Sexual Development Genes of *Neurospora crassa*

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ABSTRACT

The filamentous fungus *Neurospora crassa* undergoes a complex program of sexual development to form a fruiting body composed of several kinds of specialized tissue. Subtractive hybridization was used to isolate genes that are expressed preferentially during this sexual phase. Many such *sexual development* (*sdv*) genes were identified in a cosmid library of Neurospora genomic DNA. Fourteen of the *sdv* genes were subcloned, and their expression in mutant strains and under crossing and vegetative growth conditions was examined. All of the regulated transcripts were less abundant (and in many cases not detectable) in strains grown under vegetative (high nitrogen) conditions, suggesting that nitrogen starvation is required for their synthesis. The expression of most of the *sdv* genes also required a functional *A* mating type product, even under crossing growth conditions, suggesting that this product functions as a master control in sexual development. To determine if the products of the *sdv* genes play essential roles in the sexual cycle, a reverse-genetic approach (based on RIP (*r*epeat-*i*nduced *p*oint mutation)-mediated gene disruptions) was used to create mutations in the genes. A mutant strain (*asd-1*) with a recessive crossing defect (apparently caused by the RIP process) was isolated; in this strain, early development is normal and many asci are formed, but ascospores are never delineated. A second recessive mutant strain (*asd-2*) was apparently created by ectopic integration of the transforming DNA into a gene required for the sexual process; in this strain the sexual process was blocked at an early stage, and the ascogenous tissue underwent little development.

NEUROSPORA crassa is a heterothallic filamentous fungus that undergoes a complex pattern of sexual differentiation to form the female reproductive structure (protoperithecium) when subjected to conditions of nitrogen starvation, light and low temperature (PERKINS and BARRY 1977). The protoperithecium consists of a coiled multicellular hypha (or ascogonium) enclosed within a coiled aggregate of hyphae. A specialized hypha (trichogyne) extends from the protoperithecium and fuses with the fertilizing cell. *N. crassa* has two distinct mating types (*A* and *a*), and fusion with the opposite mating type is required for fertilization of the protoperithecium. After fertilization, the protoperithecium enlarges and differentiates to form a fruiting body (or perithecium), consisting of several specialized tissues, from which the meiotic products (ascospores) are eventually ejected.

Some of the genes whose products regulate sexual development in Neurospora have been identified by mutation. The two mating type genes, *a* and *A*, have been cloned and sequenced (GLASS, GROTELUESCHEN and METZENBERG 1990; STABEN and YANOFSKY 1990). The product of the *fmf-1* (*female-male fertility*) gene must be functional in both the male and female parent to allow normal sexual development; when one parent harbors a dysfunctional *fmf-1* gene, sexual development aborts shortly after fertilization (JOHNSON 1979). The *wc* (*white collar*) genes encode products required for all of the blue light-induced phenomena of Neurospora, including the formation of the female reproductive structure and the phototropism of the perithecial beaks (DEGLI INNOCENTI and RUSSO 1983; HARDING and MELLES 1983).

A large number of genes affecting sexual development in *N. crassa* have been identified by mutation; well over 100 such genes have been characterized and mapped (PERKINS *et al.* 1982). However, the products of most of those genes are probably not directly involved in sexual development. Many auxotrophic mutants of Neurospora cause female sterility, even when the medium is appropriately supplemented; this reflects the fact that most or all of the perithecial tissue is derived from the female parent (HOWE and JOHNSON 1976).

Since sexual development in *N. crassa* is quite sensitive to changes in basic metabolism, and we wished to study only genes directly involved in the sexual process, we chose not to analyze previously identified mutants affecting sexual development. Instead, we have isolated genes that are expressed specifically in the early stages of the sexual differentiation process. Many such *sdv* genes were identified, and the factors

This paper is dedicated to the memory of Professor WALTER PLAUT.

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TABLE 1

N. crassa strains used in transcript analyses

Strain description	Genotype	Source
Wild type	74-OR23-IV *A*	FGSC 2489
a tol	*pan-1 nic-3 tol a*	RLM 58-13[a]
a tol/A tol heterokaryon	[*pan-1 nic-3 al-1 tol A*] + [*pan-1 inl tol a*]	RLM 58-12 + RLM 58-14[a]
A^{m44} sterile mutant	*un-3 ad-3A nic-2 cyh-1* A^{m44}	FGSC 4570[b]
fmf-1	*fmf-1 pyr-3 A*	FGSC 3108[c]

[a] NEWMEYER (1970).
[b] GRIFFITHS (1982).
[c] JOHNSON (1979).

regulating their expression were examined.

Our eventual goal is the elucidation of the sexual differentiation process of *N. crassa* at the molecular level, including the identification of regulator and target genes, and a determination of their modes of action and physiological significance. We have begun with a functional analysis of fourteen *sdv* genes, most of which are target genes controlled by the *A* mating type product. To determine if the products of these crossing-specific genes play essential roles in the sexual cycle, we used a reverse-genetic approach.

CASE (1986) showed that ectopically inserted copies of the *N. crassa qa-2*$^+$ gene are often inactivated in meiotic progeny (although the transforming sequences are still present). SELKER and colleagues have shown that the duplicated sequences are disrupted premeiotically in a cross, and that the disruption is caused by multiple G-C to A-T transition mutations in the duplicated regions; this process has been termed RIP, for *r*epeat-*i*nduced *p*oint mutation (SELKER and GARRETT 1988; CAMBARERI *et al.* 1989). Neurospora is a haploid organism, so most genes are normally present in one rather than two copies within the nucleus. The RIP process probably involves a pairing of the affected sequences, since in strains harboring three copies of a gene, one of the copies often escapes unaltered, while in strains with duplicated copies, both copies are mutated (FINCHAM *et al.* 1989). Using the RIP process, one can analyze the function of any cloned gene.

We have used the RIP-based reverse genetic technique to analyze the functions of 14 *sdv* genes. To determine if the products of these crossing-specific genes play essential roles in the sexual cycle, we subjected the genes to RIP-mediated gene disruption, and examined the meiotic progeny for dominant and recessive defects in sexual development.

MATERIALS AND METHODS

Strains: The strains of *Neurospora crassa* used for transcript analyses are listed in Table 1; FGSC indicates the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, and RLM indicates laboratory strains of R. L. METZENBERG. The reason for inclusion of the *tol* mutation in some of the strains requires comment. In order to biochemically mimic the typical crossing reactions, and yet allow at least slow growth of the organism, a nutritionally forced heterokaryon between the *a* and *A* mating type strains was prepared. While such heterokaryons are formed readily by hyphal fusion, they are normally unable to grow out vegetatively because of a mutually semilethal incompatibility between the two mating types. A recessive mutation at the *tol* locus reduces the degree of incompatibility so that such mixed mating type heterokaryons survive and grow (NEWMEYER 1970), albeit more slowly than does a wild-type strain. Hence *tol* was included in both partners of the heterokaryon, and for an appropriate control, in the homokaryon to which it was compared.

The pSdv constructs were introduced into *qa-2 aro-9 inl al-2 a* (RLM 63-01) spheroplasts, and *qa-2*$^+$ transformants were selected. Derivatives of the *qa-2*$^+$ transformants were obtained by isolation of single conidia, and examination of those isolates for segregation of *qa-2*. These derivatives were judged to be homokaryotic when they failed to segregate conidia unable to grow without the aromatic amino acid supplement. The homokaryotic transformant strains are referred to as T-sdv-1a, T-sdv-1b (two independent transformants obtained with pSdv-1), etc. Restriction fragment length polymorphism (RFLP) experiments were carried out with DNAs of FGSC strains 4411, 4416 and 4450 through 4487. The *N. crassa* wild type [74-OR23-IV *A* (FGSC 2489) and ORS*a* (FGSC 2490)], multicent-2 *a* (*un-2 arg-5 thi-4 pyr-1 lys-1 inl nic-3 ars-1 a*; FGSC 4488) and *fluffy* (*fl a*; FGSC 4347 and *fl A*; FGSC 4317) strains were also used. The strains that were used for the RIP crosses are listed in Table 5. *Escherichia coli* NM522 (GOUGH and MURRAY 1983) was the host for bacterial transformations.

Materials: Cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase was obtained from Bethesda Research Laboratories. The random primers (pd$(N)_6$) and oligo(dT)-cellulose were from Pharmacia. [^{32}P]dCTP and [^{35}S]dATP were obtained from Du Pont, Zetabind membrane from AMF Cuno, Inc. (Meriden, Connecticut), and restriction and other enzymes from Promega.

