Molecular Cloning of a Gene (*cfp*) Encoding the Cytoplasmic Filament Protein P59Nc and Its Genetic Relationship to the *snowflake* Locus of *Neurospora crassa*

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ABSTRACT

P59Nc is a 59-kD polypeptide associated with 8–10-nm diameter cellular filaments in normal *Neurospora crassa* strains. Abnormally sized and shaped bundles of these structures are present in *N. crassa* strains carrying mutations at the locus *sn* (*snowflake*). By using molecular cloning and restriction fragment length polymorphism (RFLP) segregation analysis strategies we show here that *sn* is not the genetic locus of P59Nc. Several P59Nc cDNAs were cloned from a *N. crassa* λGT11 library after immunoscreening with specific polyclonal anti-P59Nc antibodies. Additional longer cDNAs were obtained from a *N. crassa* cDNA-λZAP library. When used as probes in Southern blots of total DNA from wild-type strains, multicent-2 (a multiple mutant strain), and *snowflake* mutants, the P59Nc cDNAs revealed comparable patterns of hybridizing bands for all of the restriction enzymes tested. Analysis of segregation of *Bcl*I and *Cla*I RFLPs, detected in the genomic region of the P59Nc gene (locus *cfp: c*ellular *f*ilament *p*olypeptide), among a set of strains designed for RFLP mapping, or among selected progeny of crosses involving a *snowflake* parent, respectively, indicate that (i) there is in *N. crassa* a single *cfp* locus positioned on the right arm of linkage group VII between the locus *for* and the proximal breakpoint of the translocation *T(VII → I)5936*; (ii) the *sn* mutations in the centromere region of chromosome *I* do not represent translocations of *cfp*; and (iii) the *snowflake* mutants possesses a normal copy of the P59Nc gene on their chromosomes *VII*. Taken together the results indicate that the aberrant *in vivo* arrangement of the P59Nc 8–10-nm filaments occurring in *snowflake* mutants are not due to alterations in the P59Nc gene.

MICROTUBULES (20–25 nm in diameter) and microfilaments (5–7 nm in diameter) are the most clearly defined elements from the filamentous fungi cellular matrix (McKERRACHER and HEATH 1987). Other filamentous cytoplasmic structures observed in electron microscope studies of filamentous fungi cells remain poorly characterized (see ROSA, PERALTA-SOLER and MACCIONI 1990). Recently, the isolation and characterization of bundles of 8–10-nm diameter filaments in the fungus *N. crassa* were described (ROSA *et al.* 1990). Similar filament bundles were first observed in electron microscope studies of *N. crassa* wild type, in the morphological mutant *snowflake* (ALLEN, LOWRY and SUSSMAN 1974), and in other filamentous fungi (ANDERSON and ZACCHARIAN 1974; GULL 1975; HOCH and HOWARD 1980). However, their biochemical nature remains unsettled (see ROSA, PERALTA-SOLER and MACCIONI 1990). The *N. crassa* 8–10-nm filaments are constituted of a polypeptide of 59 kD ("P59Nc"; ROSA *et al.* 1990), are profusely distributed in the cytoplasmic and nuclear compartments of the cell in either young or old mycelia (ROSA, PERALTA-SOLER and MACCIONI 1990), and are abnormal in size and shape in the *N. crassa snowflake* mutant (ALLEN, LOWRY and SUSSMAN 1974). ROSA, ALVAREZ and MALDONADO (1990) proposed that the locus *sn* (*snowflake*) may be the genetic locus for the P59Nc gene or for a gene whose product is involved in the *in vivo* assembly of the 8–10-nm filaments. We report here the molecular cloning of the P59Nc gene. Besides, by performing genomic Southern blot and RFLP segregation analyses, we have studied both (i) if the locus *sn* on the centromere region of chromosome *I* includes the P59Nc gene and (ii) if the *snowflake* mutants possess alterations in the *cfp* locus.

