GENETICS OF THE TUBULIN GENE FAMILIES OF PHYSARUM

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ABSTRACT

The organization of the α- and β-tubulin gene families in Physarum was investigated by Mendelian analysis. Restriction endonuclease-generated DNA fragments homologous to α- and β-tubulin show length polymorphisms that can be used as markers for genetic mapping. Analysis of meiotic assortment among progeny of heterozygotes allowed α- and β-tubulin sequence loci to be defined. There are four unlinked α-tubulin sequence loci (*altA*, *altB*, *altC* and *altD*) and at least three unlinked β-tubulin sequence loci (*betA*, *betB* and *betC*). The α-tubulin loci are not linked to the β-tubulin loci. —— Segregation of tubulin sequence loci with respect to *ben* mutations that confer resistance to antitubulin benzimidazole drugs was used to investigate whether any members of the α- or β-tubulin gene families are allelic to *ben* loci. The β-tubulin sequence locus *betB* is allelic to the resistance locus *benD*, the *betA* locus is probably allelic to *benA* and the α-tubulin sequence locus *altC* may be allelic to *benC*. The molecular implications of benzimidazole resistance phenotypes when only one of the expressed β-tubulin gene family members mutates to drug resistance are discussed in relation to tubulin function.

MICROTUBULES are major structural components of eukaryotic cells and function in mitosis, meiosis, motility and determination of cell shape. They are assembled from protomers composed of one α-tubulin and one β-tubulin polypeptide. Both the α- and the β-tubulin polypeptide sequences are conserved among eukaryotes (KIRSCHNER 1978; LUDUENA 1979; CLEVELAND *et al.* 1980; NEFF *et al.* 1983). Nevertheless, heterogeneity among α- and β-tubulin polypeptides is observed within an individual organism and even within a single cell type (MORRIS, LAI and OAKLEY 1979; GOZES and SWEADNER 1981; RAFF *et al.* 1982; MCKEITHAN *et al.* 1983; HALL *et al.* 1983; BURLAND *et al.* 1983).

In many eukaryotes, the tubulins are encoded by an α-tubulin gene family and a β-tubulin gene family (reviewed by CLEVELAND 1983). The organization and expression of these families are of much interest. In particular, does any individual member of a gene family encode a tubulin polypeptide that assembles into functionally distinct microtubules? A fruitful avenue for investigating expression and function of individual tubulins is to obtain mutations in tubulin genes (KEMPHUES *et al.* 1982; OAKLEY and MORRIS 1980). Tubulin gene mutations have also been valuable in Physarum, in which a mutant with an elec-

trophoretically altered β-tubulin demonstrated that one β-tubulin polypeptide is expressed in both myxamoebae and plasmodia, whereas a second β-tubulin is expressed specifically in myxamoebae (BURLAND *et al.* 1984).

This report examines the genetic organization of the α- and β-tubulin gene familes in Physarum. α- And β-tubulin sequences are detected by cleavage of Physarum DNA with restriction endonucleases, electrophoretically separating the fragments, transferring them to filters and then hybridizing with α- or β-tubulin sequence probes. These tubulin restriction fragments detected by DNA blotting show length polymorphisms between different Physarum strains. Such length polymorphisms allow restriction fragments to be used as markers for genetic analysis of α- and β-tubulin sequences. Haploid myxamoebae with different spectra of polymorphic tubulin DNA fragments are used as gametes in crosses to construct heterozygous diploid plasmodia. The plasmodia are then sporulated and clonal meiotic myxamoebal segregants obtained. From the pattern of meiotic assortment of polymorphic restriction fragments, α- and β-tubulin sequence loci can be defined and their genetic organization deduced; this technique has been used previously to analyze the organization of the actin gene family in Physarum (SCHEDL and DOVE 1982). When one of the myxamoebal gametes is resistant to antitubulin drugs (BURLAND *et al.* 1984), segregation of α- and β-tubulin sequence loci with respect to drug resistance can be examined, allowing one to determine whether any member of a tubulin gene family is allelic to a given drug resistance locus. If a tubulin DNA sequence and a drug resistance mutation are allelic, then the expression pattern and mutant phenotype of the drug-resistant mutant can be ascribed to that particular tubulin gene family member.

MATERIALS AND METHODS

Strains: The genotypes of myxamoebal strains used as gametes in crosses to construct diploid plasmodia and for Mendelian analysis are shown in Table 1.

Recombinant plasmids: The Physarum plasmodial α-tubulin cDNA clone Ppc-α125 is described by SCHEDL *et al.* 1984), SCHEDL (1984) and BURLAND *et al.* (1983). The following plasmids were generous gifts: chick α- and β-tubulin cDNA clones pT1 and pT2 from D. CLEVELAND (CLEVELAND *et al.* 1980), Drosophila α- and β-tubulin genomic clones DTA4 and DTB4 from J. NATZLE (SANCHEZ *et al.* 1980) and Drosophila actin genomic clone λDmA2 from E. FYRBERG (FYRBERG *et al.* 1980). Plasmid DNA was isolated by the method of CLEWELL (1972).

Genetic methods: Crosses, plasmodial testing, sporulation and analysis of clonal segregants were performed as described by BURLAND *et al.* (1984). Progeny clones from crosses with CLd (*benA*) and CLd (*benC*) gametes were tested for growth on 5 μM methylbenzimidazole-2yl-carbamate (MBC). Progeny clones from crosses with CLd (*benD*) gametes were tested for growth on 5, 10 and 50 μM MBC. Resistance was defined as growth on at least 5 μM MBC.

Culture methods and DNA isolation: Myxamoebae were grown on lawns of live or formalin-killed *E. coli* as described by BURLAND *et al.* (1984). Plasmodia were cultured on SDM (DEE and POULTER 1970) or MYM (SCHEDL *et al.* 1984). For isolation of DNA from each clonal myxamoebal strain, 100 plates were each inoculated with 2.5–5 × 10^5 myxamoebae. Plates became confluent in 2–3 days at 26°; myxamoebae were harvested by flooding each plate with 5 ml of distilled water, gently scraping with a glass spreader and pooling the suspension in an Erlenmeyer flask. The pooled suspension was allowed to stand 2–4 hr at 22° to facilitate excystment of any encysted myxamoebae. The suspension was passed twice through milk filters (Rapid Flow) and then concentrated by pelleting at 1000 × *g* for 10 min in a clinical centrifuge. Myxamoebae were separated from contaminating *E. coli* by three cycles of low-speed centrifugation, 200 × *g* for 5 min. This

TABLE 1

Amoebal strains

Strain	Genotype	Source/reference
CLd	*mt-h npfC⁻ matB1 fusA2 ardA2 ardB2 ardC2 ardD1 betA1 betB1 betC1 altA1 altB1 altC1 altD1*	DEE (1982); SCHEDL and DOVE (1982); SCHEDL (1984)
MA275	*mt-15* or *16 matB12* or *13 ardA3 ardB3 ardC3 ardD3 betA2 betB2 betC2 altA2 altB2 altC2 altD2*	J. A. GORMAN, unpublished data; KIROUAC-BRUNET, MASSON and PALLOTTA (1981); SCHEDL (1984)
LU862	*mt3 matB3 fusA1 eme^R ardC4*; all other *ard*, *bet* and *alt* alleles are those of CLd.	DEE (1978); SCHEDL (1984); and present report
BEN41, BEN107, BEN210, MA407	CLd(*ben*)	See BURLAND *et al.* (1984)

yields less than one *E. coli* per myxamoeba. DNA was then isolated from the purified myxamoebae as previously described (SCHEDL and DOVE 1982). Plasmodial DNA was purified as described by GORMAN, DOVE and WARREN (1981) except that 200 μg of proteinase K/ml (Beckman) replaced pronase.