Preparation of RNA: RNA isolation from *N. crassa* cultures was as described by LUCAS, JACOBSON and GILES (1977). Polyadenylated RNA was isolated by chromatography on oligo(dT)-cellulose according to AVIV and LEDER (1972).

In growth of cultures for RNA preparations, conidia were inoculated at 10^6/ml. For RNAs from crossing conditions, strains were grown in crossing medium plus 1% sucrose, 25 μg/ml ampicillin and any necessary supplements (WESTERGAARD and MITCHELL 1947). Unless otherwise noted, growth was in 125-ml Erlenmeyer flasks containing 20 ml of medium (to ensure adequate aeration and provide a large

surface area), and strains were incubated without agitation at 25° in the light. Different strains were grown until they had attained roughly the same mass, or about 10 mg wet weight per ml of culture. The heterokaryotic strain [*pan-1 nic-3 al-1 tol A* (RLM 58-12)] + [*pan-1 inl tol a* (RLM 58-14)] was grown under selective conditions (with pantothenic acid, but in the absence of nicotinamide and inositol); this strain grew slowly and required about four days to attain the desired mass. The other strains were grown in crossing medium for 2–3 days. When the heterokaryotic strain was grown with agitation in crossing medium, it was grown at 25° for 19 hr. Vegetative RNA was isolated from strains grown with agitation at 25° for 13 hr; the *pan-1 nic-3 tol a* (RLM 58-13) strain was grown in 1 × Vogel minimal medium (DAVIS and DE SERRES 1970) plus 2% sucrose, 10 μg/ml calcium pantothenate, 10 μg/ml nicotinamide and 25 μg/ml ampicillin, and the heterokaryotic strain was grown in the same medium lacking nicotinamide.

Preparation of labeled cDNAs: RNA was prepared from the heterokaryotic strain [*pan-1 nic-3 al-1 tol A*] + [*pan-1 inl tol a*] grown without agitation in crossing medium, as described above. Polyadenylated RNA was prepared by two cycles of purification on oligo(dT)-cellulose columns. Random primer cDNA of high specific activity was synthesized with M-MLV reverse transcriptase and 1 μg of poly(A)$^+$ RNA, as described by SARGENT (1987). After the reaction was stopped, the RNA was hydrolyzed and the unincorporated label was removed by chromatography on Sephadex G-50 (SARGENT 1987).

Subtractive hybridization: The driver RNA was obtained from the *pan-1 nic-3 tol a* (RLM 58-13) strain grown under vegetative conditions (above); the corresponding poly(A)$^+$ RNA was cycled twice on oligo(dT)-cellulose columns. The labeled cDNAs were precipitated with a large excess (44 μg) of driver vegetative RNA. The mixture (in an 18-μl reaction volume) was transferred to a sterile glass capillary tube, sealed, and denatured in boiling water for 5 minutes, after which hybridization was carried out at 68° for 60 hr (SARGENT 1987).

Chromatographic separation of single-stranded cDNAs: The double-stranded cDNA-RNA hybrids were separated from the unhybridized cDNAs by hydroxylapatite chromatography as described by JACKSON and FELSENFELD (1987). The single-stranded cDNAs were eluted from the hydroxylapatite by four washes with 150 mM NaH_2PO_4, and the cDNA-RNA hybrids were eluted with 500 mM NaH_2PO_4. The single-stranded cDNAs were concentrated, and short cDNA products were removed by chromatography on Sepharose CL-6B in NETS buffer (SARGENT 1987).

Northern (RNA) and Southern hybridization blots: Northern blot procedures were those of FOURNEY *et al.* (1988). Hybridization conditions were as previously described (NELSON *et al.* 1989). Probes were labeled using the random priming method (FEINBERG and VOGELSTEIN 1983). The control *am* probe (*glutamate dehydrogenase*; KINNAIRD *et al.* 1982) that was used in Northern hybridizations was a 2-kbp *Eco*RI-*Bam*HI fragment from pBJ011 (MANN *et al.* 1988). Screening of the cosmid library (ordered in 32 microtiter plates) with the crossing-specific cDNA probes was performed as described by VOLLMER and YANOFSKY (1986). Genomic Neurospora DNA was isolated as described by STEVENS and METZENBERG (1982). Southern hybridizations were performed as described by MANIATIS, FRITSCH and SAMBROOK (1982).

Construction of the pMSN1 vector and recombinant plasmids: The pMSN1 vector (Figure 1) was constructed to facilitate the cloning of the *sdv* genes and the subsequent Neurospora transformation experiments. The selectable

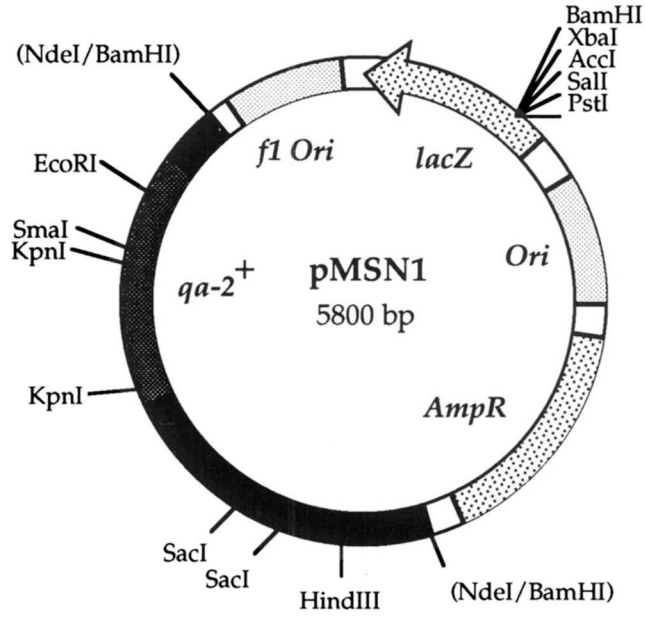

FIGURE 1.—Map of the pMSN1 vector (approximately 5.8 kbp). The light regions represent the pGEM-3Zf(+) vector, and the dark regions the Neurospora insert containing the *qa-2*$^+$ gene. The *Nde*I and *Bam*HI sites that were destroyed by filling-in the recessed 3′ ends are indicated in parentheses. The restriction enzymes within the multiple cloning site that cut the vector only once are shown (expanded on the right side of the plasmid); the *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Ava*I, *Hinc*II, *Sph*I, and *Hin*dIII sites of the multiple cloning site are not indicated, since these enzymes also cut within the Neurospora sequence. The direction of transcription of the *lacZ* (*β-galactosidase*) gene is denoted by an arrow. The positions of the origin of replication for growth in *E. coli* (Ori), the filamentous bacteriophage f1 origin of replication (f1 Ori) and the ampicillin resistance gene (*AmpR*) are shown. Selected restriction sites within the Neurospora sequence are indicated. The plasmid is drawn approximately to scale.

qa-2$^+$ gene of *N. crassa* was inserted into a noncoding region (the *Nde*I site) of the parental pGEM-3Zf(+) (Promega). The vector was digested with *Nde*I, the recessed 3′ ends were filled in with the Klenow fragment of DNA polymerase I and the blunt-ended molecules were phosphatase-treated (COBIANCHI and WILSON 1987). An approximately 2.5-kbp *Bam*HI fragment containing the *qa-2*$^+$ gene (SCHWEIZER *et al.* 1981) was made blunt with Klenow fragment, gel-purified and ligated to the prepared pGEM-3Zf(+) vector (COBIANCHI and WILSON 1987). The 5.8-kbp pMSN1 plasmid was isolated after transformation of NM522 with the ligation mixture (MILLER 1987).

Subclones (pSdv-1 through pSdv-14) of cosmids encoding crossing-specific genes were isolated by inserting *sdv* fragments into the multiple cloning site of pMSN1 and screening for white colonies on plates containing ampicillin; the identities of the inserts were confirmed by restriction mapping. The *sdv* fragments with *Eco*RI extremities were filled in with the Klenow fragment of DNA polymerase I and cloned blunt into the filled-in *Sal*I site of the pMSN1 multiple cloning region (COBIANCHI and WILSON 1987).

Transformation: Preparation of *qa-2 aro-9 inl al-2 a* spheroplasts was as described by SCHWEIZER *et al.* (1981). Transformation with the recombinant clones purified through low melting point agarose was according to LIU and DUNLAP (1988), except that transformants were regenerated in liquid medium and spread onto selection plates

(KUIPER *et al.* 1988). Transformations of *E. coli* were as described by MILLER (1987).

