bam*HI fragment and the *nia*D gene as a 3.6 kb *Bam*HI fragment into the polylinker sites of pUC19, which yielded pGW1102 and pGW1103 respectively. The *Bam*HI fragment containing the *nia*D gene from pGW1103 was cloned into the unique *Bam*HI site of pGW1102. The resulting plasmid, pGW1104, contained the entire 8.4 kb *nii*A-*nia*D region.

B. Construction of the receptor strains: An internal 7.0 kb *Xho*I-*Nco*I fragment of pGW1104 was replaced by a 2.5 kb *Sst*I-*Hin*dIII fragment containing the phleomycin resistance cassette (prc) from pAN8-1 (MATTERN and PUNT 1988). The cloning procedure included treatment of vector and insert with T4 DNA polymerase (T4Pol). The resulting plasmid pGS3 harbored prc bordered by 1.0 kb of *nii*A sequences and 0.4 kb of *nia*D sequences. *A. nidulans* strain WG427 was transformed with pGS3 and phleomycin resistant colonies were selected. Transformants showing no growth on nitrite were analyzed by Southern blotting and hybridization. One transformant showing hybridization patterns consistent with a replacement of the resident *nii*A and *nia*D genes by the pGS3 insert was designated Receptor1.

Strain construction (continued)

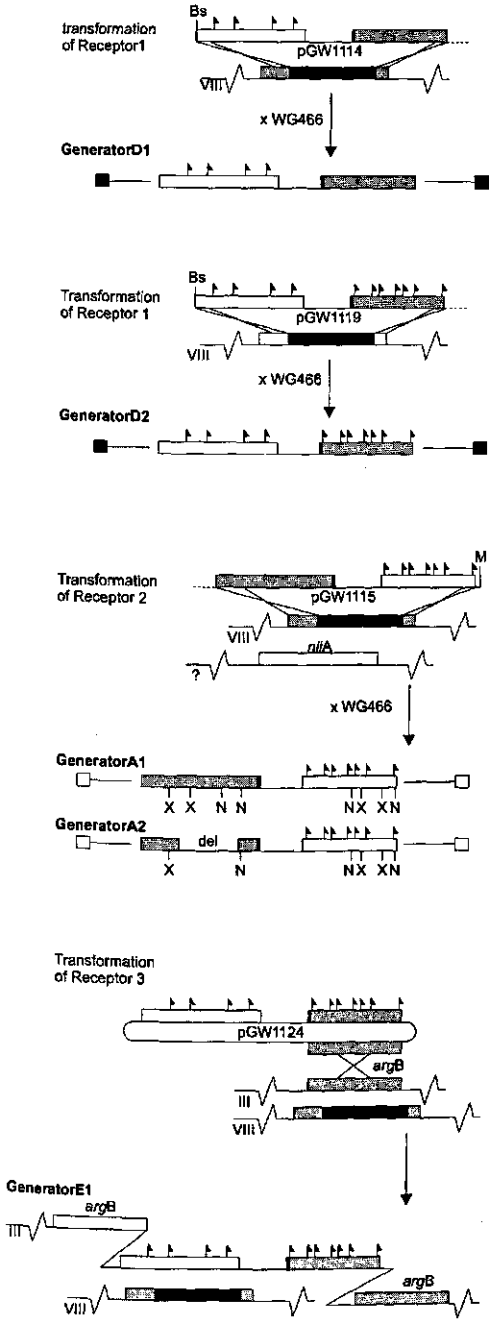
C. Mutagenesis and construction of plasmids for transformation



Receptor1 was transformed with pGW1102 and transformants were selected on nitrite as the sole nitrogen source and phleomycin. Ectopic integration of the *nii*A gene in a transformant designated Receptor2 was confirmed by Southern analysis. Receptor3, carrying the *arg*B2 allele essential for site-directed ectopic integration of the *nii*A and *nia*D genes, was selected among the progeny of a cross between Receptor 1 and WG474 (Table 5).