Restriction enzyme digestion and gel electrophoresis: Restriction endonucleases were obtained from New England Biolabs; digestion buffers were those recommended by the supplier. Physarum DNA, about 2 μg for haploids and 4 μg for diploids, was digested with a tenfold excess of enzyme for 2–3 hr at 37°. DNA fragments were fractionated by electrophoresis on 0.5% or 0.7% agarose gels (MCDONNELL, SIMON and STUDIER 1977). In some cases, 0.1 ng of phage λ DNA was included in the Physarum DNA to be digested. This allowed the efficiency of fragment transfer by blotting to be determined (see following data) and provided *in situ* molecular size markers when the λ fragments were detected with nick-translated λ probes. Size markers were λ and λ digested with *Eco*RI, *Hin*dIII, *Sal*I or *Kpn*I (SANGER *et al.* 1982).

Hybridization probes: Nonspecific hybridization to Physarum DNA in blot experiments was observed when *Pst*I inserts of cDNA clones were used as probes. Specific hybridization was obtained when the probes were internal fragments of these cDNA inserts lacking the dG-dC tails (SCHEDL 1984). Presumably, the nonspecific hybridization is the result of dG/dC-rich sequences dispersed throughout the Physarum genome.

The Physarum α-tubulin probe was an internal 1-kb *Bgl*II-*Sac*I fragment of cDNA clone Ppc-α125 (SCHEDL 1984). The interspecies α-tubulin probe was either a 750-base pair (bp) *Pst*I-*Sph*I fragment of chick cDNA clone pT1 (VALENZUELA *et al.* 1981) or a 1.5-kb *Eco*RI-*Sal*I fragment from the Drosophila genomic clone DTA4 (SANCHEZ *et al.* 1980). The interspecies β-tubulin probe was either a 950-bp *Bgl*II-*Stu*I fragment of chick cDNA clone pT2 (VALENZUELA *et al.* 1981) or a 2.35-kb *Eco*RI-*Bgl*I fragment from the Drosophila genomic clone DTB4 (SANCHEZ *et al.* 1980). The actin probe was a 1.8-kb *Hin*dIII fragment from the Drosophila genomic clone λDmA2 (FYRBERG *et al.* 1980, 1981; also see SCHEDL and DOVE 1982). Purified fragments were labeled *in vitro* by nick translation (RIGBY *et al.* 1977) to a specific activity of 1–6 × 10^8 cpm/μg. Following labeling, probes were purified by phenol extraction, Sephadex G-75 chromatography (Pharmacia) and finally filtration through 0.45-μm filters (Acrodisc, Gelman).

DNA blotting and hybridization: Following electrophoresis, DNA fragments within the gel were partially cleaved *in situ* by the method of WAHL, STERN and STARK (1979) and then transferred to nitrocellulose filters (SOUTHERN 1975). For the interspecies α- and β-tubulin probes, an aqueous dextran sulfate solution was used for the prehybridization and the hybridization. Filters were prehybridized overnight at 42° in 5 × SSC (1 × SSC is 15 mM sodium citrate, 150 mM NaCl),

200 μg/ml of sonicated *E. coli* DNA, 200 μg/ml of yeast tRNA, 100 μg/ml of polyrA, 1 mg/ml of polyvinylpyrrolidone, 1 mg/ml of Ficoll, 50 mM sodium phosphate buffer (pH 7.0), 8 mM EDTA, 0.5% SDS and 10% dextran sulfate. The prehybridization solution was removed, and an identical solution plus 0.5–2.5 × 10^6 cpm/ml of nick-translated probe was added and hybridized at 65° for 8 hr. Following hybridization, filters were washed in a vast excess of 3 × SSC, 0.1% SDS at 55° for 2 hr with agitation. For the Physarum α-tubulin probe and the actin probe, filters were prehybridized and hybridized as previously described (SCHEDL and DOVE 1982) and then washed in 1 × SSC, 0.1% SDS at 55° for 2 hr with agitation. Under the hybridization conditions employed, α-tubulin sequences do not cross-hybridize with β-tubulin sequences. Filters were air dried and exposed to Kodak XAR-5 film using a Lightning-Plus intensifying screen.

To quantitate hybridization signals from blots, autoradiographs were scanned with a soft laser densitometer (LKB) and peak areas determined with an electronic digitizer (Numonics Corporation). Linearity of film response was assessed by twofold dilutions of the samples on the gel to be analyzed. The efficiency of restriction fragment transfer to nitrocellulose filters was judged by scanning λ marker fragments detected by hybridization with a nick-translated λ probe. For λ bands, the hybridization intensity should be a linear function of molecular weight. Fragments between 2 and 10 kb were transferred with equivalent efficiencies under the conditions used. To obtain relative hybridization intensities for the *Kpn*I β-tubulin fragment alleles (Table 7), the areas of peaks in each lane were normalized to the area of the monomorphic c fragment peak in the same lane from the same exposure: (relative area of peak q) = (q peak area)/(c peak area). The relative hybridization intensity analysis in Table 7 is independent of transfer efficiency since only fragments that are the same size are compared.

RESULTS

To determine simultaneously the genetic organization of tubulin DNA sequences and the cosegregation of benzimidazole resistance phenotypes, it was necessary to cross the CLd (*ben*) strains with a partner that differed in the length of α- and β-tubulin restriction fragments. Such restriction fragment length polymorphisms (BOTSTEIN *et al.* 1980) can be used as phenotypic markers for α- and β-tubulin sequences in the genome. A haploid myxamoebal segregant from the Wis2 natural plasmodial isolate, MA275, shows considerable fragment length polymorphism relative to CLd for α-tubulin, β-tubulin and actin sequences. MA275 is benzimidazole sensitive and well behaved in crosses with standard haploid myxamoebal strains of Physarum used for genetic analysis.

Assortment of polymorphic α-tubulin, β-tubulin, actin and benzimidazole resistance was analyzed by using MA275 and CLd (*ben*) myxamoebae as gametes in crosses. The CLd (*ben*) gametes; MA407 (*benC15*), BEN41 (*benA41*), BEN107 (*benD107*) and BEN210 (*benD210*), are described in the accompanying paper (BURLAND *et al.* 1984). Heterozygous diploid plasmodia obtained from the crosses were sporulated and meiotic myxamoebal segregants isolated. Myxamoebal clones were tested for growth on MBC and for mating type phenotype. From each cross, DNA was isolated from a set of clonal myxamoebal segregants and analyzed for the assortment of polymorphic restriction fragments.

