

Mendelian Analysis of the Organization of Actin Sequences in *Physarum polycephalum*

TIM SCHEDL AND WILLIAM F. DOVE

*McArdle Laboratory for Cancer Research
University of Wisconsin, Madison, WI 53706, U.S.A.*

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The genomic organization of the multiple actin DNA sequences in the lower eukaryote *Physarum polycephalum* was investigated by Mendelian mapping. Actin-homologous restriction endonuclease cleavage fragments detected by DNA blotting showed length polymorphisms when different strains were compared. These length polymorphisms were used as phenotypic markers for actin sequences in the genome. The meiotic assortment of the polymorphic restriction fragments was analysed, revealing four unlinked actin loci. The data for three of the actin loci, *ardB*, *C* and *D*, are consistent with a single sequence or gene at each locus. The data for the other actin locus, *ardA*, is consistent with multiple linked actin sequences or genes.

1. Introduction

The genomes of eukaryotes often carry multiple genes coding for related or identical proteins. Such groups of genes have been termed families. Examples include the globins (Maniatis *et al.*, 1980), actins (Vandekerckhove & Weber, 1978a), histones (Kedes, 1979), isocytochromes *c* (Ernst *et al.*, 1981) and major histocompatibility proteins (Coligan *et al.*, 1981). The organization of multigene family sequences within the genome may reflect mechanisms by which differential expression is regulated and/or mechanisms by which the family has evolved.

Sequences related to actin have been identified in a number of organisms by recombinant DNA techniques (Kindle & Firtel, 1978; Tobin *et al.*, 1980; Ng & Abelson, 1980; Durica *et al.*, 1980; Engel *et al.*, 1981). With the exception of yeast, which has a single actin gene (Ng & Abelson, 1980), all organisms examined to date have multiple actin sequences. The organization of the actin sequences in the *Drosophila* genome has been investigated by *in situ* hybridization to polytene chromosomes, revealing that each of the six actin genes lies at a different chromosomal location (Fyrberg *et al.*, 1980; Tobin *et al.*, 1980). Analysis of chick cytoplasmic actin sequences by chromosome-purification methods suggests that the sequences reside in at least three different chromosomal fractions (Cleveland *et al.*, 1981). In contrast, Scheller *et al.* (1981) present evidence from analysis of recombinant DNA clones that at least some of the sea urchin actin genes are

clustered. Thus actin genes do not appear to be tandemly repeated like histone genes (Kedes, 1979), but can be either unlinked or clustered.

For the lower eukaryote *Physarum polycephalum*, mapping multigene sequences by *in situ* hybridization, chromosome purification or chromosome segregation in cell hybrids is not feasible. In this paper, the number and genomic organization of actin sequences in *Physarum* are investigated by Mendelian analysis of polymorphic restriction fragments. *Physarum* fragments homologous to actin, generated by restriction endonuclease cleavage (restriction fragments), show length polymorphism. This length polymorphism allows the actin fragments to be used as phenotypic markers for genetic analysis. The meiotic assortment of polymorphic restriction fragments among haploid amoebal segregants derived from a heterozygous diploid plasmodium is analyzed. Four unlinked loci are identified; one locus is complex, containing multiple actin-homologous restriction fragments. For *Physarum*, polymorphic restriction fragments will permit mapping and dissection of multigene family sequences and facilitate genetic analysis in general.

2. Materials and Methods

(a) *Physarum* strains

The genotypes and ploidy of plasmodial strains are described in Table 1. Amoebal strains are described in the text.

(b) Recombinant plasmids and bacterial strains

Plasmid pYactI carries a 3.8 kb† *Eco*RI insert containing the entire yeast actin gene and was a generous gift from R. Ng (Ng & Abelson, 1980). A plasmid carrying a 1.8 kb *Hind*III insert of the entire *Drosophila* λ DmA2 gene was a generous gift from E. Fyrberg (Fyrberg *et al.*, 1980, 1981). Both are genomic clones. Plasmids were propagated in *Escherichia coli* K-12 strain HB101 (Boyer & Roulland-Dussoix, 1969).

TABLE 1
Plasmodial strains

Strain	Relevant genotype	Ploidy	Reference
CL ^a	<i>mt-h</i> (<i>mt-2</i> like)	1 <i>n</i>	Cooke & Dee (1975)
[LU648 × LU688]	<i>mt-1/mt-2</i>	2 <i>n</i> (inbred) ^c	Cooke & Dee (1975)
Wis 1 (sublines) ^b	<i>mt-1/mt-2</i>	2 <i>n</i> (natural isolate)	Dee (1960)
Wis 2	<i>mt-15/mt-16</i>	2 <i>n</i> (natural isolate)	Kirouac-Brunet <i>et al.</i> (1980)
PpII 1-17-80	<i>mt-6</i>	1 <i>n</i> ^d (natural isolate)	Collins (1975)
[Ward 1 × Ward 10]	<i>mt-7/mt-8</i>	2 <i>n</i> (natural isolate)	Collins (1975)
[3 × 19]	<i>mt-1/mt-2</i>	2 <i>n</i>	This work
[3 × 20]	<i>mt-1/mt-2</i>	2 <i>n</i>	This work

^a CL is probably a derivative of Wis 1 (Honey *et al.*, 1979).

^b All Wis 1 sublines tested (M₃B, M₃CV and M₃CVIII) gave identical results.

^c Represents *mt-1* and *mt-2* derivatives of Wis 1 that were highly inbred with CL.

^d Derived from an amoebal culture that asexually differentiated into a plasmodium.

† Abbreviation used: kb, 10³ bases.

(c) *Culture methods*

Amoebal cultures were grown on liver infusion agar plates (LI: 0.05% Oxoid liver infusion, 1.5% agar) with live or formalin-killed *E. coli* as a food source. Microplasmidia were cultured in simplified soy medium (Laffler *et al.*, 1981). Macroplasmidia were grown on SDM agar (semi-defined medium: Dee & Poulter, 1970).