Media and culture conditions: The *qa-2*$^+$ transformants were selected on 1 × Vogel (DAVIS and DE SERRES 1970), 1% sorbose, 0.05% glucose, 0.05% fructose, 50 μg/ml inositol, 1.5% agar plates. All crosses were performed on crossing medium containing 1% sucrose and any needed supplements (WESTERGAARD and MITCHELL 1947). The progeny of the crosses were grown on 1 × Vogel, 1% sorbose, 0.05% glucose, 0.05% fructose, 1.5% agar plates.

Fertility tests: The meiotic progeny from the RIP crosses were crossed to wild type as both female (protoperithecial) and male (fertilizing) parents to detect any dominant effects on crossing. The progeny were first crossed as males. A concentrated drop of conidia of the progeny was spotted onto *fl a* and *fl A* tester strains grown on plates with crossing medium. After two days the mating types of the progeny (as evidenced by the formation of perithecia on the *fl a* or *fl A* plate) were recorded, and the perithecia were covered with small plastic caps. Fourteen days after fertilization the crosses were examined for the presence of ejected ascospores on the plastic caps. The progeny were also grown at room temperature in the light as the protoperithecial parent in small (10 × 75 mm) tubes containing 1 ml of crossing medium plus 50 μg/ml inositol, and fertilized after seven days with concentrated suspensions of wild-type conidia of the opposite mating type. The production of perithecia was noted 2 days after fertilization, and the crosses were examined for ejected ascospores fourteen days after fertilization. The meiotic progeny were also crossed with other progeny from the same cross, to detect recessive mutations.

Microscopy: Perithecia were removed from crosses seven days post-fertilization, cleaned of mycelial debris, and placed in small drops of water on microscope slides. The rosettes of asci were extruded from the fruiting bodies into the water, the perithecial debris was removed, and a cover slip was applied. Microscopic analysis was carried out shortly after sample preparation. Nomarski interference (differential interference) photomicrographs were taken using a Zeiss Universal microscope and Kodak Technical Pan film.

RESULTS

Identification of cosmids encoding *sexual development* genes: Formation of the female reproductive structure in *N. crassa* occurs efficiently only in strains grown under conditions of nitrogen starvation, low temperature (25° or less), and blue light illumination (DEGLI INNOCENTI and RUSSO 1983; PERKINS and BARRY 1977). Also, the protoperithecia form only on the surface of the growing tissue. In selecting parameters for the growth of strains under crossing conditions, we attempted to mimic these requirements as closely as possible and to at the same time choose conditions that would allow efficient RNA isolation. Given these considerations, strains were grown in liquid crossing medium (at 25° in the light) as floating mycelial mats, and the surface to volume ratio was maximized (MATERIALS AND METHODS). Different strains were harvested after 2–4 days, when they had attained roughly the same amount of total growth. Since protoperithecial development begins about 36 hr after inoculation on crossing medium (ROTHSCHILD and SUSKIND 1966), the mycelial mats should contain significant amounts of transcripts specific to sexual development.

Polyadenylated RNA was isolated from total RNA prepared from an *a tol/A tol* heterokaryotic strain ([*pan-1 nic-3 al-1 tol A*] + [*pan-1 inl tol a*]) grown under crossing conditions. Labeled cDNAs of high specific activity were synthesized [with M-MLV reverse transcriptase using random primers (SARGENT 1987)] from those mRNAs. Random primers were used instead of oligo(dT) primers to avoid any bias toward 3′-proximal sequences. The labeled cDNAs were hybridized with a large excess of mRNA prepared from a vegetatively growing culture, and the single-stranded cDNAs (those not corresponding to transcripts present during vegetative growth) were separated by hydroxylapatite chromatography. About one-half of the labeled cDNAs were present as single-stranded species after the subtractive hybridization (and the remaining half occurred in cDNA-RNA hybrid form). The large amount of unhybridized cDNAs was unexpected, and could have been due at least partially to the presence of cDNAs corresponding to the ribosomal RNAs (as discussed further below).

The labeled cDNAs (enriched for sexual development sequences) were used to probe a cosmid library containing large inserts (about 40 kbp) of genomic Neurospora DNA (VOLLMER and YANOFSKY 1986). Hybridization signals ranging from quite strong to weak were observed for about 7% of the 3,072 cosmids. Of the positive signals, 55 corresponded to ribosomal DNA-containing cosmids, and these were not further analyzed. The presence of rDNA-recognizing cDNAs among the labeled population could have been at least partly caused by the use of random primers rather than oligo(dT) primers in the cDNA synthesis. Among the other positive signals, the fifty most strongly hybridizing cosmids were selected for additional study.

Characterization of cosmids recognized by crossing-specific cDNAs: In the initial screening of the 50 strongly hybridizing non-rDNA cosmids, the entire cosmid sequences were used as probes in Northern hybridizations. Test strips containing the poly(A)$^+$ RNAs that were used in the subtractive hybridization (cycled once rather than twice on oligo(dT)-cellulose columns) were prepared. Each test strip consisted of two lanes of separated mRNAs, containing 1.5 μg of vegetative transcripts (from the *a tol* strain) or 1.5 μg of crossing transcripts (from the *a tol/A tol* heterokaryon, grown on crossing medium), respectively. The strains that were used are listed in Table 1, and details of growth conditions are described in MATERIALS AND METHODS.

Since the entire cosmids were used in the Northern hybridizations, multiple transcripts were recognized in most of the hybridizations (not shown). Many of

TABLE 2

Cosmids encoding *sdv* genes

Cosmid[a]	No. of crossing-specific transcripts[b]	*sdv* genes[c]	Overlapping cosmids
3:4D	One		
4:3A	Three	*sdv-1, sdv-2, sdv-3*	
5:6F*	Two	*sdv-4, sdv-5*	4:12G, 21:4D
6:11E	One		
8:9A*	Two	*sdv-6*	20:5E
10:4A	One		
10:10F	Two		
11:5F	One		
12:2E*	Several	*sdv-7, sdv-8, sdv-9*	1:1A, 2:2B, 5:4D, 5:11D, 7:7F, 7:10G, 9:3F, 13:12F, 14:5D, 15:6F, 22:11C
12:7B	Two	*sdv-10*	
12:9D	One		
14:1H*	One		6:11D
15:11H	Two		
19:3B	One		
20:11A*	Three	*sdv-11, sdv-12*	13:3F, 20:12F
28:11F*	Two		28:11G
28:12G	Two	*sdv-13, sdv-14*	

[a] Repeatedly isolated (overlapping) cosmids are indicated by an asterisk. Each is listed only once, regardless of the number of times isolated, and the overlapping cosmids that were also detected in the screening with crossing-specific cDNAs are indicated in the rightmost column.

[b] The number of crossing-specific transcripts (defined as those detected in poly(A)$^+$ RNA from the *a tol/A tol* heterokaryon grown in stationary crossing medium, and either greatly reduced in amount or not detectable in *a tol* grown under vegetative conditions) encoded by the respective cosmid is indicated.

[c] When restriction fragments of single *sexual development* genes were isolated and further studied, those *sdv* genes are indicated here.

the cosmids encoded transcripts that were present in equal amounts under crossing and vegetative conditions (constitutively expressed genes), as was expected given the average size of the Neurospora genomic DNA inserts (about 40 kbp). Of the 50 cosmids, 35 also encoded transcripts whose levels were increased, and in many cases only detectable, under crossing conditions (Table 2). These species were defined as sexual development transcripts.

Overlapping cosmids were defined as such based on the transcripts that they encoded and on restriction mapping results (not shown). Six of the 35 DNA sequences present in cosmids encoding sexual development transcripts were repeatedly isolated (as overlapping cosmids), accounting for a total of 24 cosmids, while 11 were isolated only once (Table 2). The remaining 15 of the 50 original cosmids (including three that were isolated twice) did not appear to encode transcripts preferentially expressed during the sexual phase; however, low abundancy crossing-specific transcripts or those comigrating with a constitutive transcript might have been overlooked in the screening (especially since entire cosmids were used as probes).

Many of the *sexual development* genes appeared to be clustered, as most of the cosmids apparently encode two or three genes that were expressed only under crossing conditions. The 17 different (nonoverlapping) cosmids that were identified seem to encode about 30 *sexual development* genes.