Physarum genomic α-tubulin sequences were detected in blot hybridization experiments using a Physarum plasmodial α-tubulin cDNA clone, Ppc-α125 (BURLAND *et al.* 1983; SCHEDL *et al.* 1984). The same set of Physarum restriction endonuclease fragments were also detected with interspecies α-tubulin

probes from Drosophila and chick (SCHEDL 1984). The conserved nature of tubulin nucleic acid sequences (CLEVELAND *et al.* 1980; NEFF *et al.* 1983) was exploited to detect Physarum β-tubulin sequences. Interspecies β-tubulin probes from Drosophila and chick (MATERIALS AND METHODS) hybridized to an identical set of Physarum genomic restriction fragments with approximately equivalent intensities for all restriction endonucleases that have been tested (SCHEDL 1984). The fact that two β-tubulin probes from phylogenetically distant species detect the identical set of Physarum fragments provides evidence that these bands contain Physarum β-tubulin sequences. Further evidence that the two probes detect Physarum β-tubulin sequences is the observation that they can select by hybridization plasmodial RNA that encodes both plasmodial β-tubulins (BURLAND *et al.* 1983). The sizes of α- and β-tubulin restriction fragments were identical in myxamoebae and plasmodia; therefore, there is no evidence for genetic rearrangement of these sequences between the two phases of the life cycle.

α-Tubulin loci defined by assortment of Eco*RV fragments*: Clonal myxamoebal segregant DNAs from the four crosses were digested with restriction endonuclease *Eco*RV, and the α-tubulin fragments were detected by blot hybridization using the Ppc-α125 fragment probe (see MATERIALS AND METHODS). The plasmodial α-tubulin exon sequence of Ppc-α125 contains an *Eco*RV cleavage site, generating two sequences that hybridize with the probe. Figure 1 shows the *Eco*RV α-tubulin fragments detected and their assortment among haploid meiotic segregants. Fifteen *Eco*RV bands, designated a through o, can be detected in the diploid plasmodium (CLd × MA275). The first two lanes show that the CLd and the MA275 gametes have no bands in common. This means that the [CLd (*ben*) × MA275] parental plasmodium in these crosses is heterozygous with respect to all *Eco*RV α-tubulin fragments. Each haploid segregant has a subset of seven or eight parental bands (Figure 1). However, in the two crosses in which the CLd gamete carried a *benD* benzimidazole resistance allele, four segregants (284, 286, 288 and 306) are ill behaved in carrying more than eight *Eco*RV fragments. These nonhaploid meiotic segregants will be discussed separately. The segregants from the four crosses (46 total) were scored for the presence or absence of parental *Eco*RV bands. The α-tubulin fragments observed in each segregant were tabulated by cross in a strain distribution pattern.

Allelism and linkage among fragments was determined by pairwise comparison of bands in the strain distribution pattern as previously described (SCHEDL and DOVE 1982). The following Mendelian assumptions were used to group the data: (1) if two fragments are alternative alleles, each haploid segregant will have only one of the two fragments; (2) if two fragments are derived from unlinked loci, they will assort independently and their strain distribution patterns will be uncorrelated; (3) if two fragments are tightly linked, their strain distribution patterns will be concordant; (4) extensive but incomplete concordance may result from loose linkage. For example, *Eco*RV fragments d and e (Figure 1) show mutual exclusion among haploid segregants indicating that the d and e fragments are alternative alleles of the same locus. The distribution

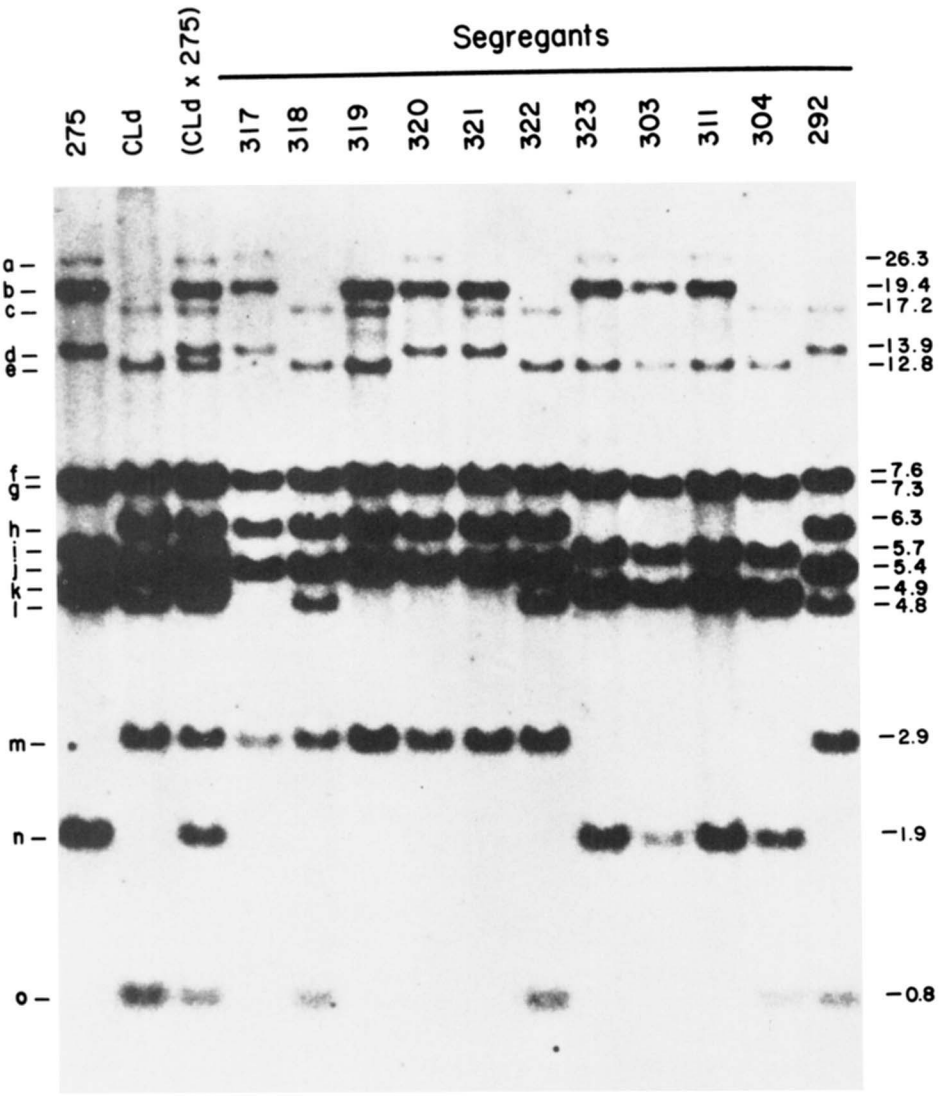

FIGURE 1.—Meiotic assortment of polymorphic *Eco*RV α-tubulin fragments among CLd (*ben*) × MA275 progeny. DNA from [CLd × MA275] (4 μg) and from myxamoebal segregants (about 2 μg) were digested with *Eco*RV, electrophoresed on a 0.7% agarose gel, blotted and then hybridized with nick-translated Ppc-α125 fragment probe (1×10^6 cpm/ml; see MATERIALS AND METHODS). Exposure shown was for 7 days. Fifteen bands (a through o) are detected in the [CLd × MA275] heterozygous diploid plasmodia. This band pattern is the sum of those found in the two haploid gametes, CLd and MA275. No monomorphic fragments are observed. Meiotic segregants from crosses 407 (*benC*) × MA275 and BEN210 × MA275 are shown. Strain designations have been abbreviated (*e.g.*, 275 = MA275).

of bands b and d among segregants is uncorrelated, as expected for independently assorting loci. By contrast, the concordant distribution of bands h and j indicates that they are linked.