For isolation of DNA from each clonal amoebal strain, 50 to 100 LI plates with either live or formalin-killed *E. coli* were used. Amoebae were purified from contaminating *E. coli* by the method of Gorman (unpublished). Each plate was flooded with 5 ml of PBS (PBS is the BSS of Goodman, 1972, without FeSO₄), scraped gently, the suspension of amoebae was passed twice through milk filters (Rapid Flow) and the amoebae pelleted at 2000 g in a clinical centrifuge. The pellets were resuspended in PBS at 2 to 5 × 10⁷ cells/ml; 0.5 ml was layered on an 8-ml step of 50% (v/v) LSM (Lymphocyte Separation Medium, Bionetics Laboratory Products) with PBS in a 12-ml centrifuge tube (Corning, 8140) and spun at 500 g for 5 min at 22°C. The top cloudy layer containing *E. coli* (1 to 2 ml) was removed and the remaining amoebae were then pelleted at 2000 g. The supernatant was removed by aspiration, and the amoebal pellets resuspended in PBS, combined and repelleted at 2000 g. The 50% LSM step was repeated and the amoebae again pelleted. Amoebae and *E. coli* were monitored with a hemacytometer. The yield was 1 to 8 × 10⁸ amoebae; contamination was 0.1 to 10 *E. coli* per amoeba.

(d) *Genetic methods*

Sporulation of Wis 1 plasmidia, cloning of amoebal segregants, matings and mating type (*mt*) testing were as described by Gorman *et al.* (1979).

(e) *DNA isolation*

Covalently closed circular plasmid DNAs were prepared essentially as described by Clewell (1972). *Physarum* microplasmoidal DNA was purified as described by Gorman *et al.* (1981). Amoebal DNA was isolated by resuspending the purified amoebae at 2.0 to 5.0 × 10⁷ cells/ml in 100 mM-EDTA, 20 mM-Tris·HCl (pH 8.0) and 100 µg Proteinase K/ml (Beckman). Sodium dodecyl sulfate was added to 0.5% and the solution was incubated at 70°C for 15 min, followed by addition of Proteinase K to a final concentration of 200 µg/ml and incubation for 12 to 14 h at 37°C. RNAses A and T₁ were added to 100 µg/ml and 100 units/ml, respectively, and the incubation continued for 1 h at 37°C. The DNA solution was extracted 3 times with buffer-saturated phenol and 3 times with chloroform/isoamyl alcohol (24:1, v/v) and then dialyzed against 10 mM-NaCl, 1 mM-EDTA and 10 mM-Tris·HCl (pH 7.5) for 24 h. To remove contaminating agar, solid CsCl was added to a density of 1.5 g/cm³ and ethidium bromide to 400 µg/ml, and the DNA was banded by centrifugation at 140,000 g in a Ti60 rotor for 48 h at 25°C. The DNA-containing band was collected through a wide-bore needle, the ethidium bromide removed by extraction with CsCl-saturated isopropanol, and the DNA dialyzed extensively against 10 mM-NaCl, 1 mM-EDTA, 10 mM-Tris·HCl (pH 7.5).

(f) *Restriction enzyme digestion and gel electrophoresis*

Restriction enzymes were obtained from New England BioLabs; digestion buffers were those recommended by the supplier. DNA fragments were fractionated by electrophoresis on agarose (LE, Seakem) gels (McDonell *et al.*, 1977). Electrophoresis buffer consisted of 40 mM-Tris-acetate (pH 7.9), 50 mM-sodium acetate, 1.0 mM-EDTA. *Physarum* DNAs (2 to 5 µg/slot) were digested with a 10-fold excess of restriction enzyme for 1.5 h at 37°C. The reaction was terminated by adding EDTA to 10 mM and heating at 70°C for 5 min. The DNA was loaded on 0.5% or 0.7% agarose gels and electrophoresed for 36 to 60 h at less than 1 V/cm.

The extent of digestion of *Physarum* DNA was monitored by including 0.1 ng of phage λ

DNA in each reaction. Following electrophoresis and transfer to nitrocellulose filters (see below), the digested λ DNA was detected using nick-translated λ DNA as a hybridization probe. Only those blots showing complete digestion of λ DNA have been reported. The λ digestion fragments also provide *in situ* molecular size markers. Size markers were: λ , and λ digested with *EcoRI*, *HindIII*, *SalI* or *KpnI* (Szybalski & Szybalski, 1979).

(g) *Hybridization probes*

The yeast actin probe was a 1.7 kb *BamHI-KpnI* fragment from plasmid pYactI, containing sequences coding for the N-terminal 301 amino acids (Ng & Abelson, 1980). The *Drosophila* actin probe was a 1.8 kb *HindIII* fragment derived from λ DmA2 containing the complete actin coding sequence (Fyrberg *et al.*, 1980, 1981). Plasmids were digested with restriction enzymes, preparatively fractionated on 1% agarose gels, and the actin fragments were purified from the appropriate slice essentially as described by Moran *et al.* (1979). The DNA was labeled *in vitro* by nick-translation (Rigby *et al.*, 1977) with [³²P]dTTP (400 Ci/mmol; Amersham). The specific activity of the nick-translated actin fragment was 3 to 10×10^7 cts/min per μ g; for λ it was 1 to 2×10^8 cts/min per μ g.

(h) *DNA blotting and hybridization*

Following electrophoresis, the DNA fragments within the gel were partially dephosphorylated, cleaved *in situ* by the method of Wahl *et al.* (1979), and then transferred to nitrocellulose filters by the method of Southern (1975). The nitrocellulose filters were prehybridized in $6 \times \text{SSC}$ ($1 \times \text{SSC}$ is 15 mM-sodium citrate, 150 mM-sodium chloride), $10 \times$ Denhardt's solution (Denhardt, 1966), 200 μ g of sonicated, denatured *E. coli* DNA/ml (a generous gift from R. Boston), 0.5% sodium dodecyl sulfate and 0.1% sodium pyrophosphate in sealed plastic bags and incubated at 65°C for 6 to 18 h. The prehybridization solution was removed and an identical solution plus 5×10^5 cts/min per ml of denatured ³²P-labeled probe was added and hybridized for 24 to 48 h. Following hybridization, filters were washed with a vast excess of $3 \times \text{SSC}/0.1\%$ sodium dodecyl sulfate at 55°C with reciprocal agitation for 2 to 3 h. Filters were dried and exposed to Kodak XAR-5 X-ray film, using a Dupont Cronex Quanta IIB intensifying screen, for 0.5 to 14 days.

Autoradiographs were scanned with a Joyce-Loebl microdensitometer. The efficiency of transfer of restriction fragments was judged by scanning λ marker fragments that were blotted and hybridized with a nick-translated λ probe. For the λ bands, the intensity should be a linear function of molecular weight if the transfer efficiency is constant.

