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Pulsed-field gel electrophoresis (PFGE) of the strains representing the 14 races of *U. hordei,* the causal agent of covered smut on barley, revealed extensive polymorphism in chromosome length and number. The purpose of this study was to determine by two approaches the exact chromosome number for each *U. hordei* race, and to ascertain whether some strains are aneuploid, using two approaches. A telomere-specific repeat from *Fusarium oxysporum* was used as a probe onto Southern blots of restriction digests of individual chromosome bands to determine the number of chromosomes contained in each band. Nineteen to twenty-three chromosomes were identified in the strains representing the 14 races of *U. hordei.* To ascertain the number of chromosomes identified by the telomere-specific probe, chromosome-specific libraries were constructed and linkage groups were established by using chromosome-specific fragments as probes. The homologous chromosomes identified by these probes were typically monosomic with

a maximum of 15 percent of variability, but cases of disomy were also observed in some strains.

The second objective of this study was to analyze a filamentous mutant of *U. hordei,* designated *fil1-1,* that was isolated following heat-shock treatment. The filamentous phenotype is of interest, because it is believed to be involved in pathogenicity. *U. hordei* is a dimorphic fungus which has yeast like cells that are non pathogenic, while dikaryons produced upon mating are filamentous and pathogenic. Molecular characterization of the *fil1-1* mutant showed that it has suffered a 50 kb deletion in a 940 kb chromosome. Genetical and physical analysis placed the *fil1* mutation near the terminus of one arm of the 940 kb chromosome. The filamentous phenotype reverted to the sporidial wild type in presence of cyclic AMP. Biochemical analyses revealed that the intracellular level of cyclic AMP is three-fold lower in the mutant phenotype than in the wild type. These results indicate that cyclic AMP is an important determinant in fungal morphogenesis.

Molecular and Genetic Analyses of Genome Variability in *Ustilago hordei*

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Molecular and Genetic Analyses of Genome Variability in *Ustilago hordei*

INTRODUCTION

The covered smut disease of barley (*Hordeum vulgare* L.), is caused by the basidiomycetous fungus *Ustilago hordei* (Pers.) Lagerh. (Fisher and Holton, 1957). Cultivated barley is infected by three smut species, and *U. hordei* is distinguished from semiloose smut (*U. nuda*) and loose smut (*U. nigra)* by its mode of infection, symptom expression and spore wall morphology. The characteristic symptom of barley covered smut is the presence of a persistent membrane enclosing the smut sorus until the plant is mature. This distinguishes it from loose and semiloose smut, which have a nonpersistent membrane that breaks while the plant is still green (Mathre, 1982).

The life cycle of *U. hordei* consists of a saprophytic and a parasitic phase. The saprophytic phase begins with the germination of a diploid teliospore and the formation of a basidium (promycelium) and four haploid basidiospores (sporidia). The basisiospores may be isolated sequentially with a micromanipulator for tetrad analysis (Pedersen and Kiesling, 1979). When grown asexually, the basidiospores bud in a yeastlike fashion and produce numerous secondary sporidia. These cells are non-pathogenic and grow on artificial media. To initiate the parasitic phase of the life cycle, two basidiospores of opposite mating type must fuse to form a conjugation bridge from which a pathogenic dikaryotic mycelium grows (Martinez-Espinoza *et al.*, 1992). Infection occurs through the coleoptile of young barley seedlings, after which the

mycelium advances through the host tissue and becomes established at the meristem of the plant (Kozar, 1969). At flower formation, the fungus permeates the ovary tissue, forming a fungal mass, which differentiates into sexual teliospores in place of the seed. The teliospores are contained within membranous sacs or sori that are generally released at harvest during threshing. The fungus survives between growing seasons as teliospores lodged on seed or in the soil, and germination coincides with the germination of barley seeds. The disease occurs worldwide, wherever barley is grown, but its economic importance has decreased with wider use of seed treatment and the availability of moderately resistant varieties (Mathre, 1982). Nevertheless, the disease is still present every year and has the potential to cause serious losses if effective control measures are not practiced diligently (Thomas, 1984).