The *Eco*RV α-tubulin fragments are organized into four unlinked loci (des-

ignated *alt* for alpha tubulin). Tables 2 and 3 illustrate the linkage relationships among *Eco*RV fragments from the crosses MA407 (*benC15*) × MA275 and BEN41 (*benA41*) × MA275. These relationships were deduced from all four crosses (data from BEN107 (*benD107*) × MA275 and BEN210 (*benD210*) × MA275, not shown). The *1* allele (*altA1*, *altB1*, *altC1* and *altD1*) represents fragments derived from the CLd gamete, whereas the *2* allele represents those derived from the MA275 gamete. The set of four *alt* loci accounts for all of the *Eco*RV fragments and thus represents a complete Mendelian characterization of α-tubulin loci detectable with the Physarum plasmodial Ppc-α125 probe and the Drosophila and chick interspecies probes.

The *alt* sequence loci are also identified by the assortment of polymorphic *Kpn*I α-tubulin fragments (SCHEDL 1984). A pair of *Eco*RV and *Kpn*I fragments that show the same distribution pattern among all of the segregants correspond to fragments from the same α-tubulin sequence and thus represent two markers for the same allele (see SCHEDL and DOVE 1982). Assortment of polymorphic *Kpn*I fragments identifies the *altA*, *altB* and *altC* loci, with a monomorphic fragment that presumably corresponds to the *altD* sequences (data not shown). In contrast to *Eco*RV, *Kpn*I does not cleave the α-tubulin exon sequence of the Ppc-α125 probe. However, two *Kpn*I fragments cosegregate for *altB1*, and three other *Kpn*I fragments cosegregate for *altB2*. The difference in number of *Kpn*I fragments between the *altB1* and *altB2* alleles can be interpreted as an intralocus *Kpn*I site polymorphism.

The *altB* locus is complex: four *Eco*RV and two or three *Kpn*I fragments cosegregate for each allele. No recombinants were observed among the 46 segregants. This indicates that the *Eco*RV fragments g, i, k and n are linked (Tables 2 and 3), as are the two *Kpn*I fragments, each of which define the *altB1* allele. Similar statements apply to the *altB2* allele. This linkage is highly significant (binomial probability < 0.001).

The linkage of the *altB* fragments suggests either that there are multiple endonuclease cleavage sites within a single α-tubulin gene or there is more than one α-tubulin sequence at the *altB* locus. To test whether the *altB* locus contains multiple α-tubulin sequences, the hybridization intensities of the *altB* *Eco*RV fragments were compared with a hybridization standard curve (LIS, PRESTIDGE and HOGNESS 1978) generated from different numbers of Ppc-α125 copies per haploid genome. The aggregate hybridization intensity of the *Eco*RV-generated *altB1* fragments in the haploid CLd corresponds to about 2.6 copies of Ppc-α125 per haploid genome. In agreement with this result, the aggregate hybridization intensity of the *altB1* plus *altB2* fragments in the diploid CLd × MA275 corresponds to about 5.1 copies of Ppc-α125 per diploid genome (SCHEDL 1984). This gives a minimum estimate for the number of α-tubulin sequences at *altB*, since it assumes that the probe hybridizes as efficiently to the *altB* sequences as it does to itself.

How tightly linked are these *altB* sequences? The limits of recombinational distance can be calculated (CROW and KIMURA 1970). At the 95% confidence level, the upper limit is 6 cM, whereas the lower limit is zero, which implies shared *Kpn*I or *Eco*RV cleavage sites for *altB* fragments.

β-Tubulin loci defined by assortment of Stu*I fragments*: The linkage arrange-

TABLE 2

Assortment of α-tubulin-homologous Eco*RV fragment alleles among [407*(benC15) × *MA275] progeny*

Locus	DNA band	Allele[a]	Gametes		[407 × MA275]	Segregants						
			407	MA275		317	318	319	320	321	322	323
altA	*Eco*RV b	*altA2*	−	+	+	+	−	+	+	+	−	+
	*Eco*RV l, o	*altA1*	+	−	+	−	+	−	−	−	+	−
altB	*Eco*RV f, h, j, m	*altB1*	+	−	+	+	+	+	+	+	+	−
	*Eco*RV g, i, k, n	*altB2*	−	+	+	−	−	−	−	−	−	+
altC	*Eco*RV a	*altC2*	−	+	+	+	−	−	+	−	−	+
	*Eco*RV c	*altC1*	+	−	+	−	+	+	−	+	+	−
altD	*Eco*RV d	*altD2*	−	+	+	+	−	−	+	+	−	−
	*Eco*RV e	*altD1*	+	−	+	−	+	+	−	−	+	+
BEN phenotype[b]			R	S	S	S	R	R	S	R	R	S

[a] Allele *1* fragment(s) derived from the CLd(*ben*) gamete; allele *2* derived from the MA275 gamete.

[b] R = MBC resistant; S = MBC sensitive.

TABLE 3

Assortment of α-tubulin-homologous Eco*RV fragment alleles among [BEN41* (benA41) × *MA275] progeny*

Locus	DNA band	Allele[a]	[BEN41 × MA275]	Segregant 277	278	279	280	281	282	298	299	300	301	302	326	327
altA	*Eco*RV b	*altA2*	+	+	−	+	+	+	−	+	+	+	−	−	+	−
	*Eco*RV l, o	*altA1*	+	−	+	−	−	−	+	−	−	−	+	+	−	+
altB	*Eco*RV f, h, j, m	*altB1*	+	+	−	+	+	−	+	+	+	−	−	+	−	+
	*Eco*RV g, i, k, n	*altB2*	+	−	+	−	−	+	−	−	−	+	+	−	+	−
altC	*Eco*RV a	*altC2*	+	+	−	+	−	+	+	−	+	−	+	−	+	+
	*Eco*RV c	*altC1*	+	−	+	−	+	−	−	+	−	+	−	+	−	−
altD	*Eco*RV d	*altD2*	+	+	−	−	−	−	−	+	+	−	+	+	+	−
	*Eco*RV e	*altD1*	+	−	+	+	+	+	+	−	−	+	−	−	−	+
BEN phenotype			S	S	S	S	R	R	R	S	R	R	S	R	S	S

[a] Allele *1* fragment(s) derived from the CLd(*ben*) gamete; allele *2* derived from the MA275 gamete.