(i) *Statistical analysis*

To decide if there is genetic linkage between any of the actin restriction fragment markers, the null hypothesis of unlinked fragments was tested. Under this hypothesis, when two bands in Table 2 are compared one expects approximately equal numbers of concordant and discordant scores for 2 bands among the set of segregants. Thus the null hypothesis is rejected if the number of discordancies between a pair of distribution patterns is sufficiently small. The number of discordancies was found for each pairwise combination of bands. For example, the strain distribution pattern of *HindIII* bands g and i (or their complementary bands h and j) in Table 2A shows 3 discordancies out of 14 haploid segregants. The (binomial) probability was calculated for obtaining no more than that number of discordancies, at least once in the total set of pairwise comparisons, under the null hypothesis. Only if this number was small ($P < 0.05$) was the concordance taken as evidence for linkage between the fragment markers in question.

3. Results

(a) *Identification of Physarum DNA sequences homologous to both Drosophila and yeast actin probes*

The amino acid sequence of the major *Physarum* plasmodial actin species (Vandekerckhove & Weber, 1978b) is very similar to that encoded by actin DNA clones from *Drosophila* (Fyrberg *et al.*, 1981) and yeast (Ng & Abelson, 1980). This evolutionary conservation of actin coding sequences has allowed detection of *Physarum* actin DNA sequences by blotting (Southern, 1975) using hybridization probes derived from both *Drosophila* and yeast. The *Drosophila* probe contains the complete actin coding sequence (Fyrberg *et al.*, 1980,1981) while the yeast actin probe contains the sequence coding for the amino-terminal 301 amino acids (Ng & Abelson, 1980; see Materials and Methods). *Physarum* DNA was isolated from an inbred diploid plasmodium [LU648 × LU688], digested with restriction endonuclease *Hind*III or *Eco*RI, electrophoresed on agarose gels, blotted to nitrocellulose filters and hybridized with ³²P-labeled actin probes from either *Drosophila* (Fig. 1, lanes 1 and 2) or from yeast (Fig. 1, lanes 3 and 4; see Materials and Methods). The term hybridization spectrum will be used to denote the pattern of bands obtained from a blot-hybridization experiment (Scherer & Davis, 1979). The actin hybridization spectra shown in Figure 1 demonstrate that both probes detect the same *Physarum* restriction fragments, as judged by mobility and pattern. Similar cross-species hybridizations have been used to detect actin sequences in other organisms (Fyrberg *et al.*, 1980; Cleveland *et al.*, 1980; Engel *et al.*, 1981). It is reasonable to conclude that these fragments represent *Physarum* actin-homologous sequences.

(b) *All Physarum actin restriction fragments are polymorphic*

The actin hybridization spectra of several natural isolates of *Physarum polycephalum* were compared. DNA from four different isolates and the inbred strain [LU648 × LU688] (see Table 1 for strain descriptions) were digested with *Hind*III (Fig. 2(a)) or *Eco*RI (Fig. 2(b)) and hybridized with the *Drosophila* actin probe. The isolates show distinct hybridization spectra: few bands are found in common between pairs of strains and no band is shared by all four strains. Similar evidence for polymorphism is obtained when still other restriction enzymes are tested (data not shown). The difference in pattern between strains can be explained by genetic variation in the positions of restriction enzyme cleavage sites surrounding actin sequences, resulting in fragments of different length. Such restriction fragment length polymorphism (Botstein *et al.*, 1980) can arise from mutational loss or creation of restriction enzyme cleavage sites, or from insertions, deletions or rearrangements.

The inbred strain [LU648 × LU688] was derived from *mt-1* and *mt-2* segregants of the natural isolate Wis 1 (Cooke & Dee, 1975). A comparison of Wis 1 and [LU648 × LU688] hybridization spectra (Fig. 2(a), lanes 1 and 2; Fig. 2(b), lanes 1 and 2) reveals that [LU648 × LU688] has a subset consisting of precisely half of the

bands found in Wis 1. A reduction in band number would be expected if the inbred strain [LU648 × LU688] is homozygous and Wis 1 is heterozygous with respect to polymorphic actin fragments. A precise halving of band number provides strong evidence that all actin restriction fragments in Wis 1 are heterozygous. The polymorphic restriction fragments can thus be used as phenotypic markers for mapping all the actin sequences. Since Wis 1 is heterozygous for actin fragment length, the linkage relationships among different actin sequences can be determined by analysis of their pattern of meiotic assortment.