Despite the fact that the host plant is required for completion of the sexual cycle, *U. hordei* has been established as a useful tool and model system for genetical analyses of host-pathogen interaction. Furthermore, a well characterized race structure, based on the gene-for-gene concept defined by Flor (1946), has been identified in this fungus and a considerable amount of information is available on the genetics of virulence and pathogenicity in *U. hordei*.

Genetics of host-pathogen interaction

At least 13 physiologic races have been described in *U. hordei*. The term physiologic race was first created by rust investigators to describe pathogenically distinct, dikaryotic uredial clones. Kniep (1919) using morphological characteristics, was the first to demonstrate physiologic specialization in the smut fungi. Stakman

(1922) described four methods to classify physiologic races that were based on cultural characteristics, physico-chemical reactions, morphology or pathogenicity. Because of economic considerations, pathogenicity became the most important of the race differential criteria, and for that reason the concept of physiologic specialization is mostly considered as one of pathogenic or host specialization. On the basis of pathogenicity on four different barley varieties (differentials), Faris (1924a) was the first to identify five physiologic races in *U. hordei*. In 1937, Tapke reported on experiments involving 200 collections of covered smut from 26 states and was able to distinguish eight races on five differentials. In subsequent work, five additional races were identified among 244 collections bringing the total to 13 races (Tapke, 1945). Three races: 1, 5 and 6 comprised 86 percent of the total collections with race 6 occurring in 61 percent of the total 444 collections. However, it is important to indicate that a positive correlation has been observed between the distribution of races observed and the type of barley grown (Fischer and Holton, 1957). Recently, two new races, designated race 0 and race 14, have been identified (Mills and Pederson, pers. communication). Race 0 represents the stabilized form of race 8, while race 14, the most virulent of all races, has been the object of molecular and genetical analyses by Abdennadher *et al.* (in preparation).

Genetic analysis of virulence and pathogenicity

According to Fischer and Holton (1957), Goldschmidt was the first to demonstrate the mode of pathogenicity inheritance in the smuts. Using the anther smut, *U. violaceae*, he obtained evidence that differences in race pathogenicity were

attributable to single genes. In *U. hordei*, Thomas (1964), and Thomas and Person (1965) were the first to study the inheritance of pathogenicity and showed that virulence is generally controlled by recessive genes. This conclusion was supported by Lade (1968) and Jensen (1971), who performed similar studies on the inheritance of pathogenicity in *U. hordei*. In subsequent studies, Sidhu and Person (1972), found that virulence on "Hannchen" and "Vantage" was both controlled by the same gene pair, while virulence on "Excelsior" was controlled by a different recessive gene pair. In addition, they were also the first investigators to show that a gene-for-gene relationship existed between barley and *U. hordei*.

In other studies, Ebba and Person (1975) reported multiple allelisms of genes controlling three levels of virulence in *U. hordei*. Using F_1 and backcross F_1 data, they described the existence of a series of multiple alleles that, control three levels of virulence in races 4, 7, and 11 on "Trebi" barley, with the higher level of virulence dominant in each case. Metcalfe (1962), using F_1, F_2, and F_3 data, showed that resistance of the cultivar "Jet" to *U. nigra* was governed by a single dominant gene. The close association between reactions producing covered and loose smut on this cultivar, indicated that the resistance to these seedling infecting pathogens could be conditioned by the same host gene. Fullerton and Nielson (1973) published similar results and demonstrated that identical avirulence genes could be detected in strains of *U. hordei* and *U. nigra*, suggesting that either fungus could serve for both in host resistance testing programs.