C. Mutagenesis of the *nii*A and *nia*D genes and construction of plasmids for transformation: The inserts of the plasmids pGW1102 and pGW1103 were cloned into the pAlter vector for subsequent mutagenesis. The pAlter vector containing the *nii*A gene was mutagenized using the primers for markers A1-4, resulting in pGW1108 and with the primer for marker A0, resulting in pGW1109. The pAlter vector containing the *nia*D gene was mutagenized with the primers for markers D1-7, resulting in pGW1110, with the primer for marker D0, resulting in pGW1111. The insert of pGW1110 was recloned into pAlter to allow a second round of mutagenesis with the primer for D0, resulting in pGW1117. The insert of pGW1111 was cloned into the *Bam*HI site of pGW1108, resulting in pGW1114. This plasmid contained the *nii*A-*nia*D region with 4 silent mutations in the *nii*A gene and a stop mutation in the *nia*D gene. The insert of pGW1110 was cloned into the *Bam*HI site of pGW1109, resulting in pGW1115. This plasmid contained the *nii*A-*nia*D region with a stop mutation in the *nii*A gene and 7 silent mutations in the *nia*D gene. The insert of pGW1117 was cloned into the *Bam*HI site of pGW1108, resulting in pGW1119, containing all eleven silent mutations and a stop mutation in the *nia*D gene. To direct integration of the mutagenized *nii*A and *nia*D genes to the *arg*B locus on chromosome III, a 3.5 kb *Xba*I fragment of pGW1507 containing a non-functional *arg*B gene was cloned into the *Xba*I site of pHSS20, resulting in pGW1120. The *arg*B gene in pGW1507 was previously mutagenized by filling in of a *Bgl*II site. Subsequently, the inserts of pGW1108 and pGW1117 were cloned into the polylinker of pGW1120. The resulting plasmid, designated pGW1124, contained the *nii*A-*nia*D region with all silent mutations and a stop mutation in the *nia*D

D. Construction of the parental strains



gene, and the non-functional copy of the *argB* gene. The direction of transcription was identical for the *argB* gene and the *niaD* gene.

D. Construction of the parental strains: Receptor1 was transformed with pGW1114, which had been linearized with *Bst*EII, and transformants growing on nitrite as the sole nitrogen source were screened for phleomycin resistance. Transformants sensitive to phleomycin were submitted to Southern analysis and one transformant showing hybridization patterns consistent with replacement of the prc by the mutagenized *niiA-niaD* region was crossed to WG466 to introduce the *facC* and *brlA* outside markers. A recombinant with the desired genetic constitution was identified among the progeny and designated GeneratorD1. To obtain the strain GeneratorD2, pGW1119 was used as the transforming plasmid in a similar set of experiments. Receptor2 was transformed with pGW1115, linearized with *Mlu*I. Transformants were selected by growth on nitrate and susceptibility to phleomycin. Four such transformants were analyzed by Southern blotting and hybridization. Two of these showed patterns consistent with a replacement of the phleomycin construct by the insert of pGW1115. The other two transformants apparently contained a deletion in the *niiA* gene of 2 kb. On the level of restriction site analysis, these deletions appeared identical and included an *Xho*I and an *Nco*I site. One of the strains without the deletion and one with the deletion were crossed to WG440 to eliminate the ectopic, functional copy on the *niiA* gene. Among the progeny of these crosses, the strains GeneratorA1 (without the deletion) and GeneratorA2 (with the deletion) were selected. No linkage between the ectopic *niiA* gene and the *niiA-niaD* locus was observed. Receptor3 was transformed with pGW1124 and transformants were selected on medium without arginine and with nitrite as the sole nitrogen source. Transformants were subjected to Southern analysis and one transformant showing patterns consistent with a single-copy integration at the *argB* locus was designated GeneratorE1. Note that it is unknown which of the two copies of the *argB* gene in GeneratorE1 is functional.

The mass was filtered over glasswool and the resulting suspension of conidiospores and ascospores was washed twice with TS (2% Tween 80, 0.9% (w/v) NaCl) and resuspended in a small volume of TS. Spores were routinely counted using a hemocytometer; approximately half of the spores were ascospores. Plates selective for recombinants able to grow on nitrate were inoculated with aliquots of the spore suspension. Parental strains in each cross carried either the *biA*1 marker (on chromosome I) or the *pyro*A4 marker (on chromosome IV); these vitamin markers were not supplemented during selection. Therefore, assuming random segregation of chromosomes at meiosis I, we expected a loss of 75% of the recombinants. However, the selective conditions also abolished background growth of conidiospores and uniparental ascospores. We further assumed a negligible frequency of mitotic recombination. Mitotic recombination would have resulted in large numbers of identical recombinant conidiospores; no such uniform subpopulations were observed among the recombinants analyzed. The number of viable ascospores was estimated by plating serial dilutions of the spore suspension on plates without vitamins and with urea as the nitrogen source. After 2-3 days at 37°, recombinants were transferred to fresh plates, which were incubated at 26° to allow sporulation of colonies harboring the temperature-sensitive *brlA*42 allele. Single spore colonies were obtained and tested for all genetic markers on chromosome VIII.