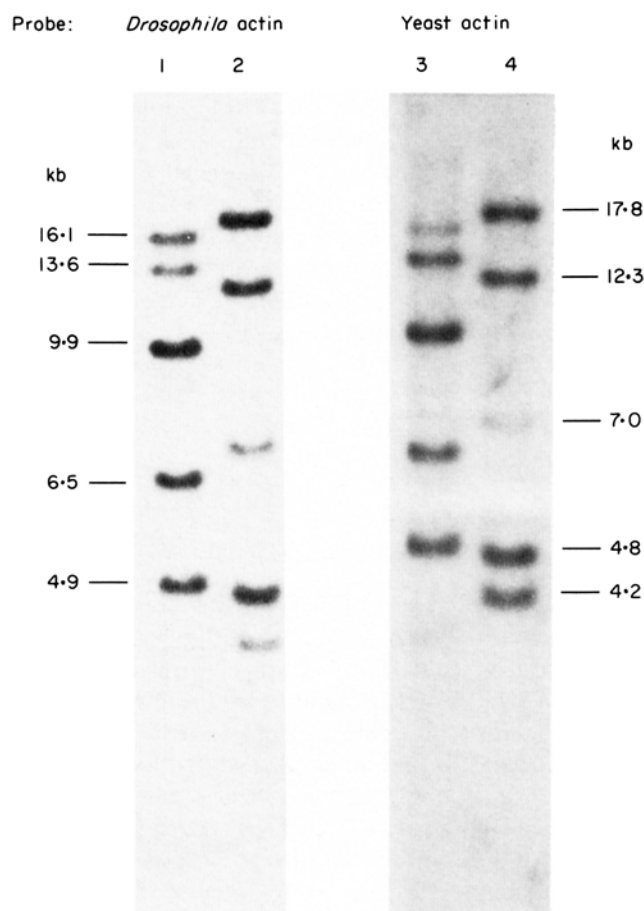


FIG. 1. *Physarum* restriction fragments detected using *Drosophila* and yeast actin hybridization probes. DNA was isolated from microplasmodia of inbred strain [LU648 × LU688]; 2 μ g was digested with *Eco*RI (lanes 1 and 3) or *Hind*III (lanes 2 and 4), electrophoresed on a 0.7% agarose gel, blotted to nitrocellulose paper and hybridized with nick-translated *Drosophila* (lanes 1 and 2) or yeast (lanes 3 and 4) probes (see Materials and Methods). Lanes 1 and 2 were run on a different gel from lanes 3 and 4, so that although the fragment positions are not the same, the relative mobilities are identical. In all, 5×10^5 cts/min per ml of each probe at approximately 6×10^7 cts/min per μ g was used. Exposure time: *Drosophila* probe, 1 day; yeast probe, 8 days.

(c) Assortment of polymorphic actin fragments

A Wis 1 plasmodium (diploid) was sporulated and haploid meiotic amoebal progeny obtained. DNA isolated from populations of each amoebal segregant was digested with *Hind*III or *Eco*RI, blotted, and actin fragments detected by hybridization with a nick-translated *Drosophila* probe. Figure 3 shows the pattern of actin fragment assortment for five representative amoebal segregants. All of the fragments found in Wis 1 are assorting (e.g. segregants 14 and 15 do not have any bands in common), and each haploid segregant possesses precisely half the number of bands found in Wis 1.

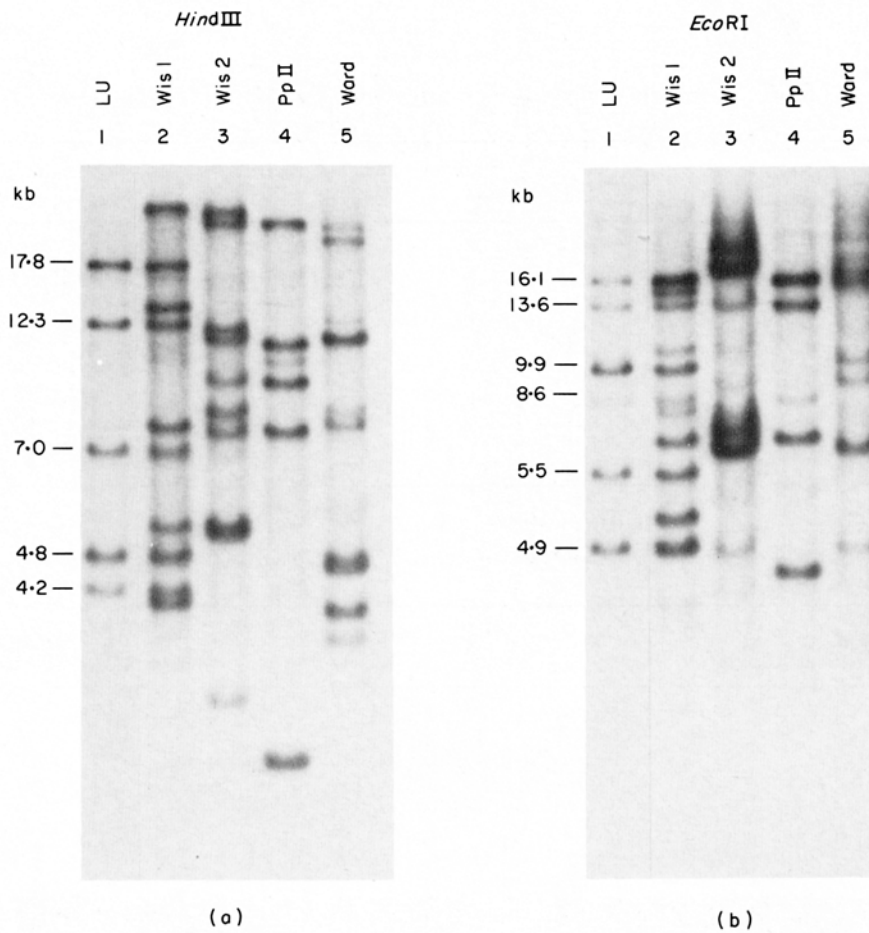


FIG. 2. Characterization of actin fragments from different isolates of *Physarum*. DNA isolated from microplasmodia of different *Physarum* strains (see Table 1 for strain description) was digested with (a) *Hind*III or (b) *Eco*RI, electrophoresed on a 0.7% agarose gel, blotted and hybridized with nick-translated *Drosophila* actin probe (see Materials and Methods). For (a) and (b), DNAs were: lane 1 [LU648 × LU688] (same as Fig. 1); lane 2. Wis 1; lane 3. Wis 2; lane 4. PpII 1-17-80; lane 5. [Ward 1 × Ward 10]. Similar results were obtained using the yeast actin probe (data not shown).

Each segregant was scored for the actin bands found in the heterozygous diploid set of Wis 1. Ten bands (a through j) comprise the *Hind*III hybridization spectrum (Fig. 3(a), also Fig. 2, lane 2), while 12 bands (a through l) comprise the *Eco*RI hybridization spectrum (Fig. 3(b), also Fig. 2(b), lane 2). The slowest-migrating *Eco*RI zone of Wis 1 is a doublet (composed of bands a and b). This is shown in Figure 3(b) in which amoebal segregant 14 displays an upper band, b, with a mobility slightly faster than the upper band, a, displayed by segregants 8 and 15. Minor bands that were not reproducibly visualized are not included in the analysis.

The distribution of actin fragments among 14 meiotic segregants of Wis 1 is compiled in Table 2A (*Hind*III) and B (*Eco*RI). The distribution for a given strain represents the pattern obtained in at least two independent experiments.