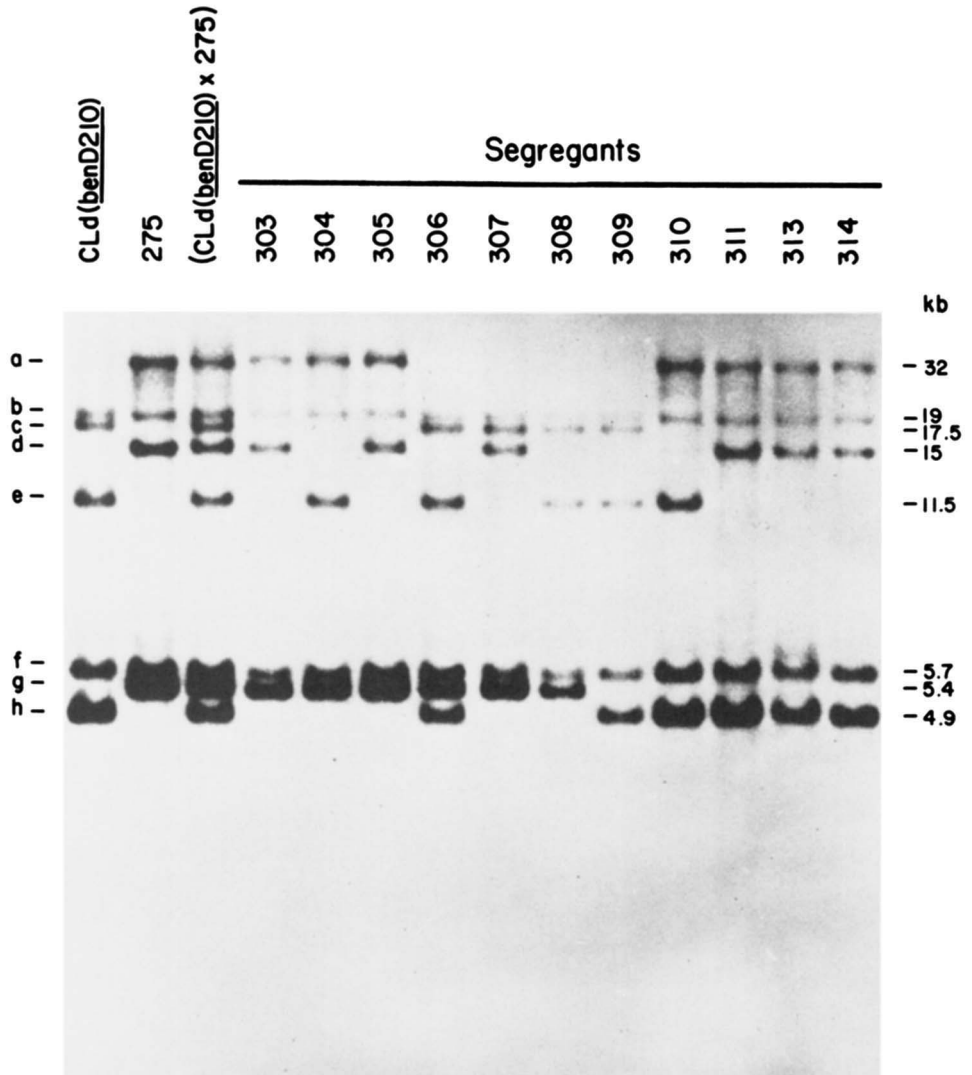

FIGURE 2.—Meiotic assortment of polymorphic *Stu*I β-tubulin fragments among CLd (*ben*) × MA275 progeny. DNA from [CLd (*benD210*) × MA275] (4 μg), and from clonal myxamoebal segregants (about 2 μg), was digested with *Stu*I, electrophoresed on a 0.7% agarose gel and then blotted to nitrocellulose filters. Filters were hybridized, using the aqueous dextran sulfate method, with 2×10^6 cpm/ml of nick-translated chick β-tubulin probe (see MATERIALS AND METHODS). Exposure shown was for 2 days. Eight bands (a through h) are detected in the [CLd × MA275] diploid. The CLd and the MA275 gametes each have three bands that are assorting, while two bands (b and f) are monomorphic. All of the five MBC-resistant haploid segregants have the *Stu*I h fragment from the CLd (*benD*) gamete. All five MBC-sensitive haploid segregants have the *Stu*I g fragment from the MA275 gamete. The nonhaploid segregant, 306, has both the g and h fragments. Equivalent results are obtained with the Drosophila β-tubulin probe.

ment of β-tubulin sequences was examined by meiotic assortment of polymorphic *Stu*I fragments from the four CLd (*ben*) × MA275 crosses. Figure 2 shows the *Stu*I β-tubulin fragments detected with the chick β-tubulin probe

and their assortment among meiotic progeny from the cross BEN210 (*benD210*) × MA275. The fragments detected for the CLd (*benD210*) and MA275 gametes (first two lanes, Figure 2) show that six bands are assorting, whereas two bands (b and f) are monomorphic, so that their assortment cannot be observed. Minor bands that were not reproducibly visualized are omitted from the analysis. Haploid segregants have five *Stu*I fragments. However, four segregants (284, 286, 288 and 306 shown in Figure 2) are ill behaved, with more than five bands; these are discussed separately.

The linkage relationships among *Stu*I β-tubulin fragments was determined as described earlier and are shown for crosses BEN41 (*benA41*) × MA275 and BEN210 (*benD210*) × MA275 in Tables 4 and 5, respectively (data for crosses MA407 (*benC15*) × MA275 and BEN107 (*benD107*) × MA275 not shown). Three unlinked β-tubulin loci (designated *bet* for beta tubulin) are identified. Each allele of the *betA*, *betB* and *betC* loci has a single *Stu*I fragment. As described earlier, allele *1* is derived from the CLd gamete, and allele *2* is from the MA275 gamete. Three is a lower limit for the number of β-tubulin sequence loci since the monomorphic *Stu*I fragments may be derived from additional loci. Alternatively, the monomorphic *Stu*I fragments could also be fragments from one of the three identified *bet* loci. Assortment of polymorphic β-tubulin fragments generated by cleavage of genomic DNA with ten other restriction endonucleases (each with six-base specificity) reveals no additional loci; each of these enzymes generates at least two monomorphic fragments.

Cosegregation of ben *loci and tubulin DNA sequence loci*: The assortment of benzimidazole resistance phenotype and polymorphic tubulin restriction fragments was compared for each cross. Data presented in the accompanying report (BURLAND *et al.* 1984) demonstrate that the *benD* locus encodes a β-tubulin. One might then expect that the resistance phenotype of *benD* mutants would cosegregate with a *Stu*I β-tubulin fragment from the CLd gamete that carried the *benD* mutation but not with a fragment from the MA275 gamete. Assortment of the *benD*-encoded resistance phenotype and the *Stu*I β-tubulin fragments are shown for BEN210 (*benD210*) × MA275 in Table 5 (data for the BEN107 (*benD107*) × MA275 segregants not shown). Resistance cosegregated with the CLd-derived *Stu*I h fragment (*betB1* allele) in 13 of 13 haploid segregants. Sensitivity cosegregated with the MA275-derived *Stu*I g fragment (*betB2*) in nine of nine haploid segregants. This linkage is significant ($P < 0.001$). Based on cosegregation of *benD* resistance phenotype and *betB* fragment alleles in 22 of 22 haploid segregants, the estimated recombinational distance is 0 cM (upper 95% confidence level is 16 cM). Thus, *benD* and *betB* are recombinational alleles. The independent, strong criterion used to indicate that the *benD* mutant phenotype is determined by a β-tubulin structural gene mutation is that the *benD210* allele encodes an electrophoretically altered β-tubulin polypeptide denoted β1-210 (BURLAND *et al.* 1984). Thus, cosegregation of the resistance phenotypes and *betB* β-tubulin sequences allows identification of the member of the β-tubulin gene family that encodes the *benD210* mutant polypeptide.