It is also important to note that many of the earlier studies on pathogenicity inheritance in *U. hordei,* as well as in *U. nigra,* produced numerous varying and

inconclusive results. Most of those results, were attributed to heterozygous fungal isolates, poor choice of avirulent parental strains, improper methods of analysis, and the occurrence of morphological factors such as abnormal infection hyphae which prevented infection and thereby biased results. For example, Lade (1968) reported increases in virulence of *U. hordei* on the cultivar "Pannier". The data, however, was inconclusive and had to be explained as being the result of contamination, mutation, variation in the host or pathogen, or undetected virulence genes.

Jensen (1971) first demonstrated a stepwise increase in virulence in progeny obtained by inbreeding race 8, virulent only on the cultivar "Odessa" which has no known resistance genes. After three generations of consecutive inbreeding, an isolate was obtained that was virulent on all of the barley differentials. To investigate whether repetitive inbreeding causes increase in virulence, Pedersen and Kiesling (1979) repeated Jensen's experiment and identified an additional isolate with increased virulence. Jensen (1971) attributed the shift to virulence to a heterozygous parent, while Pedersen and Kiesling (1979) hypothesize that only recessive regulator or mutator genes could explain this increase in virulence.

Jensen (1971) also first described a morphological factor of smut fungi that inhibits infection of a susceptible host. Data from selfed tetrads and from backcrosses, indicate that the *ihi* factor, which inhibits the infection of the susceptible cultivar "Odessa", is controlled by a single gene pair. Pedersen *et al.,* (1977) claim that it is not a recessive gene for avirulence, but rather a gene conditioning a morphological character in the fungus which prevents penetration. Microscopic examination of the non-pathogenic compatible mating *ihi* X *ihi* confirmed this

conclusion, and showed that dikaryotic infection hyphae were present but reduced in both number per area and length of the hyphae when compared to the wild type (*IHI*) (Pederson *et al.*, 1977).

While all of the previous studies focused uniquely on the presence or absence of virulence, Groth *et al.* (1976) were able to distinguish between different degrees of disease expression, reporting that in addition to variation in virulence among races in *U. hordei*, variation also exists in the degree of disease severity. The term aggressiveness has been chosen to describe variability in severity of disease reactions among virulent biotypes of a pathogen in a host (Burnett, 1975). It is believed that aggressiveness is a component of parasitic fitness that describes the relative ability of a parasitic genotype to persist successfully in a population over time (Nelson, 1979). Emara (1972), Emara and Sidhu (1974) and Caten *et al.* (1984) interpreted aggressiveness as being the percentage of smutted heads when a susceptible barley cultivar ("Odessa") is inoculated with a virulent isolate of *U. hordei*. They established the genetic nature of aggressiveness and found that the percentage of smutted plants was poly-genetically controlled. However, detailed experiments that analyze the genetic nature of variability in aggressiveness and disease severity components within races of *U. hordei* have not been extensively performed. Gaudet and Kiesling (1991) studied aggressiveness components such as pedoncule compaction, dwarfing, number of tillers infected and extent of sorus formation in spikes, leaves, and nodes of barley in compatible and incompatible interactions involving the 13 physiological races of *U. hordei*. They showed that races 7 and 12 were the most variable for aggressiveness, while races 1 and 8 were the

least. Little is known about the number and functions of genes contributing to aggressiveness.

In addition to genetical components, there are several environmental factors that are known to affect the type and percentage of infections by *U. hordei*. They include soil temperature (Schafer *et al.*, 1962), moisture, acidity and fertility levels (Fischer and Holton, 1957), as well as depth of planting (Taylor and Zetner, 1931; Tapke, 1940) and spore load on the seed (Faris, 1924b).

Although classical genetic studies were facilitated by the well-characterized race structure in *U. hordei*, analyses of pathogenicity and virulence at the molecular and chromosomal level were difficult because of the small size of the fungal chromosomes and the lack of appropriate techniques for those studies. It is only recently, with the development of a technique called pulsed-field gel electrophoresis (PFGE) in 1984, that karyotypic analysis and determination of chromosome number and genome size has become possible for *U. hordei*.