TABLE 2

A. Strain distribution pattern of actin-homologous *Hind*III fragments

Band	Diploid		Haploid segregants														Fragment size (kb)
	Wis 1	CL ^a	2	3	4	5	7	8	10	11	14	15	19	20	22		
a	a	—	—	a	—	—	a	—	—	—	a	—	—	—	a	36.2	
b	b	b	b	—	b	b	—	b	b	b	—	b	b	b	—	17.8	
c	c	—	—	c	c	c	—	—	c	c	c	—	c	—	—	13.5	
d	d	d	d	—	—	—	d	d	—	—	—	d	—	d	d	12.3	
e	e	—	—	e	—	—	e	—	—	—	e	—	—	—	e	7.8	
f	f	f	f	—	f	f	—	f	f	f	—	f	f	f	—	7.0	
g	g	—	—	g	—	g	g	g	—	g	—	g	g	—	g	5.3	
h	h	h	h	—	h	—	—	—	h	—	h	—	—	h	—	4.8	
i	i	i	—	i	—	i	—	i	i	i	—	i	i	—	i	4.2	
j	j	—	j	—	j	—	j	—	—	—	j	—	—	j	—	4.0	

B. Strain distribution pattern of actin-homologous *Eco*RI fragments

Band	Diploid		Haploid segregants														Fragment size (kb)
	Wis 1	CL ^a	2	3	4	5	7	8	10	11	14	15	19	20	22 ^b		
a	a	a	a	—	a	a	—	a	a	a	—	a	a	a	—	16.1	
b	b	—	—	b	—	—	b	—	—	—	b	—	—	—	b	16.0	
c	c	—	—	c	—	—	c	—	—	—	c	—	—	—	c	14.9	
d	d	d	—	d	—	d	—	d	d	d	—	d	d	—	d	13.6	
e	e	—	e	—	e	—	e	—	—	—	e	—	—	e	—	10.9	
f	f	f	f	—	f	f	—	f	f	f	—	f	f	f	—	9.9	
g	g	g	g	—	g	g	—	g	g	g	—	g	g	g	—	8.6	
h	h	—	—	h	—	—	h	—	—	—	h	—	—	—	h	8.3	
i	i	—	—	i	—	i	i	i	—	i	—	i	i	—	i	7.4	
j	j	j	j	—	j	—	—	—	j	—	j	—	—	j	—	6.5	
k	k	—	—	k	k	k	—	—	k	k	k	—	k	—	—	5.5	
l	l	l	l	—	—	—	l	l	—	—	—	l	—	l	l	4.9	

^a The strain distribution pattern for CL is identical to [LU648 × LU688] for both *Hind*III and *Eco*RI.

^b Segregant 22 has an additional 25 kb *Eco*RI fragment not found in the Wis 1 parent or in any other haploid segregant.

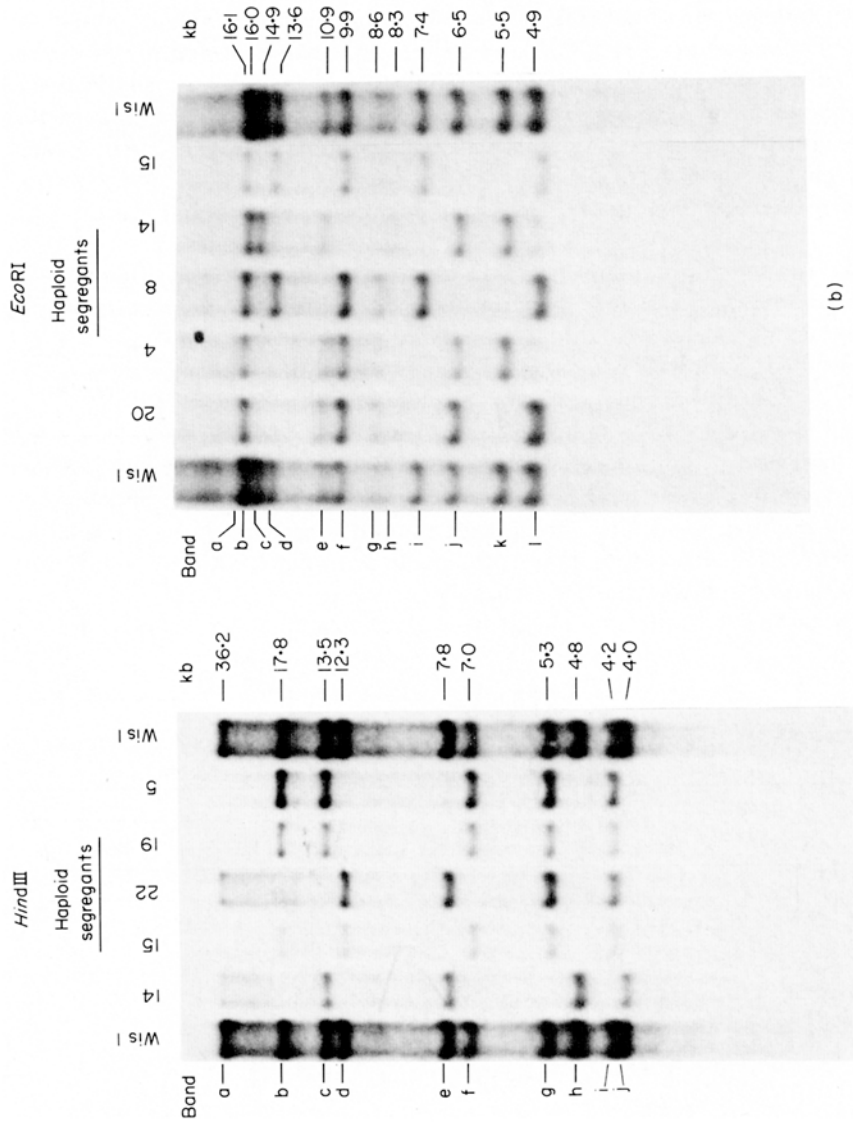


FIG. 3. Meiotic assortment of polymorphic actin fragments. DNA was isolated from clonal amoebal segregants, and from Wis I microplasmodia, digested with (a) *Hind*III or (b) *Eco*RI, electrophoresed on a 0.7% agarose gel, blotted and hybridized with nick-translated *Drosophila* actin probe (see Materials and Methods). (a) *Hind*III digestion of Wis I diploid and amoebal segregants 14, 15, 22, 19 and 5. (b) *Eco*RI digestion of Wis I diploid and amoebal segregants 20, 4, 8, 14 and 15.