Identification of *sexual development* (*sdv*) genes: Northern analyses were used to identify cosmid restriction fragments that encode sexual development transcripts. For this analysis, four different strains (wild type, the *a tol/A tol* heterokaryon, an A^m sterile mutant and *fmf-1*; Table 1) were grown under crossing conditions, and one of the strains (the *a tol/A tol* heterokaryon) was also grown under vegetative conditions and with agitation in crossing medium. Northern blots containing separated mRNAs from these six conditions were prepared, and the blots were probed with random primer-labeled isolated restriction fragments from seven of the cosmids encoding crossing-specific RNAs. Those fragments recognizing crossing-specific transcripts were considered to contain *sdv* (or *sexual development*) genes (Table 3); the fragments could contain all or just part of the respective *sdv* gene. The Northern hybridization results that were obtained with *sdv-1* through *sdv-14* are shown in Figure 2. This figure also shows that the transcript from the constitutively expressed *am* gene (*glutamate dehydrogenase*; KINNAIRD *et al.* 1982) was present at similar levels in all of the mRNA populations that were examined.

Characterization of *sdv* transcripts: The Northern analysis (Figure 2) allowed us to distinguish different patterns of expression of the *sdv* genes. The amounts of all of the *sdv* transcripts were reduced (and in many cases not detectable) in strains grown with adequate nitrogen, suggesting that nitrogen starvation is one of

TABLE 3

***Sexual development* (*sdv*) genes and their regulated transcripts**

Gene	Fragment[a]	Cosmid[b]	Transcript size (kb)
sdv-1	3-kbp *Bam*HI-*Pst*I	4:3A	1.4
sdv-2	2.6-kbp *Pst*I	4:3A	2.5
sdv-3	5.5-kbp *Bam*HI	4:3A	1.4
sdv-4	12-kbp *Sal*I	5:6F	2.2
sdv-5	2.8-kbp *Sal*I	5:6F	2.2
sdv-6	12-kbp *Eco*RI-*Bam*HI	8:9A	2.0
sdv-7	7-kbp *Bam*HI	12:2E	1.5
sdv-8	3.4-kbp *Bam*HI	12:2E	1.6 and 1.8[c]
sdv-9	0.8-kbp *Bam*HI	12:2E	3.3
sdv-10	1.4-kbp *Bam*HI	12:7B	2.0
sdv-11	4.8-kbp *Pst*I	20:11A	4.0
sdv-12	1.8-kbp *Pst*I	20:11A	2.8
sdv-13	12-kbp *Eco*RI	28:12G	2.6
sdv-14	3-kbp *Eco*RI	28:12G	2.4

[a] Restriction fragment containing at least part of the crossing-specific gene; the sizes are approximate.
[b] Cosmid source of the fragment.
[c] These two transcripts are probably overlapping.

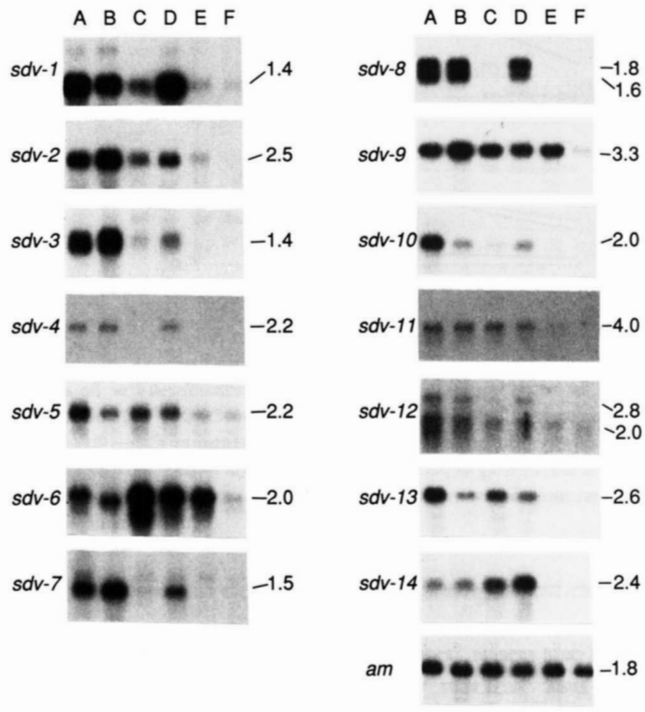

FIGURE 2.—Northern (RNA) blots showing the expression of the crossing-specific (*sdv*) genes and the constitutively expressed *am*$^+$ gene. Poly(A)$^+$ RNA was isolated from the designated strains grown under crossing or vegetative growth conditions (see MATERIALS AND METHODS). Samples (1 μg) of each RNA were separated on 1% agarose-0.66 M formaldehyde gels (FOURNEY *et al.* 1988), transferred to Zetabind membranes, and hybridized with the probes noted. The approximate sizes of major transcripts (in kb) are indicated to the right. RNAs in lanes A, B, C and D were prepared from strains grown without agitation in crossing medium, RNA in lane E from the heterokaryotic strain grown with shaking in crossing medium, and RNA in lane F from the heterokaryotic strain grown with shaking in vegetative (Vogel) medium (see MATERIALS AND METHODS). (A) Wild type, (B) *fmf-1*, (C) A^{mi1} sterile mutant, (D, E and F) *a tol*/*A tol* heterokaryon (see Table 1 for detailed strain descriptions).

the signals required for their synthesis and/or accumulation. One of the *sdv* genes (*sdv-9*) appeared to require only nitrogen starvation (and not functional *A* mating type or *fmf-1* genes or other stimuli) for its expression.

Most of the *sdv* genes encode transcripts that required both nitrogen starvation and a functional *A* mating type gene for their expression, and that were not expressed even in crossing medium if the flasks were shaken (see DISCUSSION). The *sdv-1* gene encodes such a transcript of 1.4 kb; a weak transcript of about 2.8 kb could be a precursor of the smaller, more abundant transcript, or could correspond to a flanking crossing-specific gene. The transcript band recognized by *sdv-3* was quite broad, and could correspond to two different species; the amount of this transcript(s) was also reduced in the *a tol*/*A tol* heterokaryon grown without agitation in crossing medium. Two different crossing-specific transcripts were encoded by the *sdv-8* gene. Since smaller fragments of *sdv-8* also recognized both transcripts (not shown), these transcripts are most likely overlapping.

Efficient transcription of the *sdv-6* gene (or accumulation of its transcript) required both starvation for nitrogen and a functional *fmf* gene (see DISCUSSION). The levels of four transcripts (those encoded by the *sdv-5*, *sdv-11*, *sdv-13* and *sdv-14* genes) were reduced in the *a tol*/*A tol* heterokaryon grown in crossing medium with agitation (and also in the *a tol* strain grown with adequate nitrogen). (However, the accumulation of the *sdv-5* and perhaps *sdv-13* transcripts might also be affected by the *fmf-1* product.)

Three *sdv* genes (*sdv-1* to *sdv-3*) were isolated from the 4:3A cosmid. Even though the *sdv-1* and *sdv-3* genes encode transcripts of similar size, they probably correspond to different genes, since the patterns of transcription of the genes were fairly different. Likewise, the two transcripts of about 2.2 kb encoded by the *sdv-4* and *sdv-5* genes (both derived from the 5:6F cosmid) showed quite different transcription patterns, and are probably unrelated sequences.