MATERIALS AND METHODS

Strains, growth conditions and crosses: *Escherichia coli* K802 (RALEIGH and WILSON 1986), Y1089 and Y1090 (YOUNG and DAVIS 1983a), and BB4 (SHORT *et al.* 1988) were used for plasmid, λGT11 and λZAP propagation, respectively. *N. crassa* strains used in this work are listed in Table 1. The "Set1" include 38 (FGSC 4450–87) progeny individuals selected from the cross *un-2; arg-5; thi-4; pyr-1;*

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TABLE 1

Genotypes and origins of *N. crassa* strains

Strain/Genotype	Remarks	FGSC No.	Source	Reference[a]
Wild-type *74-OR23-1A*	"Oak Ridge"	987	FGSC	1
Mauriceville-1-c - A	"Exotic"	4416	FGSC	2
snC136 - a	*snowflake*	947	FGSC	1
snJL301 - a	*snowflake*	4338	FGSC	1
T(VII → I)5936 - a	Translocation VII → I	2105	FGSC	4
un-2;arg-5;thi-4;pyr-1;lys-1;inl; nic-3;ars - 1-a	Multiple mutant strain	4488	FGSC	3
Set 1 (*01-38*)	RFLP mapping	4450–4487	FGSC	3
Set 2 (*RT01–RT10*)	RFLP mapping		This work	

[a] 1, Fungal Genetics Stock Center (FGSC) Catalog, Ed. 3 (1990); 2, METZENBERG *et al.* (1985); 3, METZENBERG *et al.* (1984); and 4, PERKINS *et al.* (1982).

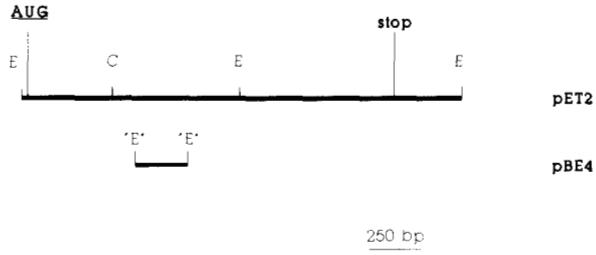

FIGURE 1.—Molecular cloning of P59Nc-cDNAs. The short horizontal line (*pBE4*) represents the first P59Nc-cDNA (250 bp) characterized. The upper line (*pET2*) represents a P59Nc-cDNA of about 2.0 kb obtained from the λZAP-cDNA library. Position of the initiation codon (*AUG*) is indicated. The probable end of the P59Nc open reading frame is indicated by *stop*. Restriction enzyme sites are: *C*, *Cla*I and *E*, *Eco*RI. "*E*" indicates *Eco*RI linkers.

lys-1; inl; nic-3, ars-1-a ("multicent-2-a"; FGSC 4488) × Mauriceville-1c-A (FGSC 4416) (METZENBERG *et al.* 1984). The set was designed for restriction fragment length polymorphism (RFLP) mapping in *N. crassa* (METZENBERG *et al.* 1984, 1985). These strains were generously supplied by C. WILSON from the Fungal Genetics Stock Center. All of the stocks were maintained at 4° in silica gel tubes (DAVIS and DE SERRES 1970). Working stocks were grown in agar slants of Vogel's minimal medium (DAVIS and DE SERRES 1970) supplemented with 2% (w/v) sucrose and nutritional requirements when necessary. Liquid cultures were grown in an orbital shaker at 100–120 rpm at 25° or 30°. "Duplication coverage" genetic mapping, by studying the presence of heterozygous RFLPs in partially duplicated strains, was performed as described (METZENBERG *et al.* 1985; PERKINS 1986). Crosses were performed at 25° in the dark using a synthetic crossing medium (DAVIS and DE SERRES 1970) with 2% (w/v) sucrose as the carbon source. Strains carrying a duplication of a large fragment from the right arm of chromosome *VII* were constructed and characterized by the "barren" phenotype as indicated (PERKINS 1986).

***N. crassa* cDNA libraries:** *N. crassa* λGT11 and λZAP cDNA libraries were prepared by SACHS *et al.* (1986) and ORBACH, SACHS and YANOFSKY (1990), respectively. Methods for immunological screening of λGT11 libraries have been described (YOUNG and DAVIS 1983b). A rabbit anti-P59Nc serum (ROSA, PERALTA-SOLER and MACCIONI 1990) was used after dilution 1:40 in 3% (w/v) bovine serum albumin in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.02% (w/v) NaN_3. ^{125}I-labeled protein A from *Staphyloccocus aureus* (CUATRECASAS 1973) was used as secondary ligand. Phage from each primary positive signal were eluted in 10 mM Tris-HCl (pH 7.5) with 10 mM $MgCl_2$ and about 500 plaque-forming units were spotted on a lawn of *E. coli* Y1090 to characterize phages producing a foreign protein that strongly binds anti-P59Nc antibodies. Small (1 × 1 cm) isopropyl-1-thio-β-D-galactopyranoside-saturated (10 mM) nitrocellulose squares were used to induce and fix the antigen produced by the clones. The filters were used as solid supports to affinity-purify epitope-specific antibodies from the anti-P59Nc serum. The antibodies were used in Western blot analyses (TOWBIN, STAEHELIN and GORDON 1979) of total *N. crassa* proteins (ROSA *et al.* 1990). Construction of λGT11 lysogens, purification of λ DNA from induced lysogens and phage plaque hybridization screening (BENTON and DAVIS 1977) of the λZAP library were performed as indicated (SAMBROOK, FRITSCH and MANIATIS 1989).