(d) *Grouping of actin restriction fragments into loci*

To determine allelism and linkage relationships among fragments, bands in the strain distribution pattern (Table 2A and B) were compared pairwise. The following Mendelian assumptions were used to group the data: (1) if two fragments are alternative alleles, each haploid strain will have only one of the two fragments (e.g. *Hind*III bands i and j in Table 2A are allelic); (2) if two fragments are derived from unlinked loci, they will assort independently and their strain distribution patterns will be uncorrelated (e.g. *Hind*III band b and c, Table 2A, are unlinked); (3) if two fragments are tightly linked (belong to the same locus) their strain distribution patterns will be concordant (*Eco*RI bands f and g, Table 2B, are linked). Extensive, but incomplete, pattern concordance may result from loose linkage.

The linkage relationships among actin fragments are summarized in Table 3. The actin bands are organized into four loci (*ard* for *actin, restriction fragment defined*) based on their independent assortment. The behavior of each of the loci, *ardB*, *C* and *D*, is consistent with that of a single allelic pair of actin sequences. The *ardA* locus, which has multiple actin fragments, will be discussed separately.

For each allele of the *ardB*, *C* and *D* loci there is one fragment for a given restriction enzyme. In addition, *Hind*III and *Eco*RI fragments show the same distribution pattern (e.g. *Hind*III h, Table 2A; *Eco*RI j, Table 2B; and Table 3, *ardC*), and thus co-segregate. The simplest interpretation of the co-segregation of *Hind*III and *Eco*RI bands is that they correspond to fragments that overlap the same actin sequence and are thus two markers for the same allele.

For each allele of the *ardB*, *C* and *D* loci, allelic fragments are found to be

TABLE 3
Locus and allele assignment for actin-homologous HindIII and EcoRI fragments

Locus	DNA band	Allele	CL	Haploid segregants															
				2	3	4	5	7	8	10	11	14	15	19	20	22			
<i>ardA</i>	<i>Hind</i> III a, e <i>Eco</i> RI b, c, h	<i>ardA1</i>	-	-	+	-	-	+	-	-	-	+	-	-	-	+			
	<i>Hind</i> III b, f <i>Eco</i> RI a, f, g	<i>ardA2</i>	+	+	-	+	+	-	+	+	+	-	+	+	+	-			
	<i>Hind</i> III c <i>Eco</i> RI k	<i>ardB1</i>	-	-	+	+	+	-	-	+	+	+	-	+	-	-			
<i>ardB</i>	<i>Hind</i> III d <i>Eco</i> RI l	<i>ardB2</i>	+	+	-	-	-	+	+	-	-	-	+	-	+	+			
	<i>Hind</i> III g <i>Eco</i> RI i	<i>ardC1</i>	-	-	+	-	+	+	+	-	+	-	+	+	-	+			
<i>ardC</i>	<i>Hind</i> III h <i>Eco</i> RI j	<i>ardC2</i>	+	+	-	+	-	-	-	+	-	+	-	-	+	-			
	<i>Hind</i> III i <i>Eco</i> RI d	<i>ardD1</i>	+	-	+	-	+	-	+	+	+	-	+	+	-	+			
<i>ardD</i>	<i>Hind</i> III j <i>Eco</i> RI e	<i>ardD2</i>	-	+	-	+	-	+	-	-	-	+	-	-	+	-			

adjacent to each other in the hybridization spectrum (e.g. *ardB*: *Hind*III c and d; *Eco*RI k and l; see Fig. 3(a) and (b)). In addition, comparison of the mobility of the allelic fragments of any one locus reveals a shift in the same direction for both the *Hind*III and *Eco*RI spectra: the *Hind*III d and *Eco*RI l fragments (*ardB*, allele 2) migrate faster than their alternative allelic fragments *Hind*III c and *Eco*RI k (*ardB*, allele 1). Thus allele 1 represents the slower migrating and allele 2 the faster migrating *Hind*III and *Eco*RI fragments. This co-ordinate shift in mobility suggests that a single insertion or deletion event in one chromosome gives rise to polymorphic fragments for both enzymes (but see below).

The *ardA* locus is complex: two *Hind*III and three *Eco*RI bands co-segregate (Table 3). This indicates that *Hind*III fragments a and e, and *Eco*RI fragments b, c and h are linked (syntenic) as are the counter markers *Hind*III b and f and *Eco*RI a, f and g. This linkage was statistically confirmed ($P < 0.001$; see Materials and Methods). The limits of recombinational distance between the *ardA* locus fragments can be calculated (Crow & Kimura, 1970). At the 95% confidence level, the upper limit is 22 centimorgans while the lower limit is zero. Thus, at the present level of analysis one cannot decide whether the *ardA* fragments abut (share restriction sites), or whether a sequence up to 22 centimorgans in length separates them. The fact that there are three *Eco*RI fragments and only two *Hind*III fragments suggests that one of the *Hind*III fragments contains an *Eco*RI site, yielding two actin-homologous fragments upon cleavage (see below). Because there are multiple co-segregating bands, individual allelic pairs cannot be assigned until recombinants within the locus are detected.

Loci *ardC* and *D* show discordant band patterns for only three of the 14 haploid segregants. Given the number of band patterns being compared, this degree of concordance is within the limits expected by chance alone ($P = 0.25$) for unlinked loci (see Materials and Methods). Thus there is no evidence for linkage of the *ardA*, *B*, *C* and *D* loci. All four loci are also unlinked to the mating type (*mt*) locus, based on four or more discordancies (data not shown).

To estimate the amount of actin-homologous sequence in each fragment, the relative intensities of the actin hybridization bands were determined by microdensitometry. When allelic fragments of the *ardB*, *C* and *D* loci were compared, all showed approximately the same relative intensity, with the exception of *ardD* *Eco*RI fragments d and e. Fragment e was less intense than its counter allele d. Restriction enzyme sites within actin coding or intervening sequences will produce fragments that have reduced intensities relative to uncleaved fragments. A minor band migrating slightly slower than *Eco*RI fragment l can be observed in the seven segregants in which it is not obscured by band l. The pattern of this minor band is concordant with that of band e (present twice, absent five times), suggesting that e and the minor band are linked. Thus, band d may be equivalent to band e plus the minor band. The relative intensities of *ardA* *Hind*III (a, e; b, f) and *Eco*RI (b, c; a, f) fragments are equal to or greater than *ardB*, *C* and *D* fragments. In our hands, however, the efficiency of fragment transfer by blotting was constant only below 10 kb. Since these *ardA* fragments are 10 kb or greater in length, their intensities probably give underestimates. The intensities of the *ardA* *Eco*RI fragments g and h are reduced

relative to the other *EcoRI* fragments. Fragments g and h are allelic and each linked to two other *EcoRI* fragments. Thus, the extra *EcoRI* fragment in each *ardA* allele may be a cleavage product of one of the two *HindIII* fragments in the allele. This analysis must be tempered by the possibility that reduced hybridization intensities may be the result of divergence of the *Physarum* actin sequences in some fragments with respect to the *Drosophila* and yeast actin probes.