Three nonhaploid segregants from the cross BEN107 (*benD107*) × MA275 (284, 286, 288) and one from the cross BEN210 (*benD210*) × MA275 (306)

TABLE 4

Assortment of β-tubulin-homologous StuI *fragment alleles among [BEN41* (benA41) × *MA275] progeny*

Locus	DNA band	Allele[a]	[BEN41 × MA275]	Segregants[b]												
				277	278	279	280	281	282	298	299[c]	300	301	302	326	327
betA	*Stu*I d	*betA2*	+	+	+	+	−	−	−	+	−	−	+	−	+	+
	*Stu*I e	*betA1*	+	−	−	−	+	+	+	−	+	+	−	+	−	−
betB	*Stu*I g	*betB2*	+	+	+	−	−	+	+	+	+	+	+	−	+	−
	*Stu*I h	*betB1*	+	−	−	+	+	−	−	−	−	−	−	+	−	+
betC	*Stu*I a	*betC2*	+	−	+	+	+	−	+	+	−	+	−	−	+	+
	*Stu*I c	*betC1*	+	+	−	−	−	+	−	−	+	−	+	+	−	−
	*Stu*I b, f	Monomorphic	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BEN phenotype			S	S	S	S	R	R	R	S	R	R	S	R	S	S

[a] Allele *1* fragment(s) derived from the CLd(*ben*) gamete; allele *2* derived from MA275 gamete.

[b] An additional segregant of BEN41 × MA275, 297 (MBC resistant), has the *betA2* (sensitive) allele. However, the test cross 297 × BEN41 yielded 10/48 MBC sensitive progeny. One concludes that 297 carries a resistance mutation different from *benA41*. The origin of this anomaly is unknown.

[c] Segregant 299 shows some growth on 5 μM MBC but less than BEN41. The cross 299 × CLd yields some fully resistant progeny. One concludes that the incomplete resistance of 299 is created by the Wis1 × Wis2 mixed natural isolate background.

TABLE 5

Assortment of β-tubulin-homologous StuI *fragment alleles among [BEN210* (benD210) × *MA275] progeny*

Locus	DNA band	Allele[a]	[BEN210 × MA275]	Segregants											
				303	304	305	306	307	308	309	310	311	312	313	314
betA	*Stu*I d	*betA2*	+	+	−	+	−	+	−	−	−	+	−	+	+
	*Stu*I e	*betA1*	+	−	+	−	+	−	+	+	+	−	+	−	−
betB	*Stu*I g	*betB2*	+	+	+	+	+	+	+	−	−	−	−	−	−
	*Stu*I h	*betB1*	+	−	−	−	+	−	−	+	+	+	+	+	+
betC	*Stu*I a	*betC2*	+	+	+	+	−	−	−	−	+	+	−	+	+
	*Stu*I c	*betC1*	+	−	−	−	+	+	+	+	−	−	+	−	−
	*Stu*I b, f	monomorphic	+	+	+	+	+	+	+	+	+	+	+	+	+
BEN phenotype			S	S	S	S	S	S	S	R	R	R	R	R	R

[a] Same as Table 4.

TABLE 6

The alt, bet *and* ard *alleles among the nonhaploid* [*BEN107* (benD107) × *MA275*] *and* [*BEN210* (benD210) × *MA275*] *segregants*

Locus/allele	Segregant[a] 284	286	288	306
altA1	+	+	+	+
altA2	+	+	+	+
altB1	−	+	+	−
altB2	+	+	+	+
altC1	−	+	+	−
altC2	+	+	+	+
altD1	+	+	+	+
altD2	+	−	+	+
betA1	+	−	+	+
betA2	−	+	+	−
betB1	+	+	+	+
betB2	+	+	+	+
betC1	+	+	+	+
betC2	−	+	+	−
ardA3	+	+	+	+
ardA2	+	+	+	+
ardB2	+	−	+	+
ardB3	+	+	−	−
ardC2	+	+	+	+
ardC3	+	−	+	+
ardD1	−	+	+	+
ardD3	+	+	−	+
No. of loci heterozygous	6/11	7/11	9/11	6/11
BEN phenotype	S	S	S	S

[a] Segregants 284, 286 and 288 are from the cross BEN107 (*benD107*) × MA275, whereas 306 is from the cross BEN210 (*benD210*) × MA275.

are all heterozygous at *betB* and are sensitive to MBC (Table 6). Two-dimensional gel analysis of segregant 306 showed that both the mutant polypeptide β1-210, as well as the normal β1 polypeptide are expressed (not shown). As expected, segregant 310 (MBC-resistant) also expresses β1-210, whereas segregant 303 (MBC-sensitive) does not (T. G. BURLAND, unpublished observation). The benzimidazole sensitivity of these nonhaploid segregants containing both the *betB1*(*benD*) resistance and *betB2* sensitivity alleles indicates that resistance is recessive (see DISCUSSION).

In the cross BEN41 (*benA41*) × MA275, the *benA41* resistance mutation cosegregates with the *betA* β-tubulin sequence locus (Table 4). All six benzimidazole-resistant segregants have the CLd allele, *betA1*, whereas all seven sensitive segregants have the MA275 allele, *betA2*. This linkage is statistically significant ($P < 0.001$), suggesting that *benA* and *betA* are allelic. The 95% confidence limits of recombinational distance are from 0 to 26 cM. Two-dimensional gel analysis of BEN41 (*benA41*) and BEN46 (*benA46*) myxamoebal proteins does not reveal any altered α- and β-tubulin polypeptides (BURLAND *et al.* 1984).

Benzimidazole resistance from the cross 407 (*benC15*) × MA275 did not cosegregate with any of the *bet* loci. However, the *benC15* resistance phenotype does cosegregate with the α-tubulin locus *altC* (Table 2). The four segregants with the *altC1* allele are MBC resistant, whereas the three *altC2* segregants are MBC sensitive. The linkage of *altC* and *benC* is significant ($P < 0.01$), suggesting that they are allelic. The limits of recombinational distance between *altC* and *benC* is from 0 to 46 cM (95% confidence level). BEN15 was not found to have any electrophoretically altered α- or β-tubulin polypeptides (BURLAND *et al.* 1984).

The assortment of tubulin sequence loci has not been analyzed for strains carrying *benB* mutations.

Linkage relationships between α-tubulin, β-tubulin and actin loci: Linkage between *alt*, *bet* and *ard* (actin) loci was examined by pairwise comparison of strain distribution patterns from all of the crosses (SCHEDL 1984). The four α-tubulin loci are each unlinked to any of the three β-tubulin loci. The β-tubulin loci are each unlinked to any of the four actin loci. However, one of the α-tubulin loci (*altD*) is linked to one of the actin loci (*ardC*). The CLd alleles *ardC2* and *altD1* cosegregate as do the MA275 alleles *ardC3* and *altD2*. One recombinant, segregant 293 (*ardC3*, *altD1*) was observed among 46 segregants. Thus, *ardC* and *altD* are separated by about 2 cM (0.5–9 cM, 95% confidence limits). None of the *alt*, *bet* or *ard* loci was found to be linked to the mating type locus (*mt*).