DNA manipulations: Total *N. crassa* DNA was obtained from frozen and powdered mycelia using either the procedure of RAEDER and BRODA (1985) or OAKLEY *et al.* (1987). Restriction of the DNA was performed overnight in a final volume of 200 μl (3–6 μg of total DNA) with 5–10 units of the appropriate restriction enzyme per μg of DNA. Conditions for Southern blot transfer of DNA to nitrocellulose or nylon solid supports (Hybond C and N, respectively, from Amersham) were as described (SAMBROOK, FRITSCH and MANIATIS 1989). Probes were labeled with ^{32}P by oligolabeling (FEINBERG and VOGELSTEIN 1983) using [α-^{32}P]dATP (3000 mCi/mmol) from Du Pont. Hybridization was at 62° in 6 × SSC for 24–48 hr; washing steps were in 6 × SSC with 0.1% (w/v) sodium dodecyl sufate at 62°.

RESULTS

Molecular cloning of P59Nc-cDNAs: A rabbit anti-P59Nc polyclonal antibody (ROSA *et al.* 1990) was used for immunoscreening of about 30,000 λGT11 clones (80% recombinants as judged from the percentage of Lac$^-$ phages in X-Gal plates) (not shown) of a mycelial cDNA *N. crassa* library (SACHS *et al.* 1986). Six positive signals were obtained in the primary screening (λNc1 to 6; see MATERIALS AND METHODS). A 250-bp cDNA present in λNc4 was subcloned into the plasmid Bluescript M13(+) to construct the plasmid pBE4 (Figure 1) and used as a probe to obtain several longer P59Nc cDNAs from a mycelial specific λZAP *N. crassa* library (ORBACH, SACHS and YANOFSKY 1990). This, and further additional screening of