(e) *Heritable phenotype of actin restriction fragments*

To demonstrate that the actin restriction fragment markers are heritable properties of actin-related sequences in *Physarum*, amoebae of different mating types were crossed, plasmodia obtained, and their hybridization spectra compared (Fig. 4). It is clear that the pattern of fragments found in the diploid plasmodium is the sum of that found in the two haploid amoebal strains from which it was derived. The cross of amoebal strains 3 and 20, which have alternative alleles at all loci, regenerates the Wis 1 heterozygous diploid pattern of actin bands. Strains 3 and 19 have the same alleles at the *ardB*, *C* and *D* loci, so that the plasmodium [3 × 19] is homozygous at these loci, but heterozygous at the *ardA* locus. This demonstrates that the polymorphic restriction fragments display a codominant phenotype throughout the *Physarum* life cycle.

(f) *Insertional polymorphism*

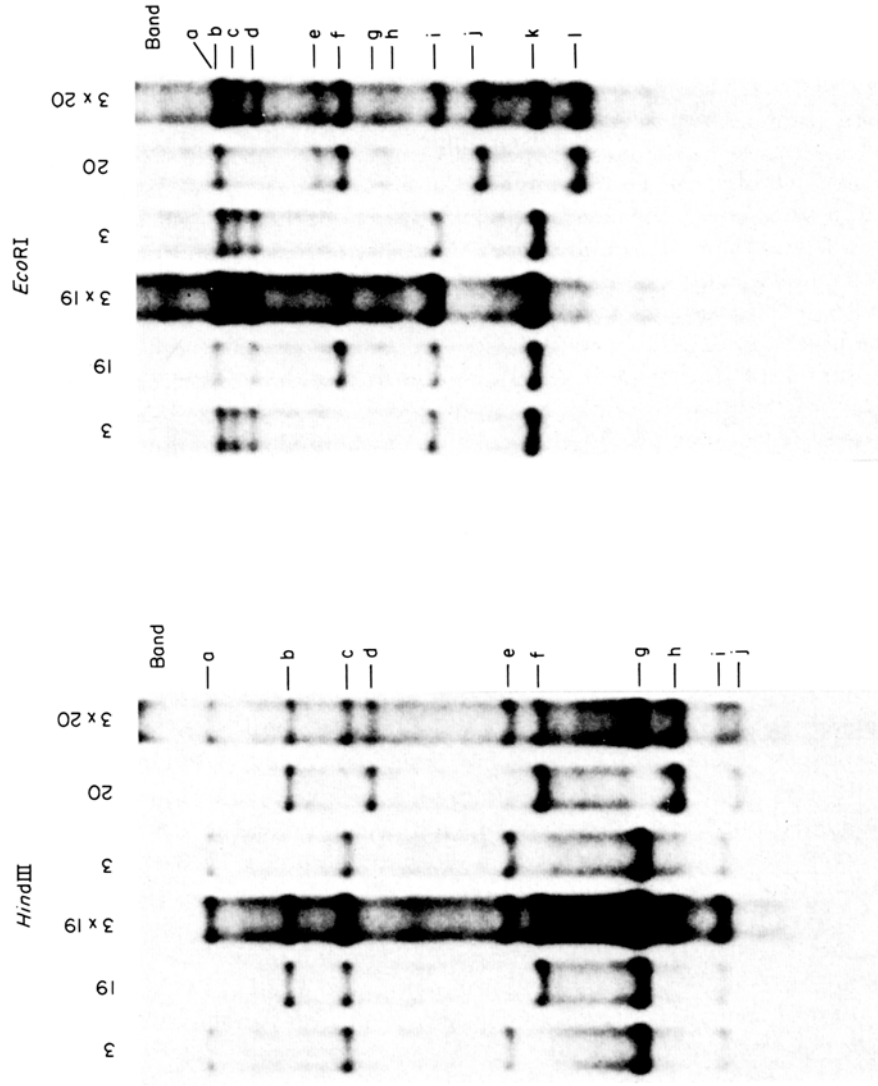
Because there is a co-ordinate shift in the mobility of the *HindIII* and *EcoRI* fragments from one allele to its alternative, and because actin fragment-length polymorphism is observed with several restriction enzymes, it is reasonable to suggest that the polymorphism is the result of a DNA insertion. For an insertion lacking *HindIII* and *EcoRI* sites the size of the inserted sequence would become the

TABLE 4
Allelic fragment lengths

Locus	Enzyme	Size (kb) ^a		Difference ^b
		Allele 1	Allele 2	
<i>ardB</i>	<i>HindIII</i>	13.5 ± 0.6	12.3 ± 0.6	1.2 ± 0.2
	<i>EcoRI</i>	5.5 ± 0.1	4.9 ± 0.1	0.6 ± 0.04
<i>ardC</i>	<i>HindIII</i>	5.3 ± 0.1	4.8 ± 0.1	0.5 ± 0.06
	<i>EcoRI</i>	7.4 ± 0.2	6.5 ± 0.2	0.9 ± 0.1
<i>ardD</i>	<i>HindIII</i>	4.2 ± 0.1	4.0 ± 0.1	0.2 ± 0.05
	<i>EcoRI</i>	13.6 ± 0.6	10.9 ± 0.4	2.7 ± 0.5

^a Mean ± standard deviation of size determinations from 12 (*HindIII*) and 9 (*EcoRI*) independent gels.

^b Mean ± standard deviation of size difference (allele 1 – allele 2).