RFLP mapping of the *sdv* genes: The map positions of the seven cosmids encoding *sdv-1*–*sdv-14* were determined using the RFLP technique. This technique involves the use of both conventional genetic markers and restriction site differences to map essentially any cloned fragment of Neurospora DNA (METZENBERG *et al.* 1985; METZENBERG and GROTELUESCHEN 1990). The segregation of genetic markers and RFLPs is analyzed in the ordered progeny from a cross of a multiply marked laboratory strain (multicent-2 *a*, in Oak Ridge genetic background) with a wild-collected strain (Mauriceville-1c *A*). For this analysis, the cosmids encoding crossing-specific transcripts were linearized with restriction enzymes, random primer-labeled, and hybridized with Southern

TABLE 4

RFLP mapping of the *sdv* genes

	A 1	A|B 4|6	B|C 7|1	C|D 4|5	D|E 7|1	E 3	E 5	E|F 7|1	F|G 3|1	G|H 4|5	H|I 7|6	I|J 8|1	J 4	J|K 4|1	K|L 4|1	L|M 4|5	M|N 8|2	N|O 3|2	O|P 4|1	P|Q 4|2	Q|R 4|1	R 4
LG I																						
Fsr-1	M	O|M	M|M	O|O	M|M	M	O	O|-	O|M	O|M	M|M	M|M	M|O	M|M	O|O	O|O	-|M	O|O	M|O	O|O	M
5:6F	M	O|M	O|M	O|O	M|M	M	O	O|M	O|M	O|M	M|M	M|M	M|O	M|M	O|O	O|O	M|M	O|O	M|O	O|O	M
Fsr-18	M	O|M	O|O	O|O	M|M	M	O	O|M	O|O	M|M	M|O	M|M	O|O	O|M	O|M	O|O	M|O	M|O	M|O	O|O	M
LG IV																						
SPIAE	O	M|O	O|M	O|M	O|O	M	M	O|M	O|M	M|O	O|O	M|M	M|M	M|M	O|M	O|M	O|M	O|O	O|M	M|O	M
4:3A	M	O|O	O|M	M|O	O|M	M	O	O|O	O|M	M|O	O|M	M|M	M|M	M|M	M|M	M|O	O|O	O|O	O|M	M|M	M
Fsr-63	M	M|M	O|M	M|O	O|M	M	O	O|O	O|M	M|O	O|M	M|M	M|M	M|M	M|M	M|O	-|O	O|M	O|M	M|M	M
pyr-1	M	M|M	O|M	M|O	O|M	M	O	O|O	O|M	M|O	O|M	M|M	M|M	M|M	M|M	M|O	O|O	O|M	O|M	O|M	M
20:11A	M	O|M	O|M	M|O	O|M	M	O	O|O	O|M	M|O	M|M	O|M	O|M	M|M	M|M	M|O	O|O	O|M	O|M	O|M	M
Fsr-62	M	O|M	O|M	O|O	O|M	M	O	O|O	O|M	M|O	M|O	O|M	O|M	M|M	M|M	M|M	O|O	M|M	O|M	O|M	M
Fsr-13	M	O|M	O|M	O|O	O|M	M	O	O|O	O|O	M|O	M|O	O|M	O|M	M|M	M|M	M|O	O|O	M|M	O|M	O|M	M
12:2E	M	O|M	O|M	O|O	O|M	M	O	O|O	O|O	O|O	M|O	M|M	O|O	M|M	M|M	M|O	O|O	M|M	O|M	O|M	M
Fsr-4	M	O|M	O|M	O|O	M|M	O	O	M|O	M|O	M|O	O|O	M|O	O|O	M|O	M|O	M|O	-|M	O|O	M|M	M|M	O
LG V																						
Fsr-20	O	M|O	O|M	M|O	M|O	O	M	M|M	M|M	M|M	M|O	M|O	M|M	M|O	M|M	M|M	-|O	M|M	O|O	M|M	O
8:9A	O	M|O	O|M	O|O	M|O	O	M	M|M	M|M	M|M	O|O	M|O	M|M	M|O	M|M	M|M	M|O	M|M	O|O	M|M	O
00015	O	M|O	M|M	O|O	M|O	O	M	M|M	M|M	M|M	O|O	M|O	M|M	M|O	M|M	M|M	M|O	M|M	O|O	M|M	O
LG VI																						
12:7B	O	M|M	M|M	M|O	M|O	O	M	M|M	M|O	O|O	O|O	O|O	M|O	M|M	M|O	O|O	M|M	M|M	M|O	O|M	O
Fsr-50	M	M|M	M|M	M|M	M|O	O	M	M|M	M|O	O|O	O|O	O|O	O|O	O|M	M|O	O|O	O|M	M|M	M|O	O|M	M
con-11a	M	O|-	M|M	M|M	M|O	O	M	M|M	M|-	O|O	O|M	O|O	O|O	O|O	M|O	O|O	M|M	O|M	M|O	O|O	M
LG VII																						
ars-1	O	O|O	O|M	M|M	M|O	O	M	M|O	O|O	O|O	O|O	O|O	O|O	O|M	M|M	M|O	O|M	M|O	O|M	M|O	O
28:12G	O	O|O	O|M	M|M	M|O	O	M	M|O	O|O	O|O	O|O	O|O	O|O	O|M	M|M	M|O	O|M	M|O	O|M	M|O	O

Results are shown for 38 ordered progeny of the multicent-2 *a* (in Oak Ridge background) × Mauriceville-1c *A* cross (abbreviated O and M, respectively). The results obtained with the seven cosmids encoding crossing-specific genes (5:6F, 4:3A, 20:11A, 12:2E, 8:9A, 12:7B and 28:12G), mapping to LG I, IV, V, VI and VII, are shown, as are the results for the markers flanking those cosmids.

blots of digested genomic DNAs from the mapping strains. The results that were obtained are summarized in Table 4.

The cosmids were mapped to five of the seven Neurospora chromosomes. Three of the cosmids mapped to linkage group (LG) IV, but they were not closely linked to each other. The cosmids showed from 3% to 40% recombination with the flanking markers noted, with the exception of 28:12G, which showed less than 3% recombination with the *ars-1* gene (no recombinants among 38 progeny).

Isolation of transformants containing multiple copies of the *sdv* genes: The fourteen *sdv* fragments were cloned into the multiple cloning site of pMSN1, to form the pSdv-1 through pSdv-14 constructs (MATERIALS AND METHODS). In order to obtain strains containing multiple copies of the *sdv* genes (T-sdv strains) for use in RIP-mediated gene disruption experiments, we transformed *qa-2 aro-9 inl al-2 a* spheroplasts to *qa-2*$^+$ with pSdv-1 through pSdv-14. Strains able to grow in the absence of aromatic amino acid supplement were selected, and those strains were screened for the presence of duplicated *sdv* genes. In Neurospora, transformation is usually by nonhomologous integration of the transforming DNA at apparently random sites within the genome. Homologous transformants (in which the transforming DNA is substituted for the corresponding genomic DNA or inserted in tandem at the normal genomic site) are also obtained, but usually at a lower frequency (ASCH and KINSEY 1990).

The number of copies of transforming DNA was examined in 53 independent homokaryotic *qa-2*$^+$ transformants. Nearly half (43%) of the transformants appeared to contain single inserts of the transforming DNA sequences. An additional 17% seemed to have integrated two to three copies of the transforming vectors, while 9% contained multiple (about 4–10) copies. Many of the transformants (25%) appeared to have been generated by homologous (replacement-type) integration at the normal *qa-2*$^+$ locus. Three transformants apparently arose by the insertion of pMSN1 vector sequences alone; duplicated *sdv* insert sequences were not detected in these transformants.

Gene disruption experiments to inactivate the *sdv* genes: In the gene disruption (RIP) experiments, transformants that appeared to contain two copies of an *sdv* gene (the endogenous *sdv* gene plus one extra copy that was introduced by transformation) were crossed with a normal sequence strain (no duplicated

TABLE 5

Strains used in crosses to disrupt the crossing-specific genes by the RIP process

Cosmid[a]	Map position[b]	Plasmid used to transform female parent	Male parent[c]
4:3A	LG IVL	pSdv-1, pSdv-2, pSdv-3	*pyr-1 A* (RLM 16-21)
5:6F	LG IR	pSdv-4, pSdv-5	*arg-6 A* (FGSC 3591)
8:9A	LG VR	pSdv-6	*pab-2 cyh-2 al-3 inl A* (FGSC 4133)
12:2E	LG IVR	pSdv-7, pSdv-8, pSdv-9	*pan-1 al-2 A* (FGSC 2658)
12:7B	LG VIL	pSdv-10	*ad-1 pan-2 A* (FGSC 3121)
20:11A	LG IVR	pSdv-11, pSdv-12	*met-1 A* (RLM 10-34)
28:12G	LG VII	pSdv-13, pSdv-14	*met-7 A* (RLM 05-10)

[a] Sources of the respective *sdv* subclones.

[b] Map positions were determined by restriction fragment length polymorphism mapping; see Table 4. L and R refer to the left and right arms of the chromosomes, respectively.