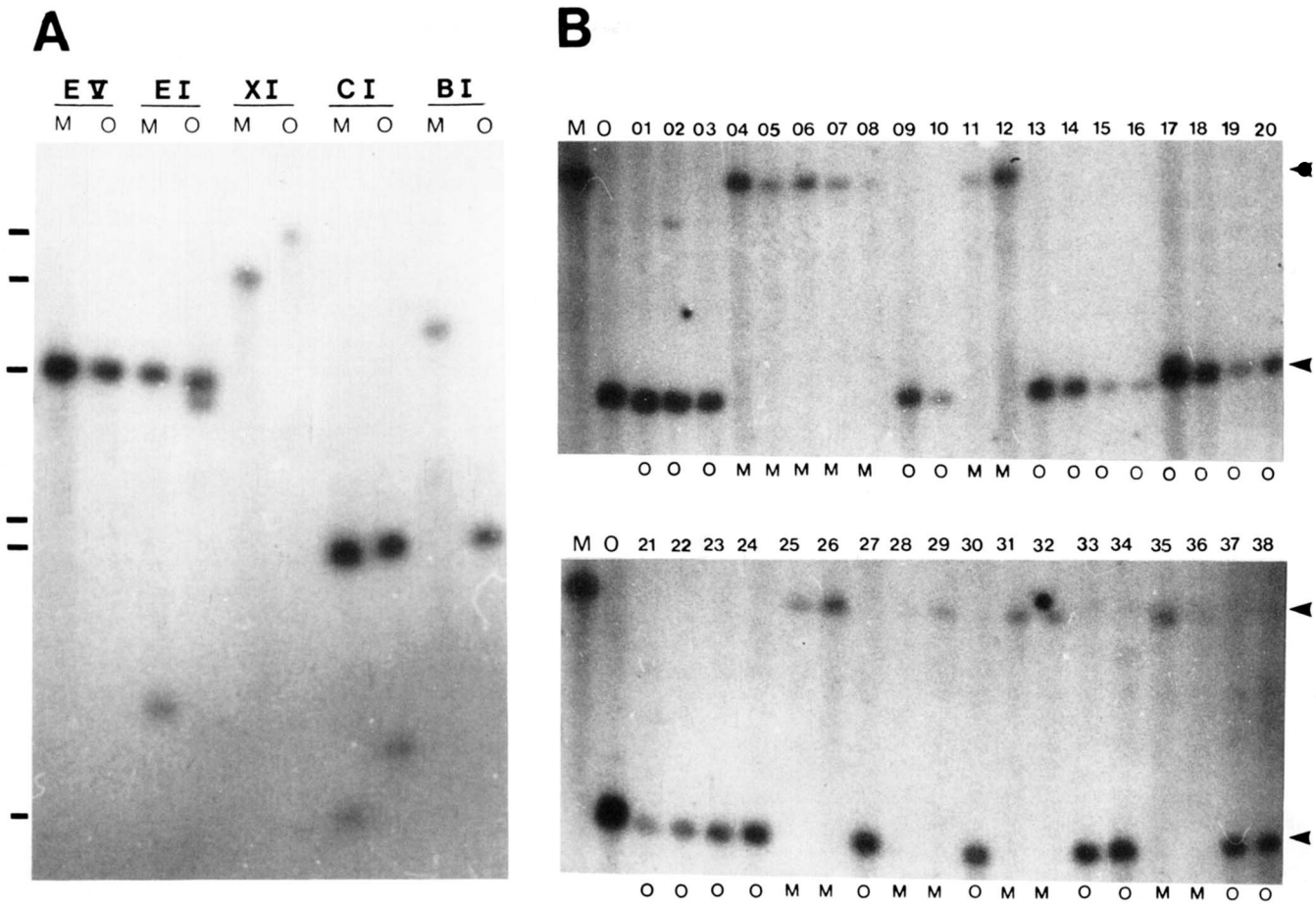

FIGURE 2.—RFLPs in the genomic region of the *cfp* locus. (A) Southern blot analyses of total DNAs from Mauriceville-1-c (*M*) or multicent-2 (*O*) strains digested with *Eco*RV (*EV*), *Eco*RI (*EI*), *Xba*I (*XI*), *Cla*I (*CI*), and *Bcl* (*BI*), and hybridized with the 2.0 kb (pET2) P59Nc-cDNA probe. The short horizontal lines at the left indicate the position of molecular size standards (λDNA, *Hin*dIII digested). RFLPs are detected for the *Bcl*I, *Cla*I, *Eco*RI and *Xba*I enzymes. (B) Southern blot showing the segregation of the *Bcl*I RFLP (indicated by arrows and represented as *M* or *O* below the photographs) among 38 individuals (*01, 02, 03, . . . ,38,* indicated above the photographs) of a selected progeny from the cross Mauriceville-1-c (*M*) × multicent-2 (*O*) (METZENBERG *et al.* 1984). For details see text.

a total of *ca.* 30,000 phages from the λZAP library, using some of the isolated cDNAs as probes, yielded a total of eight positive P59Nc-cDNA λZAP clones (λET 1–8). Figure 1 shows the position of the pBE4-cDNA insert relative to a selected, nearly full length P59Nc cDNA of 2.0 kb (pET2) obtained from the λZAP library.

The coding strand and the start codon corresponding to the P59Nc open reading frame (Figure 1) were defined by partial protein and cDNA sequencing. Microsequencing of 23 N-terminal amino acid residues of the purified native P59Nc polypeptide rendered the following sequence: Ala-Gln-Gln-Gln-Gly-Lys-Phe-Thr-Val-Gly-Asp-Tyr-Leu-Ala-Glu-Arg-Leu-Ala-Gln-Val-Gly-Val-Arg. The sequence was positioned on the following cDNA sequence: 5′-ATG (start codon; Figure 1) GTA GCC CAA CAA CAA GGA AAG TTC ACG GTG GGC GAC TAC CTC GCC GAG CGT CTT GCT CAG GTC GGC GTC CGC-3′. Although the cDNA sequence predicts the residues Met and Val at the N terminus, they were absent in the purified polypeptide. Analyses of additional sequence data are in progress (M. E. ALVAREZ, E. TEMPORINI, H. J. F. MACCIONI and A. L. ROSA, unpublished). The 2.0-kb cDNA, representing the entire coding region of the P59Nc gene, was used in the experiments reported below.