(a)

(b)

FIG. 4. Codominance of actin fragments. Amoebal segregant 3 (*mt-2*) was crossed either to 19 (*mt-1*) or to segregant 20 (*mt-1*), yielding plasmodia from which DNA was isolated. DNA was digested with (a) *Hind*III or (b) *Eco*RI, electrophoresed on a 0.5% agarose gel, blotted to nitrocellulose paper and hybridized with nick-translated *Drosophila* actin probe (see Materials and Methods). For (a) and (b), DNAs were from: amoebal segregants 3, 19, and plasmodia [3 x 19]; amoebal segregants 3, 20, and plasmodia [3 x 20].

difference between the two alleles and would be identical for each restriction enzyme. This type of insertional polymorphism has been observed in the human genome (Wyman & White, 1980; Higgs *et al.*, 1981; Bell *et al.*, 1981). Table 4 shows a comparison of allelic fragment lengths for the *ardB*, *C* and *D* loci. For a given locus, the allelic difference in size for the two restriction enzymes is not conserved ($P < 0.005$ by Student's *t*-test). This is true for all loci including all possible combinations at the *ardA* locus (data not shown). The data are thus not consistent with the polymorphism resulting from an insertion of a sequence lacking *Hind*III or *Eco*RI sites.

4. Discussion

The linkage relationship of the multiple actin sequences in *Physarum* was investigated by analysis of the meiotic assortment of actin-homologous restriction fragments. Allelic actin restriction fragments were identified based on their mutual exclusion among haploid meiotic progeny. Linkage was assessed by the extent of co-assortment of actin restriction fragments among haploid meiotic progeny. Four unlinked actin loci, *ardA*, *B*, *C* and *D*, were identified.

The resolution of the genetic analysis does not allow an unambiguous determination of the number of actin genes in *Physarum* and must be combined with analysis of recombinant DNA clones. However, the following arguments can be made about the number of actin sequences or genes at each locus. The results for the *ardB*, *C* and *D* loci are consistent with a single sequence or gene at each locus; each allele is represented by only one *Hind*III and one *Eco*RI fragment. The possibility that each fragment contains more than one actin sequence, or that the fragments are part of a tandemly repeated unit, cannot be ruled out.

The data for the *ardA* locus fragments cannot distinguish rigorously between a single gene and multiple clustered genes. A multiple gene model is supported by the fact that band intensities for the *A* locus fragments (except for *Eco*RI g and h) are equal to or greater than those of the *ardB*, *C* and *D* loci. Although the *Hind*III fragments and the major *Eco*RI fragments may abut, they could instead be separated by as much as 22 centimorgans. The relationship between recombinational distance and DNA segment length is not known in *Physarum*, but is perhaps greater than that in yeast, 2.5 kb/centimorgan (Clarke & Carbon, 1980) and less than that in mice, 2×10^3 kb/centimorgan. Two linked actin genes separated by 0.5 kb (in *Dictyostelium*; Firtel *et al.*, 1979) and 3 to 9 kb (in sea urchin; Schuler & Keller, 1981; Scheller *et al.*, 1981) have been reported. A single-gene model might be supported by the failure to find recombinants between allelic fragments at the *A* locus. But to explain one actin sequence spanning two *Hind*III and three *Eco*RI fragments it would be necessary to postulate the presence of intron(s). Moreover, the *A* locus sequence would exceed 9 kb (the smallest *Eco*RI fragment). Although some genes of this length have been reported (Numberg *et al.*, 1980; Wahli *et al.*, 1980) it would be novel for actin genes (Ng & Abelson, 1980; Bender *et al.*, 1978; Fyrberg *et al.*, 1981; Scheller *et al.*, 1981). The most reasonable conclusion is that the *ardA* locus contains at least two actin sequences or genes, while the *ardB*, *C* and *D* loci each contain a single sequence or gene.

In a number of organisms there are apparently more actin DNA sequences than

there are different actin protein species (Vandekerckhove & Weber, 1980; Tobin *et al.*, 1980; Fyrberg *et al.*, 1980; Scheller *et al.*, 1981; Engel *et al.*, 1981). A similar situation is suggested for *Physarum*; there are at least four actin DNA sequences, but only one actin protein species has been observed in the plasmodial phase of the life cycle (Vandekerckhove & Weber, 1978b). The other *Physarum* sequences may represent pseudogenes, as has been suggested for one of the *Dictyostelium* actin sequences (McKeown & Firtel, 1981). Some genes may code for amoebal or sporulation-specific actins, or minor species that were below the limit of detection of earlier studies (<5%). Taniguchi *et al.* (1979) have preliminary evidence for an amoebal-specific actin based on a glycine content different from that of plasmodial actin. Finally, there may be several actin gene sequences capable of encoding the same polypeptide, some or all of which are expressed.

The restriction fragment polymorphisms are codominant phenotypes throughout the *Physarum* life cycle. Hence, the polymorphisms cannot be the result of a *trans*-acting modification system. Actin coding sequences are highly conserved, but the 5' and 3' flanking sequences are divergent (Firtel *et al.*, 1979; McKeown & Firtel, 1981; Fyrberg *et al.*, 1981; Scheller *et al.*, 1981). The *Physarum* restriction fragments are all greater in length than the actin coding sequence. This suggests that the variations giving rise to the polymorphisms may reside in regions flanking the actin genes rather than within the coding sequences. The basis of the polymorphisms is unknown, but cannot be the result of a single insertion lacking *Hind*III and *Eco*RI cleavage sites. One cannot distinguish between any of the following causes: multiple single-base changes, insertions containing restriction sites, inversions or multiple insertions. Engel *et al.* (1981) and Scheller *et al.* (1981) report that they have not observed any polymorphic actin restriction fragments in the human or sea urchin genomes. This may be a reflection of a greater plasticity of DNA sequences surrounding the actin genes in *Physarum*.

Restriction-fragment polymorphisms are useful as genetic markers only if they are transmitted stably, and are frequent enough to detect in small population samples. Furthermore, frequent intralocus recombination would limit their usefulness by generating restriction fragments differing in size from those of the parents. This might limit their usefulness for genetic mapping in organisms such as yeast where recombination frequency is high (0.4 centimorgan/kb, from Clarke & Carbon, 1980) but would be less of a problem, for example, in mice (5×10^{-4} centimorgan/kb). In the present study no intralocus recombination was detected.

For *Physarum* with its paucity of genetic markers, polymorphic restriction fragments will greatly facilitate genetic mapping. They may also be useful for the genetic characterization of different strains. For multigene families, non-allelic sequences can be identified and, as demonstrated by Steinmetz *et al.* (1981), linkage relationships can be determined over distances that are beyond current recombinant DNA technology.

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