[c] The map positions of the relevant auxotrophic markers of the male parents are as follows: *pyr-1*, LG IVR (centromere proximal); *arg-6*, LG IR; *pab-2*, LG VR; *pan-1*, LG IVR; *ad-1*, LG VIL; *met-1*, LG IVR; *met-7*, LG VIIR.

sequences) harboring an auxotrophic mutation mapping near the respective *sdv* gene (Table 5). *sdv-1*, *sdv-2* and *sdv-3* mapped to LG IVL (near the centromere), and T-sdv-1 through T-sdv-3 strains were crossed with a strain harboring the *pyr-1* mutation on LG IVR; RFLP mapping showed about 10.5% recombination between these clustered *sdv* genes and the *pyr-1* mutation (not shown). *sdv-13* and *sdv-14* might map to either the left or right arm of LG VII, but they are quite close to the centromere and tightly linked to *met-7* [*ars-1* shows less than 1% recombination with *met-7* (PERKINS *et al.* 1982; see Table 4)]. In all other cases the T-sdv transformants were crossed with strains harboring auxotrophic mutations mapping on the same arm of the appropriate LG; the closest auxotrophic mutants with tight phenotypes were selected.

The progeny of the RIP crosses were plated on medium lacking the supplement required by the auxotrophic normal sequence parent, thus enriching for progeny containing the endogenous crossing-specific gene present in the T-sdv strain; this *sdv* gene of the transformant strain had the potential to be disrupted by the RIP process. Only duplicated sequences of the T-sdv strains should suffer the multiple GC to AT transition mutations; the corresponding sequences of the normal sequence mate will remain intact (CAMBARERI *et al.* 1989). The T-sdv transformants were crossed as female (protoperithecial) parents in the RIP crosses, but gene disruption appears to be equally efficient when the strain harboring duplicated sequences is crossed as the male parent. In all of these crosses, large numbers of ascospore progeny were formed, suggesting that the parental transformants did not harbor dominant or female-specific defects affecting the sexual process.

Analysis of progeny for possible dominant defects in sexual development: To determine if disruption of the *sdv* genes yielded a phenotype, we analyzed the selected (prototrophic) progeny of the RIP crosses for crossing defects; progeny that showed vigorous vegetative growth were examined. Disruption of the *qa-2*$^+$ gene was not a problem, as a normal *qa-2*$^+$ and/or *aro-9*$^+$ gene could be contributed by the male parent, and either is sufficient for growth on non-supplemented medium.

Transformants containing multiple copies of the fourteen *sdv* genes were analyzed in these experiments. The progeny (36 from each cross) were first examined for male fertility in crosses to *fl* (*fluffy*) strains (MATERIALS AND METHODS); these crosses also served to determine the mating type of the meiotic progeny. The *fl a* and *fl A* strains were used as female parents in these crosses because of their high fertility and inability to form macroconidia (LINDEGREN, BEANFIELD and BARBER 1939). Nearly all of the progeny showed fair to excellent fertility as the male parent; no strains harboring male-specific defects were found in this screening.

The meiotic progeny were also crossed as protoperithecial parents to a wild type strain (MATERIALS AND METHODS). A number of strains with female-specific defects were noted in this analysis. Progeny of the RIP crosses with T-sdv-2a, T-sdv-6a, T-sdv-9a, T-sdv-13a and T-sdv-14a showed female-specific defects (including the production of small fruiting bodies, low numbers of progeny and aberrant ascospores). However, when the RIP crosses were repeated with independent transformants (T-sdv-2b, T-sdv-6b, T-sdv-9b, T-sdv-13b and T-sdv-14b) obtained with the same *sdv* genes, no additional progeny with female-specific defects were isolated. Since even mutations causing small alterations in basic metabolism (when present within the female parent) can interfere with sexual development in Neurospora (PERKINS *et al.* 1982), and since the female-specific defects were not detected in independent *sdv* transformants, we suggest that the alterations causing the female-specific defects reflected mutations caused by random integration of plasmids or by the transformation process itself, and

not by RIP-mediated disruption of the corresponding *sdv* genes.

The meiotic progeny of either one (T-sdv-1, -3, -4, -7, -8, -11, -12) or two to three (T-sdv-2, -5, -6, -9, -10, -13, -14) independent transformants were examined. In no case did we discover a consistent dominant defect in sexual development among the progeny. For any given *sdv* gene, we can conclude that either: (1) the *sdv* gene product is not required for sexual development; (2) the *sdv* gene product is essential for sexual development, but it can be contributed by either parent; (3) the disruption of the *sdv* gene caused a lethal defect (one that disallowed even vegetative growth), so that strains harboring the disrupted *sdv* gene could not be tested; or (4) the disruption of the *sdv* gene occurred at a frequency too low to detect. To determine if some of the meiotic progeny harbored recessive crossing defects (the second possibility), we examined the phenotypes of the progeny in self crosses (below). The other possibilities are considered in the DISCUSSION.

Analysis of progeny for recessive defects in sexual development: Meiotic progeny from all of the transformants were crossed with other progeny of the same cross (sibling crosses) to detect strains harboring recessive defects in sexual development. In most cases, 20 progeny from a given cross were crossed against each other. In about 25% of the sibling crosses, most of the ascospore progeny were white (and inviable), rather than black (like normal spores). Many different mutations can cause the production of white ascospores (PERKINS *et al.* 1982); in this case, control crosses showed that the white spore phenotype was an artifact caused by the effects of homozygosity of the *aro-9* mutation in the cross (not shown).

In most cases, defects other than the production of white spores were not detected in the sibling crosses, suggesting that disruption of the *sdv* genes had not generated recessive mutations. However, in two sibling crosses, recessive mutations that caused dramatic defects in sexual development were identified. Those mutations were detected in the T-sdv-10 and T-sdv-5 progeny sibling crosses. The affected genes were named *asd-1* and *asd-2*, respectively (for *ascus development*); their properties are discussed below.

Characterization of the *asd-1* mutant strain: The meiotic progeny of two independent transformants containing multiple copies of the *sdv-10* gene (T-sdv-10a and T-sdv-10b) were examined for their behavior in sibling crosses. In both cases, large numbers of the progeny (27 of 36, and 18 of 36, respectively) harbored the recessive *asd-1* mutation that interfered with sexual development and disallowed the delineation of ascospores. The parental transformants T-sdv-10a and T-sdv-10b showed no crossing defects, either when crossed with wild type or *asd-1* mutant strains.

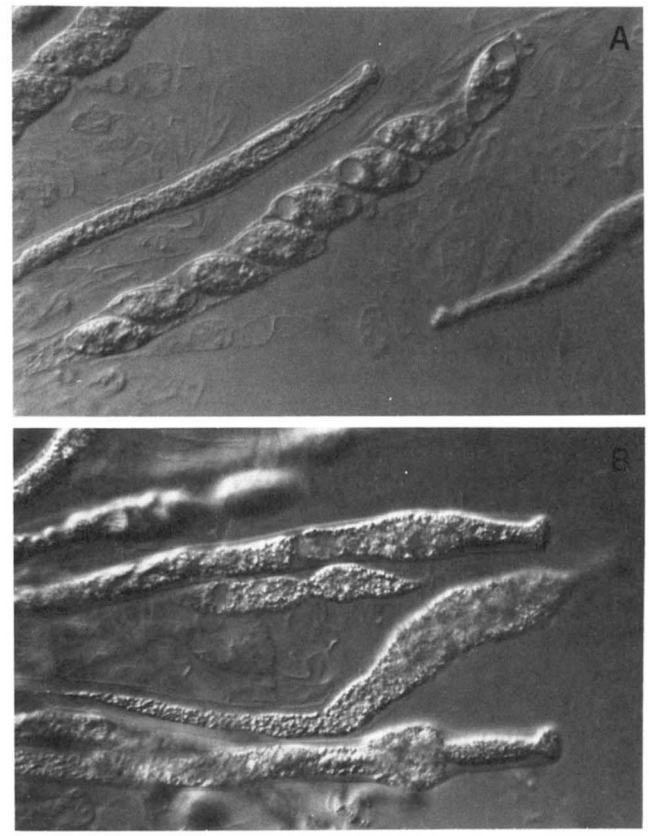

FIGURE 3.—Ascus development in wild type and *asd-1* crosses, seven days after fertilization. (A) Asci from a 74-OR23-IV *A* × ORS*a* (wild type) cross. A fairly mature ascus (at the center) and two neighboring immature asci are shown. (B) Asci from an *asd-1* × *asd-1* cross. Nomarski interference (differential interference) photomicrographs were taken at 300×.

(About 50% of the ascospore progeny of the T-sdv-10 × *ad-1 pan-2 A* crosses were white rather than black, but this defect was caused by the *pan-2* mutation (THRELKELD 1965)). Thus the *asd-1* gene appeared to have been disrupted during the crosses, presumably by the RIP process.