Characterization of genomic RFLPs in the region of the P59Nc gene and RFLP mapping of the *cfp* locus: The search for RFLPs in the genomic region of the *cfp* locus was carried out in two *N. crassa* strains having large differences in nucleotide sequences scattered in the genome. The strains are designed *M* (Mauriceville-1-c) and *O* (multicent-2) (Table 1; METZENBERG *et al.* 1984, 1985). RFLPs for *Bcl*I, *Cla*I and other enzymes were detected with the 2.0-kb P59Nc-cDNA (Figure 1) as probe (see Figure 2A; five examples among the enzymes tested are shown). The pattern of segregation of the *Bcl*I RFLP was studied among the genomes of 38 selected individuals (numbered in this work as *01–38*) from the progeny of the cross *M* × *O* (Table 1, "Set 1"; METZENBERG *et al.*

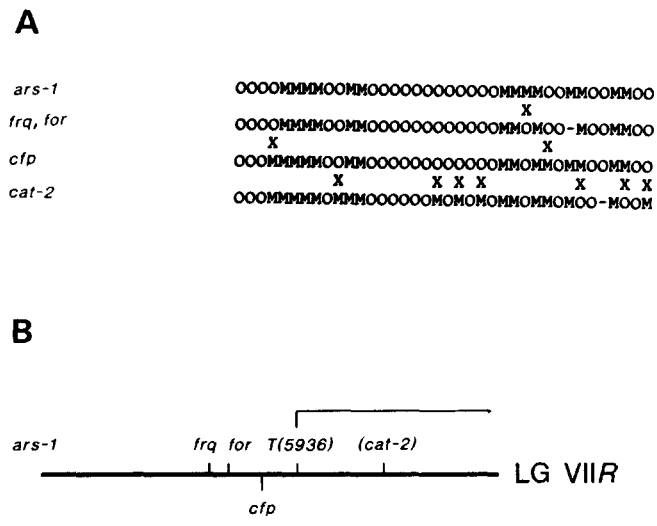

FIGURE 3.—The *cfp* locus is linked to the loci *frq* and *for* on *N. crassa* linkage group VII. (A) The horizontal rows show the segregation pattern (*M* or *O*) of several *N. crassa* genetic markers (indicated at the left column) among 38 individuals from a selected progeny of the cross Mauriceville-1-c × multicent-2. By considering the patterns *M* and *O* in a vertical sense it is possible to detect genetic intervals in the chromosome *VII* of each individual where cross over (*X*) has taken place. The sign (–) indicates that this information is not available. Data were taken from Figure 2B (for *cfp*) and from METZENBERG and GROTELUESCHEN (1989). (B) Putative linkage relationships of the *cfp* locus (indicated below the map) with classical genetic markers in the right arm of linkage group VII. The circle at the left indicates the centromere. *T(5936)* marks the proximal breakpoint of the translocation *T(VII → I)5936* and the chromosomal fragment involved.

1984). The segregation pattern (Figure 2B), compared with that of about 100 genes and anonymous DNA fragments followed in the same cross (METZENBERG and GROTELUESCHEN 1989), indicates that the *cfp* locus was on linkage group VII, probably at the right of the loci *frq* and *for* (see Figure 3A).

To better position the *cfp* locus we analysed whether the P59Nc-*Cla*I RFLP (see Figure 2A), and a *Bam*HI RFLP detected by the pET2 probe (not shown), were present in a heterozygous form in *N. crassa* strains carrying a duplicated (*M/O*) fragment of the chromosome *VII* distal to the loci *for* and *frq* (Figure 3B; see MATERIALS AND METHODS section). The expected forms of the P59Nc RFLPs were present among the DNAs of the parental strains (*M* and *O*) but only the *M* form was present in the five duplicated progeny strains analyzed (not shown). This result indicates that the *cfp* locus is not included in the translocated fragment *T(VII → I)5936*.

Taken together the results suggest that a single *cfp* locus is positioned between the *for* locus and the proximal breakpoint of the translocation *T(VII → I)5936*. Figure 3B shows the putative linkage relationship of the *cfp* locus with the mentioned additional genetic markers on the right arm of linkage group VII.