Pulsed-field gel electrophoresis (PFGE) : principle

Pulsed-field gel electrophoresis (PFGE) is one of the key technological advances of the past 15 years that has revolutionized the study of fungal genetics, making it possible to resolve DNA fragments and chromosome-size DNA molecules ranging to over 12,000 kilobase (kb). In the past, only DNA molecules less than 20 kb were efficiently fractionated by unidirectional electrophoresis in agarose gels. The mechanism of molecular-weight separation in this size range is based on the sieving effect of the macromolecules by the agarose matrix (Stellwagen, 1985, 1987) and

larger DNA molecules cannot not be separated by conventional gel electrophoresis, because they exhibit nearly constant electrophoretic mobilities in agarose gels (McDonell *et al.*, 1977). However, separation of large DNA molecules can be achieved if pulsed electric fields are applied to the gel (Cantor *et al.*, 1988). PFGE is a technique in which two different electric fields are generated by alternating the voltage between two different sets of electrodes. Each time one configuration is on, a pulse is generated, thus the name "pulsed field". The directional switching of the electric field causes molecules to change direction in the gel, and separations are possible because the time each fragment takes to reorient to move into a new direction depends on its size.

Originally, PFGE stood for pulsed-field gradient gel electrophoresis (Schwartz and Cantor, 1984). However, later it was recognized that field gradient or inhomogenous fields were not necessary to separate large DNA fragments, and presently, PFGE is used to describe pulsed-field gel electrophoresis and other kinds of methods involving switching (pulsing) of fields to separate large DNAs.

Satisfactory separation of DNA larger than 100 kb was first achieved by Schwartz and Cantor (1984), who first reported this technique. Using the same principle, Carle and Olsen (1985) developed a procedure called orthogonal-field alternation gel electrophoresis (OFAGE), which was used to resolve chromosomes of the yeast *Saccharomyces cerevisiae*. However, due to the inhomogenous electric field generated by this procedure, DNA molecules migrate along curved paths, and makes it very difficult to compare large numbers of samples. Investigators have attempted to alter the geometry of the electric field to achieve straight migration of DNA. For

example, Carle *et al.* (1986) have devised a computer-aided switch to invert the electric filed in a time gradient mode (FIGE), while Chu *et al.* (1986) constructed a hexagonal array of voltage-clamped electrodes, which was used to generate homogenous alternating-fields with a constant reorientation angle of 120°, an approach they denoted Contour-Clamped Homogenous Electric Field (CHEF) gel electrophoresis. Commercial CHEF devices currently employ a hexagonal electrode array, but other types of contour such as circles or squares, can also produce alternating homogenous electric fields (Vollrath *et al.* 1992).

Transverse-alternating field gel electrophoresis (TAFE) also produces straight lanes by suspending the gel vertically in a buffer tank with electrodes positioned parallel to the two large faces of the gel (Gardiner *et al.*, 1986). The electric field produced is inhomogenous, decreasing in strength from the top to the bottom of the gel, producing a focusing effect whereby bands near the bottom are sharp and closely spaced. Although this can be useful for certain specialized applications, the unique electrode geometry constrains the thickness and overall size of the gel, limiting the usefulness of TAFE for preparative applications,.

More recently, additional PFGE systems have been introduced such as the programmable, autonomously controlled electrodes (PACE), in which 24 electrodes are independently controlled, allowing faster and better resolution of larger DNA. The electrophoresis device (ED), the most affordable of all PFGE systems, was proposed by Schwartz *et al.* (1989) as an inexpensive alternative for the investigators willing to assemble their own PFGE apparatus.