The results of a microscopic analysis of the phenotype observed in *asd-1* × *asd-1* crosses are shown in Figure 3. Unfixed asci from wild type and homozygous *asd-1* crosses were examined. In the central ascus of Figure 3A (the wild-type cross), the eight linearly arranged ascospores are apparent. Also, the structure of the pore through which the ascospores are ejected is clearly shown (upper right); this and other structures that are evident within the central ascus had not yet developed within the two flanking immature asci. Figure 3B shows asci from a homozygous *asd-1* cross; development of these asci is grossly aberrant, although the lengths of the asci are comparable to those of wild type asci (and similar numbers of asci, about 200 per fruiting body, are formed in wild-type and homozygous *asd-1* crosses). No delineation of ascospores occurred in the *asd-1* asci, and the morphology of the asci was quite irregular. Also, the development of the

pores through which asci are normally ejected was aberrant; the pores in the *asd-1* asci resemble those observed in immature wild-type asci (Figure 3A), as though this particular aspect of sexual development were blocked at that earlier stage. The development of the outer tissues of the *asd-1* fruiting bodies was also abnormal, in that only short perithecial beaks were formed. Many homozygous *asd-1* crosses have been examined, and in no case has any production of ascospore progeny been observed.

Characterization of the *asd-2* mutant strain: The meiotic progeny of three independent transformants containing multiple copies of the *sdv-5* gene (T-sdv-5a, T-sdv-5b and T-sdv-5c) were examined for their behavior in sibling crosses. In crosses between progeny of T-sdv-5a, many (14 of 36) were found to harbor the recessive *asd-2* mutation that blocked sexual development at an early stage. However, no crossing defects were observed in sibling crosses of T-sdv-5b and T-sdv-5c progeny (0 of 36 tested for each transformant). The parental transformants were tested for their crossing behavior in crosses to wild type and *asd-2* strains; T-sdv-5b and T-sdv-5c were indistinguishable from wild type, but the T-sdv-5a strain showed a recessive crossing defect identical to that observed in the *asd-2* mutant progeny of the the RIP cross. Since the parental T-sdv-5a strain already harbored the *asd-2* defect, it seems unlikely that the *asd-2* mutation in the progeny of T-sdv-5a had been created by the RIP process.

The *asd-2* mutation was mapped in a conventional cross to a multiply marked strain (multicent-2 *a*). An *asd-2* isolate (*asd-2(N18) A*) was crossed as the female parent with multicent-2 *a*. Random ascospore progeny were isolated and cultured on supplemented medium (METZENBERG *et al.* 1985), and the segregation of auxotrophic markers and the *asd-2* mutation was analyzed. The *asd-2* mutation showed linkage only to the *nic-3* and *ars-1* mutations, which map on LG VII (PERKINS *et al.* 1982). The recombination frequencies of *asd-2* with *nic-3* and *ars-1* were 15% and 36%, respectively, suggesting that *asd-2* maps centromere-distal to *nic-3* on the left arm of LG VII. Since the *sdv-5* gene maps on LG I, the map position of *asd-2* constituted additional evidence that the mutation had not arisen as the result of RIP-induced gene disruption. Instead, we suggest that the *asd-2* mutation most likely arose as the result of a transformation-induced mutation in the *asd-2*$^+$ gene, caused by the ectopic integration of the transforming DNA. Experiments are now in progress to recover the transforming plasmid and its flanking DNA sequences from T-sdv-5a genomic DNA, so that the structure and properties of the *asd-2*$^+$ gene can be analyzed.

The developmental defects characteristic of homozygous *asd-2* crosses have been examined, and in no case has any production of ascospore progeny been observed. Instead, development is blocked at an early stage, but presumably after karyogamy, since the development of ascogenous tissue is initiated. However, only small numbers of asci are ever formed, and they are very short (about 50 μm long, or one-fourth the length of normal asci); development ceases after that point and the ascogenous tissue degenerates (not shown). The growth of the external tissues of the fruiting body is also blocked, and only small perithecia lacking perithecial beaks are formed.

DISCUSSION

The process of sexual development in *N. crassa* is quite complex, and no doubt requires the concerted action of a number of regulator genes and many target genes. Classical genetic studies have been carried out to identify mutants that are blocked at various stages in the sexual development pathway. Mutants in many genes have been characterized, but it is certain that many other important genes have not yet been identified. For example, in most cases researchers have screened for only dominant mutations, and the crossing-specific genes with recessive phenotypes were missed in these studies. Also, since sexual development in *N. crassa* can be disrupted by many changes in basic metabolism, it is often not possible to identify with any certainty those mutations directly affecting this process. For these reasons, we chose a different approach to study sexual development.

Genes of *N. crassa* that are expressed preferentially during sexual development were identified by probing a genomic cosmid library with labeled cDNAs enriched by subtraction hybridization for the differentially expressed genes. Of the 50 non-rDNA cosmids that were studied, 35 encode transcripts that are preferentially expressed under crossing conditions. Eleven of the 35 cosmids selected for in depth analysis were identified only once, while six of the cosmids were repeatedly isolated, overlapping cosmids. Clearly, we have not isolated all of the crossing-specific genes that could be identified using the subtractive hybridization technique.

We noted a lot of clustering of the crossing-specific genes, in that many cosmids encode two or three genes that are expressed only under crossing conditions. Similar clustering of genes specific to the asexual cycle (conidiation-specific genes) has been noted in both *N. crassa* (BERLIN and YANOFSKY 1985) and *Aspergillus nidulans* (ORR and TIMBERLAKE 1982). The clustering may be related to the transcriptional control mechanisms that operate on the *sdv* genes. For example, the multiple *sdv* genes present on the 4:3A cosmid all require a functional *A* mating type product for expression, as do the two crossing-specific transcripts encoded on 12:7B (M. A. NELSON and R. L. METZEN-

BERG, unpublished results). However, it is not simply the case that similarly regulated *sdv* genes map in clusters. Instead, the 5:6F, 12:2E and 20:11A cosmids encode *sdv* genes whose expression is differentially regulated. Clearly, transcriptional regulation during sexual development in *N. crassa* is very complex. Similar transcriptional complexity has been observed in clusters of the conidiation-specific genes of *A. nidulans* (the *SpoC1* cluster; MILLER *et al.* 1987).

Since the female reproductive structure forms only on the surface of cultures, it was thought that growth in submerged culture (with agitation) might inhibit the expression of some or all of the crossing-specific genes. In fact, transcripts from nearly all of the *sdv* genes were greatly reduced in amount or absent in the *a tol/A tol* heterokaryon grown with agitation in crossing medium. However, it remains possible that the transcripts were absent due to the (unavoidably) shorter period of growth of agitated cultures than of stationary ones, and not because the cultures were submerged.

We chose to analyze the transcripts present in the *a tol/A tol* heterokaryon strain, in addition to those of the wild-type strain, in the expectation that perhaps the presence of both mating type genes in the heterokaryotic strain might more accurately simulate normal sexual development. However, only minor differences in gene expression between the wild type and the *a tol/A tol* heterokaryon strain grown under crossing conditions were detected.

The expression of the crossing-specific transcripts was examined in two mutant strains, the A^{m44} mating type mutant and the *fmf-1* or *female-male fertility* strain. The A^{m44} mutant is completely sterile as either the male or female parent, and in attempted crosses, fruiting body formation is not even initiated (GRIFFITHS 1982). In addition, it fails to display the vegetative heterokaryon incompatibility normally observed between strains of opposite mating type. The A^{m44} mutation most likely causes inactivation of the *A* mating type function, since sequence analysis has shown A^{m44} to be a frameshift mutation in the predicted *A* coding sequence (GLASS, GROTELUESCHEN and METZENBERG 1990). Most of the *sdv* genes were not expressed at significant levels under crossing conditions in the A^{m44} mating type mutant. These results suggest that the product of the *A* mating type gene functions as a master control in sexual development, as its effect is seen at the molecular level in the induction of transcripts specific to the crossing process. However, it is not known whether this control is direct, or mediated through other effectors. The effect of the *a* mating type product on the expression of the *sdv* genes is currently under investigation.

The *fmf-1* mutation confers both male and female sterility on the host strain, indicating that the *fmf-1* gene product is required in both the protoperithecial and male parent (JOHNSON 1979). In crosses with *fmf-1* as one of the parents, very small perithecia lacking asci and ascogonial material are produced. One transcript (that encoded by *sdv-6*) was shown to require a functional *fmf-1* gene for normal expression or accumulation under crossing conditions. This result suggests that the product of the *fmf-1* gene, as well as those of the mating type genes, might also act as a regulator during sexual development. Again, such control could be direct or could be mediated through other effector molecules.