Does *snowflake* represent a translocation of an altered form of the P59Nc gene to the centromere of chromosome *I*? The simplest interpretation of the available genetic data about the *sn* locus (PERKINS *et al.* 1982 and references cited therein; E. TEMPORINI and A. L. ROSA, unpublished results) is that the two mutant *sn* alleles isolated do not represent gross chromosomal rearrangements, and correspond to a gene naturally resident on the centromeric region of linkage group I. Even so, experiments were carried out to study the formal genetic possibility that the *sn* alleles represent the translocation to the centromere I of altered forms and/or abnormally expressing versions of the P59Nc gene.

Figure 4A shows the Southern blot analyses of *Cla*I digests of total DNAs from the standard wild-type *74-OR23-1A* ("Oak Ridge" genetic background), Mauriceville-1-c, multicent-2 and *snowflake* C136 and JL301 strains (Table 1), by using the 2.0-kb P59Nc-cDNA as a probe. Identical patterns of hybridizing fragments were observed among the DNAs from wild type, Mauriceville-1-c, and C136, or multicent-2 and JL301 strains (Figure 4A); no aberrant bands were detected in the *snowflake* mutants. The finding of the *Cla*I RFLP difference in the region of the *cfp* locus between *snowflake* strains C136 and JL301 (compare lanes *C136* and *JL301* in Figure 4A) shows that they do not possess an identical genetic background. C136 is essentially "Oak Ridge" (compare lines *Wild type* and *C136* in Figure 4A) and JL301 and multicent-2 "Not Oak Ridge" (compare lines *JL301* and *multicent-2* in Figure 4A). This interpretation was supported and extended by analyses of several P59Nc RFLP types (Table 2) and additional RFLPs in the chromosome I of these strains (S. HAEDO, M. MAUTINO and A. L. ROSA, unpublished results).

From these experiments it was not possible to eliminate the presence of two copies of the P59Nc gene in the *snowflake* genomes which render similar restriction fragment patterns. Figure 4B shows the segregation pattern of the P59Nc *Cla*I RFLP (Figures 1 and 4A) among 10 progeny individuals, 5 morphologically wild type and 5 *snowflake*, selected at random from the cross Mauriceville-1-c × *snowflake* (allele JL301). The result shows that (i) there is a unilocus segregation pattern of the *Cla*I RFLP, and (ii) the pattern corresponds to a hybridizing *Cla*I DNA fragment not linked to the *sn* locus. Besides, as the P59Nc gene is on linkage group VII in the Mauriceville-1-c strain (see above), and no *Cla*I-heterozygous progeny were isolated, we conclude that the P59Nc gene of the *snowflake* parental strain is also on linkage group VII.

DISCUSSION

The recent isolation and characterization of P59Nc, the polypeptide of 59-kD constituent of the 8–10-nm diameter cytoplasmic filaments in *N. crassa* cells (ROSA