Nonhaploid meiotic segregants: Three segregants from the cross BEN107 × MA275 and one segregant from the cross BEN210 × MA275 have both restriction fragment alleles for some loci. The *alt*, *bet* and *ard* alleles found in these four nonhaploid segregants are shown in Table 6. For each segregant, at least half of the loci are heterozygous for fragment alleles, and the patterns of linked *ardC* and *altD* alleles are concordant. This is explained most simply by the presence of both homologues (one from CLd and the other from MA275) in these segregants. Thus, these segregants are either aneuploids or diploids.

Are the nonheterozygous loci in these segregants haploid or homozygous diploid? This was assessed by densitometric analysis of autoradiograms of *Kpn*I β-tubulin fragments. Endonuclease *Kpn*I allows detection of both alleles of *betA* and *betB* with two monomorphic fragments. Table 7 shows the relative hybridization intensity of *Kpn*I β-tubulin fragments from [CLd × MA275] and nine haploid and nonhaploid segregants. The hybridization intensities were normalized to the monomorphic c fragment to correct for any difference in the

TABLE 7

Relative hybridization intensity of β-tubulin alleles detected after digestion with KpnI

Locus/allele	*Kpn*I band	Diploid plasmodium [CLd × MA275]	Haploid segregants						Nonhaploid segregants		
			290	298	299	302	303	311	284	288	306
betA1	a	2.5[a]	1.9		2.0	2.2	2.1		4.8	2.4	5.0
		2.2[a]	2.2		2.3	2.3	2.4		4.9	2.1	4.5
betA2	b	2.1		2.1				2.2		2.2	
		2.2		2.1				2.1		2.4	
Monomorphic	c	2[b]	1	1	1	1	1	1	2	2	2
betB2	d	5.8		5.9	5.6		5.8		6.1	6.3	6.0
		6.1		5.3	5.9		5.9		6.3	5.9	5.5
betB1	e	4.4	4.9			4.5		4.7	4.9	4.4	4.4
		4.5	4.8			4.7		4.8	5.1	4.3	4.7
Monomorphic	f	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, not determined.

[a] Densitometric data derived from two autoradiographic exposures of the same Southern blot in a single experiment: upper, 3 days; lower, 7 days. The film exposures were judged to be in the linear range when the normalized peak areas of bands of varying intensity (≈threefold) have equivalent values from the two different exposures.

[b] The monomorphic normalizing band (c) was assigned a value of 1 in haploids and a value of 2 in diploids and non haploids.

amounts of Physarum DNA in each lane. This fragment is assigned a value of 1 in haploids and a value of 2 in diploids. Table 7 shows that segregants that are thought to be haploid (have only one fragment allele for all 11 loci) have hybridization intensities for *betA* and *betB* alleles equivalent to those found for each allele in heterozygous diploid plasmodia [CLd × MA275]. Strain 288, which has both alleles of *betA* and *betB*, has relative hybridization intensities equivalent to those of [CLd × MA275]. Strains 284 and 306 have only one *betA* allele. However, the relative hybridization intensity of *betA1* in 284 and 306 is twice that found for *betA1* in the heterozygous diploid plasmodium and in haploid segregants (290, 299, 302 and 303). This indicates that strains 284 and 306 are homozygous diploid for the *betA1* allele. Although densitometric analysis has not been performed for other loci, the presence of both heterozygous and homozygous *bet* loci in strains 284 and 306 suggests that these four segregants may be fully diploid. Diploidy is also suggested by the mitotic stability of the fragment allele genotype when independent DNA isolates from these four strains are compared.

Are the heterozygous loci randomly distributed among all of the segregants? The distribution of heterozygous loci among the segregants was significantly different ($P < 0.001$) from that predicted if occurrences were random (Poisson). This suggests that whatever event (mechanism) caused a segregant to be diploid at one locus (heterozygous or homozygous) also caused diploidy at all

other loci. This is discussed further in the accompanying paper (BURLAND *et al.* 1984).

Comparison of relative DNA hybridization intensities suggests that benzimidazole resistance is not the result of gene amplification. BEN210 is resistant to 50 μM MBC, whereas BEN107 is resistant to 10 μM MBC. Segregants 290 (*benD107*) and 311 (*benD210*) have equivalent relative hybridization intensities for the *betB1 Kpn*I e fragment as the [CLd × MA275] plasmodium (Table 7). Thus, it seems that neither the *benD* resistance nor the allelic difference in resistance level is the result of *betB* sequence amplification. The two *benA41* (resistant to 5 μM MBC) segregants, 299 and 302, also have relative hybridization intensities for the *betA1 Kpn*I a fragment equivalent to the [CLd × MA275] plasmodium, suggesting that the *benA41* resistance phenotype is also not a result of *betA1* sequence amplification.

DISCUSSION

Meiotic assortment of polymorphic restriction fragments defines four unlinked α-tubulin sequence loci (*altA* through *altD*) and at least three unlinked β-tubulin loci (*betA* through *betC*) in Physarum. The *altB* locus contains multiple linked α-tubulin sequences. Four *alt* loci and three *bet* loci is a lower limit for the number of tubulin loci in Physarum, since divergent tubulin sequences may not have been detected (LOPATA *et al.* 1983; BAUM, LIVNEH and WENSINK 1983). Furthermore, at least two monomorphic β-tubulin restriction fragments were always observed on blots; therefore, these fragments could represent other β-tubulin loci. The α-tubulin loci are unlinked to the β-tubulin loci. The α-tubulin locus *altD* maps about 2 cM from the actin locus *ardC*, but there is no linkage between any other members of the tubulin and actin gene families.

The organization of the Physarum α- and β-tubulin gene families is similar to that found for Drosophila and chick. These species have four or five genes for both α- and β-tubulin, and the tubulin genes are dispersed throughout the genome (SANCHEZ *et al.* 1980; CLEVELAND *et al.* 1981). This organization is in contrast to the tandemly duplicated α/β gene pairs found in *Trypanosome brucei* (THOMASHOW *et al.* 1983; SEEBECK *et al.* 1983) and the tandemly repeated α-tubulin genes and separate, tandemly repeated β-tubulin genes found in *Leishmania enriettii* (LANDFEAR, MCMAHON-PRATT and WIRTH 1983).

The number of α- and β-tubulin sequence loci observed in Physarum is close to the number of tubulin polypeptide species observed on two-dimensional gels of whole cell lysates. Electrophoretic tubulin species α1, α3 and β1 are detected in myxamoebae, whereas α1, α2, β1 and β2 tubulins are detected in plasmodia (BURLAND *et al.* 1983). Different mRNAs encode α1, α2, β1 and β2 tubulins (BURLAND *et al.* 1983), whereas α3 tubulin appears to be a posttranslationally modified form of α1 (L. L. GREEN, personal communication). The accompanying manuscript (BURLAND *et al.* 1984) shows that the myxamoebal β1 tubulin species, which appears electrophoretically homogeneous, consists of the products of at least two different genes. Thus, there are at least three different β-tubulin polypeptides and three or more β-tubulin sequence loci, while there are at least two different α-tubulin polypeptides and four α-tubulin sequence

loci. This suggests that, unlike *Homo sapiens* (see GWO-SHU LEE *et al.* 1983), Physarum does not have a large number of tubulin pseudogenes.