Few genes expressed during sexual development in the filamentous fungi have yet been identified. However, two genes (*Sc1* and *Sc4*) that are expressed preferentially in fruiting dikaryons of *Schizophyllum commune* (a basidiomycete) have been characterized. The *Sc1* and *Sc4* genes encode related cysteine-rich hydrophobic proteins, which may be involved in the formation of the aerial fruiting body hyphae (WESSELS *et al.* 1991).

It is not known how the various control pathways (including nitrogen starvation and control by regulator gene products) interact in the sexual differentiation of *N. crassa*. To begin to answer this question, we have examined the functions of the crossing-specific genes. A reverse-genetic approach, based on the RIP process of Neurospora (SELKER and GARRETT 1988; CAMBARERI *et al.* 1989), was used to disrupt the *sdv* genes, and the effects of those disruptions were examined. In RIP-mediated gene disruption, multiple copies of the gene of interest are introduced by transformation into a recipient strain, and that strain is put through the sexual cycle. In many of the progeny, the duplicated sequences will have been altered by multiple G-C to A-T transition mutations. We subjected the *sdv* genes to RIP-mediated gene disruption, and examined the progeny for any defects in sexual development.

Two recessive mutations causing dramatic defects in sexual development were isolated in this study; these mutations were named *asd-1* and *asd-2* (for *ascus development*). Neither of these genes had been previously identified using classical genetic techniques. The *asd-1* mutation was probably generated by RIP-mediated gene disruption. The *asd-1* mutant has a recessive defect that causes the production of normal numbers of normal-sized but grossly aberrant asci. Ascospores are never delineated, and the development of both the asci and the external tissues of the fruiting body is abnormal; the asci are irregular in structure and never form the pores through which ascospores are normally extruded, and the lengths of the perithecial beaks never reach those of wild-type perithecia (below).

The *asd-1* mutant was created by disruption of the

crossing-specific *sdv-10* gene, and renamed to indicate its mutant properties. The *asd-1* (or *sdv-10*) transcript is present in large amounts in a wild type strain grown under crossing conditions, and is greatly reduced (barely detectable) in an *A* mating type mutant grown under the same conditions; the transcript was not detected in a vegetatively growing wild-type strain. Thus both nitrogen starvation (or growth on crossing medium) and a functional *A* mating type product are required for normal expression of the *asd-1* gene. The levels of the *asd-1* transcript were also reduced, although less drastically, in an *fmf-1* mutant (*female-male fertility*; JOHNSON 1979) and an *a tol/A tol* heterokaryotic strain grown under crossing conditions, suggesting a complex regulation of its transcription and/or accumulation.

Although the *asd-2* mutant strain was also isolated in this reverse-genetic analysis, the *asd-2* mutation was probably not caused by the RIP process. Instead, this mutation appears to have been created by the insertion of the transforming DNA ectopically into the *asd-2*$^+$ gene. In the recessive *asd-2* mutant strain, sexual development is blocked at an earlier stage than in *asd-1* mutants. There is some early (albeit abnormal) development of the ascogenous hyphae, suggesting that the block is after karyogamy. Small numbers of short asci with abnormal morphologies are formed; shortly thereafter, development aborts and the ascogenous tissue degenerates. The growth of the external tissues of the fruiting body is also blocked (below).

In both *asd-1* and *asd-2* mutants, development of the external features of the fruiting bodies never attained that seen in wild type crosses, and in fact seemed to reflect well the state of the internal ascogenous tissue. Homozygous *asd-1* crosses proceeded much further in ascogonial tissue development than did homozygous *asd-2* crosses, and the development of the external perithecial features paralleled their respective levels of internal development. The *asd-1* perithecia were nearly as large as wild type perithecia, but only short perithecial beaks were formed. Growth of the *asd-2* fruiting bodies was blocked at an earlier stage, when they were still fairly small and had not yet formed perithecial beaks. Thus in both cases, formation of outer perithecial tissues seemed to be linked to and coregulated with internal ascogonial development.

Did we fail to detect certain categories of genes required for sexual differentiation because of a low frequency of RIP-induced mutation? The efficiency of RIP is affected by a number of factors, including linkage, the number of copies and the length of the duplicated sequences. Closely linked duplicated sequences are efficiently disrupted (at a frequency approaching 100%) by the RIP process, as are fairly long (greater than 2.5 kbp) unlinked duplications, which can be disrupted with a frequency of about 50% (SELKER *et al.* 1987, 1989). In strains containing two copies of a sequence, both copies undergo RIP disruption concomitantly, while in strains with three copies, one copy often escapes untouched (FINCHAM *et al.* 1989). We attempted to select transformants with just two copies of the *sdv* genes for our analysis. The smallest *sdv* inserts in the transforming vectors were about 0.8 kbp (*sdv-9*) and 1.4 kbp (*sdv-10*); the other inserts ranged in size from 1.8 kbp to about 12 kbp. We observed a high efficiency of RIP-mediated disruption in two different strains harboring duplicate copies of the *sdv-10* gene (50 to 75% of the selected, or *ad-1*$^+$, progeny), even though the length of the duplicated sequences was only about 1.4 kbp. Therefore, it seems probable that in most of our crosses enough progeny were examined to allow the identification of any mutant strains; *sdv-9* might be the only exception. Southern hybridization analyses showed that the *sdv* sequences in many of the progeny had altered restriction sites (not shown), suggesting that RIP-induced disruptions had actually occurred (even when no crossing defects were observed).

Since the RIP process causes multiple G-C to A-T transition mutations in the duplicated sequences (CAMBARERI *et al.* 1989), it is unlikely to cause leaky mutations or anything less than total disruption of the affected gene. If a gene essential for vegetative growth is inactivated by the RIP process, many of the ascospore progeny should be inviable. However, the variable (and unknown) frequency of RIP in different transformants makes any conclusions about essential genes (drawn from the number of inviable spores) unreliable at best. Selected insertional translocations or disomics could be used to "shelter" any essential genes and permit their recovery among the progeny of the RIP cross (SELKER *et al.* 1989); such experiments have not been undertaken, so we do not know if essential genes are included among the crossing-specific genes that have been examined. Most of the crossing-specific genes are probably not required for vegetative growth, since genes with transcripts present specifically during sexual development are unlikely to code for proteins that function in the asexual phase. However, if the fragments containing the *sdv* genes also contain flanking genes that are essential for vegetative growth (a possibility especially with the larger *sdv* fragments), the creation of lethal mutations would have disallowed the detection of genes required for sexual development.

Dominant RIP-induced mutations were not found in our analysis of the *sdv* genes. This may reflect the fact that the ascogenous hyphal tissue is necessarily a heterokaryon containing the two parental nuclei, so that most mutations will be complemented (*fmf-1* (JOHNSON 1979) is an obvious exception). However, it

is also possible that the reflexiveness of the system prevents recovery of such mutants, in that any gene whose continuous activity is necessary for the completion of meiosis will be lost as a result of the RIP process.

We examined the phenotypes of fourteen potentially disrupted crossing-specific genes in this analysis. Only one mutation with a distinct phenotype, *asd-1*, was apparently created by RIP-mediated gene disruption. Another mutation, *asd-2*, was fortuitously isolated. We have tentatively concluded that the majority of the 14 *sdv* genes encode products that are not essential for sexual development. The failure to identify mutations affecting sexual development in most of the *sdv* genes does not demonstrate that these genes play no role in sexual differentiation. Instead, these genes might encode products whose functions are shared with other genes, or products whose roles in sexual development are important but not essential; mutations with subtle effects on sexual development might have been missed in our screening. The observed clustering of the *sdv* genes suggests that the genes are important; the clustering may be related to the mechanisms of transcriptional control of these genes. The further analysis of these and other *sdv* genes will be necessary to characterize the molecular control of sexual differentiation in Neurospora.

This paper is dedicated to the memory of WALTER PLAUT. He was responsible for sharing some of the mysteries of microscopy with the two geneticists who authored this paper (he helped prepare Figure 3), and he was a dear friend and colleague. We are grateful to MARY CASE for sharing strains and for helpful discussions. This research was supported by U.S. Public Health Service grant GM 08995 to R.L.M. The authors are grateful for Postdoctoral Fellowship support to M.A.N. from Merck Sharp and Dohme Research Laboratories during most of the course of this work.

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