Isolation of mitotic recombinants

Recombinant diploid derivatives of the parental diploid were isolated by means of a plate-assay (Osman *et al.* 1993). Spores of the parental diploid were placed in the center of an SN plate. The phenotype of the parental diploid on SN is a sparse growth almost without sporulation. Recombinants were detected as mycelial patches producing conidiospores after 1-2 weeks of incubation at 37°C. SN plates with approximately 10 recombinant conidiospore patches were selected for further analysis and the recombinants were purified on fresh SN plates. Recombinants were haploidized on CM plates with 3 mg/ml benlate at 37°C; haploid sectors (recognized by their yellow or white spore color, or their bristle conidiophores) were purified on SM plates (supplemented for all genetic markers of DIP1). At least ten haploid derivatives of each diploid recombinant were tested for all genetic markers.

MOLECULAR ANALYSIS OF RECOMBINANTS

Isolation of genomic DNA (miniprep)

Conidiospores of individual recombinant colonies (or haploid derivatives of diploid mitotic recombinants) were used to inoculate 2 ml MM in a 10 ml tube. Cultures were incubated overnight at 37°. The mycelial layer was blotted dry on filter paper, transferred to a fresh tube, and frozen in liquid nitrogen. The frozen material was disrupted with a grinder and 500 µl extraction buffer (500 mM NaCl, 10 mM EDTA, 1% SDS, 10 mM Tris-HCl (pH 7.5)) was added. The suspension was incubated at 65° for 30 min with occasional mixing, extracted with phenol/chloroform (1:1), and the nucleic acid fraction was precipitated with 2 volumes of ethanol. The solution was centrifuged to collect the precipitate and the pellet was dissolved in 40 µl water. One µl of this solution was used as a template in each of two PCR reactions to amplify the *niiA* gene and the *niaD* gene.

Amplification of the *nii*A and *nia*D genes

PCR reaction mixtures contained 100-500 ng genomic *A. nidulans* DNA as template, 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1 unit Taq polymerase (Gibco BRL), 0.2 μM of each primer in a volume of 100 μl. The reaction mixture was overlaid with 100 μl of silicon oil. For amplification of the *nii*A gene, primers MBL312 and OHT2B were used under the following cycling conditions: 2 minutes at 94°C, 30 sec at 54°C, 7 minutes at 72°C (1 cycle), 30 sec at 94°C, 30 sec at 54°C, 7 minutes at 72°C (33 cycles), 30 sec at 94°C, 30 sec at 54°C, 10 minutes at 72°C (1 cycle). For amplification of the *nia*D gene primers MBL516 and OHT1B were used under the following cycling conditions: 2 minutes at 94°C, 30 sec at 59°C, 5 minutes at 72°C (1 cycle), 30 sec at 94°C, 30 sec at 59°C, 5 minutes at 72°C (33 cycles), 30 sec at 94°C, 30 sec at 59°C, 7 minutes at 72°C (1 cycle).

Analysis of PCR products

The amplification products were concentrated by ethanol precipitation and subjected to restriction enzyme analysis. Presence or absence of the point mutations introduced into the *nii*A gene could be detected by digestions with *Xho*I (markers A2 and A4) or *Eag*I (markers A1 and A3), those in the *nia*D gene by digestions with *Apa*I (markers D6 and D7), *Eag*I (markers D3 and D4) or *Nsp*V (markers D1, D2, and D5) and subsequent gel electrophoresis.

Statistical analysis

The computer program Prism 2.1 (GraphPad Software, San Diego, CA, USA) was used to calculate P values.

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CURRICULUM VITAE

Johannes Pieter Thijs werd op 25 december 1964 te Amsterdam geboren. Na het behalen van het VWO-B diploma aan het Herman Wesselink College te Amstelveen begon hij in 1983 aan zijn studie Biologie aan de Vrije Universiteit te Amsterdam (VU). Praktijkervaring werd opgedaan bij de vakgroepen Moleculaire Microbiologie en Toegepaste Genetica van de VU, bij het bedrijf Mogen B.V. te Leiden, en (met een EMBO beurs) bij het John Innes Institute te Norwich (GB). In 1989 werd zijn studie afgerond met het doctoraal examen. In 1990 werd hij aangesteld als wetenschappelijk medewerker bij het Instituut voor Toepassing van Atoomenergie in de Landbouw (ITAL-DLO) te Wageningen, en met een reorganisatie verplaatst naar het Instituut voor Plantenziektekundig Instituut (IPO-DLO) te Wageningen. Tijdens deze periode heeft hij gewerkt binnen een projekt dat de bestrijding van wortelknobbelaaltjes in landbouwgewassen tot doel had. Met een OECD fellowship werd 2 maanden onderzoek gedaan bij de afdeling Genetica van de Rijksuniversiteit Gent. In 1992 begon hij als AiO aan het onderzoek dat beschreven is in dit proefschrift bij de Vakgroep Erfelijkheidsleer (nu Laboratorium voor Erfelijkheidsleer) van de Landbouwuniversiteit Wageningen.