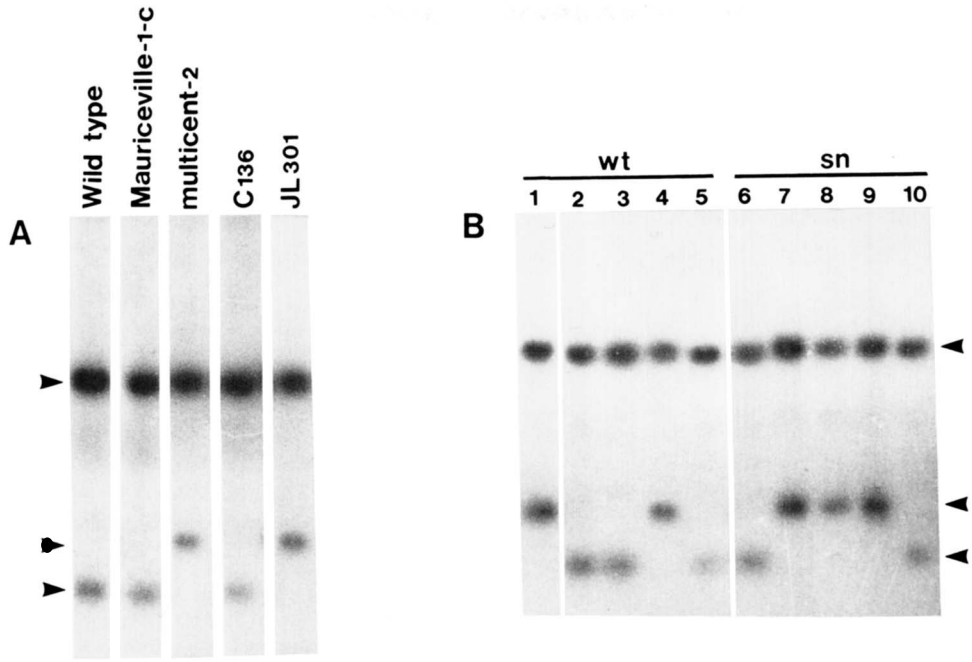

FIGURE 4.—(A) P59Nc-*Cla*I RFLP types in DNAs from wild-type *74-OR23-1A* ("Oak Ridge"), Mauriceville-1-c, multicent-2, or *snowflake* C136 and JL301, *N. crassa* strains. DNAs were digested with *Cla*I and hybridized, after Southern blotting, with the P59Nc-2.0-kb probe (pET2; see Figure 1). The arrows at the left indicate *Cla*I hybrydizing fragments of 2.26, 0.91 and 0.59 kb, from top to bottom, respectively. (B) Southern blot showing the segregation of the P59Nc-*Cla*I RFLP among morphologically wild type (*wt, 1–5*) and *snowflake* (*sn, 6–10*) progeny strains selected at random from the cross Mauriceville-1-c × *snowflake* JL301.

TABLE 2

P59Nc- RFLP types in *N. crassa* strains

RFLP for the restriction enzyme	Strain and RFLP type[a]				
	Wild type	multicent-2	Mauriceville-1-c	snC136	snJL301
Bam HI[b]	I	I	III	I	I
Cla I[c]	I	II	I	I	II
Eco RI[d]	I	II	I	I	II
Pvu II[e]	I	I	I	I	I

[a] RFLP types are arbitrarily defined as I ("Oak Ridge"), II ("No Oak Ridge"), and III ("exotic").
[b] RFLP types = "I": 4.48 kbp; "III": 3.98 kbp.
[c] RFLP types = "I": 0.59 kbp; "II": 0.91 kbp.
[d] RFLP types = "I": 1.03 kbp; "II": 3.75 kbp.
[e] RFLP type = "I": 3.44 kbp.

et al. 1990; ROSA, PERALTA-SOLER and MACCIONI 1990), opened the question about the cellular role(s) of these structures. Interestingly, it was found that the *N. crassa* morphological mutants *snowflake* showed a dramatic alteration in the *in vivo* array of the P59Nc 8–10-nm filaments (ROSA, ALVAREZ and MALDONADO 1990). We hypothesized that (i) the defect in *snowflake* could be related to a mutation in the P59Nc gene which modifies the properties of the polypeptide for *in vivo* supramolecular assembly, or (ii) the abnormal bundles of filaments observed in *snowflake* could be due to a mutation in a different gene whose product modifies the *in vivo* assembly-disassembly properties of the P59Nc 8–10-nm filaments (ROSA, ALVAREZ and MALDONADO 1990).

To distinguish between these possibilities we first cloned the P59Nc gene. In a second step, by using RFLP segregation analyses (BOTSTEIN *et al.* 1980; METZENBERG *et al.* 1985), we mapped the *cfp* locus in wild-type and *sn* mutant strains. The study of the segregation of a *Bcl*I RFLP showed that the *cfp* locus is on the right arm of chromosome *VII*. Analysis of P59Nc *Cla*I and *Bam*HI RFLPs, in strains partially duplicated for a distal fragment of the right arm of chromosome *VII*, indicated that the *cfp* locus is positioned at the left of the proximal breakpoint of the translocation *T(VII → I)5936* roughly at about 5 map units of the locus *for*.

The mapping of the P59Nc gene to the linkage group VII strongly supported the notion that the *snowflake* locus (*sn*), on the centromere region of linkage group I, is not the genetic locus of P59Nc. RFLP mapping studies demonstrate that *sn* mutations did not represent a translocation of the P59Nc gene. Besides, the experiments indicate that the *snowflake* mutants possess a single, apparently normal, copy of the P59Nc gene at its normal locus on linkage group VII.

Taken together our results indicate that the aberrant bundles of 8–10-nm filaments observed in the *snowflake* mutants are not produced by alterations in the primary sequence of the P59Nc polypeptide. The possibility that the putative product of the *sn* locus is a post-translational modifier of the P59Nc polypeptide and/or of the *in vivo* properties of the P59Nc filaments to form bundles still remains.

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