Resistance to antitubulin benzimidazoles can be conferred by mutation at any one of at least four unlinked loci in Physarum, *benA*, *benB*, *benC* and *benD* (BURLAND *et al.* 1984). It was expected that at least some of these mutations may be in tubulin genes, and one way to investigate this possibility was to test for cosegregation of benzimidazole resistance and any of the polymorphic restriction fragment size markers for the tubulin genes. The *benD107* and *benD210* resistance alleles cosegregated with the *betB* β-tubulin sequence marker, indicating that *benD* and *betB* are allelic. The fact that *benD210* encodes an electrophoretically altered β-tubulin polypeptide is strong independent evidence that *benD* is a structural gene for β-tubulin (BURLAND *et al.* 1984). Together, these observations establish the usefulness of restriction fragment length polymorphisms for mapping mutations to identifiable structural genes. To facilitate further discussion, the β1-tubulin encoded by the wild-type *betB* allele will be called β1-B, and the mutant, electrophoretically altered β1-tubulin encoded by the *betB(benD210)* allele will be called β1-B210. The concordant expression of both the drug resistance phenotype and the β1-B210 polypeptide indicates that *betB* is the member of the β-tubulin gene family that is expressed in both myxamoebal and plasmodial stages of the life cycle (see BURLAND *et al.* 1984). Note that the electrophoretic shift of the β1-B210 polypeptide uncovers a second myxamoebal β1-tubulin that is unaltered by the *betB(benD210)* mutation. This second β1-tubulin polypeptide will be called β1-A (see below).

Cosegregation of benzimidazole resistance mutations with restriction fragment markers for tubulin sequence loci can thus be used to investigate whether other benzimidazole resistance mutations may be located in any of the other tubulin genes, even when the tubulins of the mutants are electrophoretically normal (BURLAND *et al.* 1984). In this way, evidence was obtained that the benzimidazole resistance locus *benA* is allelic to *betA*. The benzimidazole-resistance phenotype of *benA* mutants is expressed in myxamoebae but not in plasmodia. The β1-A tubulin, which is unaltered by the *benD210* mutation, is similarly expressed in myxamoebae but not in plasmodia (BURLAND *et al.* 1984). The simplest interpretation of the cosegregation of *benA* with *betA* and the concordant pattern of expression of *benA* resistance and the β1-A polypeptide is that the β-tubulin gene family member *betA* encodes β1-A tubulin. To assign *benA* unequivocally to *betA* by recombination would require analysis of perhaps 10^4 myxamoebal progeny, with DNA to be extracted from each progeny clone and analyzed by blotting, clearly an impracticable undertaking. Stronger evidence that *benA* is a β-tubulin locus would be isolation of *benA* mutants with electrophoretically altered β1-A tubulin, or an altered *betA1* DNA sequence in a *benA* mutant.

The *benC15* benzimidazole resistance mutation cosegregated with the α-tubulin locus *altC* marker, suggesting that *benC* may be an α-tubulin structural gene. Since α- and β-tubulin polypeptides clearly interact in the tubulin protomer (LUDUENA 1979; MORRIS, LAI and OAKLEY 1979; OAKLEY and MORRIS 1981), it seems reasonable to propose that mutation in an α-tubulin gene could

also confer benzimidazole resistance. Although the linkage of *altC* to *benC* is significant, the data are also consistent with loose linkage of these two loci, since so few progeny were analyzed. The one known *benC* mutant, BEN15, does not have an electrophoretically altered α-tubulin (BURLAND *et al.* 1984). Thus, of the known *ben* loci, *benD* is certainly allelic to *betB*, *benA* is probably allelic to *betA* and *benC* may be allelic to *altC*.

An important issue concerns the function of tubulins where multiple forms are expressed. Are the different α- and β-tubulins each specific for a particular microtubule function (*e.g.*, participation in the mitotic spindle *vs.* the cytoskeleton), or can any of the α- and β-tubulins carry out any of the different microtubule functions? Furthermore, are individual microtubules assembled using only one α-tubulin and one β-tubulin gene product, or can all of the different α- and β-tubulins be assembled into the same microtubule? The Physarum *ben* mutations may provide clues to resolving this issue.

The following discussion makes the reasonable assumption that *betA* and *benA* are allelic. However, many of the points raised are valid even if this assumption is incorrect. Benzimidazole resistance mutations at one *bet* locus are epistatic to drug-sensitive wild-type alleles at the other *bet* loci. This is necessary given the way the mutants were selected. Thus, a mutation in only one of two expressed β-tubulin genes is sufficient to confer benzimidazole resistance. In benzimidazole-resistant, haploid *betB*(*benD210*) myxamoebae, both the resistant β1-B210 and sensitive β1-A tubulins are expressed. Similarly, in benzimidazole-resistant, haploid *betA*(*benA41*) myxamoebae, both the resistant β1-A and the sensitive β1-B tubulins are expressed. Likewise, haploid *betB*(*benD210*) plasmodia are benzimidazole resistant and express both resistant β1-B210 and wild-type plasmodium-specific β2-tubulins. Silver staining of two-dimensional gels of *benD210* myxamoebal and plasmodial proteins to compare relative amounts indicates that there is less resistant β1-B210 tubulin present than wild-type β1-A or β2 (T. G. BURLAND, unpublished results). These observations indicate that benzimidazole resistance is not a result of overabundance of resistant β-tubulin subunits. Thus, the β-tubulins encoded by *betA* (*benA*) and *betB* (*benD*) are redundant, at least for functions such as mitosis that are essential for growth in the presence of benzimidazoles.

At the molecular level, one could interpret this redundancy of *betA* and *betB* if both gene products are found in the same microtubules. Above a threshold level of resistant β-tubulin subunits, microtubules containing both resistant and sensitive β-tubulins would become resistant to benzimidazoles, regardless of whether the β1-A or β1-B subunits are resistant. This could then account for the recessive nature of *benD* mutations, since a heterozygous diploid *benA+/benA+ benD+/benD210* myxamoeba, for example, will have only half the level of resistant β1-B210 tubulin subunits as the corresponding *benA+ benD210* haploid, so that the level of β1-B210 in the diploid heterozygote would be below the threshold. Thus, these interpretations would contradict the multitubulin hypothesis, which states that the different α- and β-tubulin isoforms coexpressed have different functions (FULTON and SIMPSON 1976). However, the recessive nature of *benD* mutations and the epistasis between drug resist-

ance mutations at *benA* and *benD* are observations that can be interpreted equally well by a modified form of the multitubulin hypothesis. In this modified hypothesis, individual microtubules are specialized in composition, containing either β1-A subunits from the *betA* locus or β1-B subunits from the *betB* locus, but not both. But the functions of the β1-A microtubules would be redundant to those of the β1-B microtubules. The mitotic spindle in this example would normally contain both types of microtubule. In the presence of benzimidazoles, mitosis would be carried out by the microtubules containing only resistant β-tubulin subunits. It should be informative in this respect to determine whether both resistant and sensitive β-tubulins are found in mitotic spindle and cytoskeletal microtubules extracted from cells grown in the presence of benzimidazoles.

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