Chromosome length polymorphism and aneuploidy

Since the introduction of PFGE by Schwartz and Cantor (1984), rapid advances in this technology have allowed the genomic characterization of an impressive number of fungi and lower eukaryotes. This technique has especially become a valuable tool for investigating the genetic structure of fungi for which classical cytological analyses were difficult, due to a lack of a sexual stage, or for which comprehensive genetic data were not available (for review on PFGE: Mills and McCluskey, 1990; Skinner *et al.*, 1991; Mills *et al.*, 1995). In the context of modern molecular biology, PFGE facilitates the use of a cloned gene or a cloned random DNA fragment to identify the physical location, in a genome, of a corresponding gene or homologous (Orbach *et al.*, 1988; McCluskey and Mills, 1990). The physical maps, generated by PFGE, have become an important tool in a variety of fields including genetics, plant pathology and clinical pathology.

The electrophoretic karyotypes, or banding patterns of chromosome-sized DNAs separated by PFGE, revealed an extraordinary degree of chromosomal polymorphism in chromosome size and/or number, altering our view of fungal genomes as typicological entities forever. Magee and Magee (1987) were the first to report chromosomal-length-polymorphisms for a pathogenic fungus and showed that each of the five isolates of the fungus *Candida albicans* examined, had a unique karyotype. Since then, numerous reports of chromosome-length-polymorphisms have been published for many other pathogenic fungi, including *U. hordei* for which the electrophoretic karyotypes of strains representing the 14 races were established by

McCluskey and Mills (1990). They reported extensive chromosome polymorphism and showed that each race has a unique karyotype with chromosome number varying between 15 and 18.

In spite of the reported chromosomal polymorphisms, the electrophoretic karyotypes generated by PFGE are stably inherited and transmitted and are strain, race and species specific. This property is commonly used in clinical pathology where PFGE is used to distinguish virulent and avirulent *Candida* strains (Merz, 1990). Furthermore, results of comparative PFGE and other electrophoretic separation studies have provided the basis for identification of taxon-specific fungal genes, examinations of fungal phylogeny, and resolution of taxonomic problems. For example, Russell and Mills (1993) were able to show by using CHEF PFGE that *Tilletia contraversa*, which causes common bunt on wheat, and *T. caries*, which causes dwarf bunt on wheat (*Triticum aestivum*), have very similar karyotypic profiles. These data in addition to biochemical, physiologic and morphologic studies, suggest that these bunt pathogens are not different species but variants of a single species. The opposite case was argued for the closely related yeasts *Kluyveromyces marxinanus* var. *marxianus* and *K. m.* var. *lactis* (Steensma *et al.*, 1988), where the very dissimilar karyotypes were used as supporting evidence that these organisms are different species. A similar conclusion was drawn for the highly virulent and weakly virulent isolates of *Leptosphaeria maculans*, causal agent of blackleg on oilseed rape, where the dissimilar molecular karyotypes indicate that they are different species (Taylor *et al.*, 1991).

It has been hypothesized that the variability in chromosome length and number is the norm for phytopathogenic fungi and could result from gross genome arrangements such as chromosome duplications, translocations deletions or insertions of nonessential sequences (Orbach *et al.*, 1988; Mills and McCluskey, 1990). Evidence for duplication, deletion and insertion was obtained in *Gibberella fujikuri* (Xu *et al.*, 1995) and *Fusarium oxysporum* (Boehm *et al.*, 1994), while examples of translocations have been reported by Tzeng *et al.* (1992) in *C. heterostrophus.* Several other models have been proposed attempting to explain the occurrence of chromosome-length-polymorphism. Kistler and Miao (1992) suggested a "meiotic maintenance" hypothesis in which the extent of chromosomal polymorphism is inversely correlated to the frequency of meiosis. Repetitive DNA and tandem repeats are known to be involved in meiotic recombination and/or mitotic instability and have been proven to be useful for genetic mapping and DNA fingerprinting strains (Kim *et al.*, 1995, Daboussi and Langin, 1994; Goodwin *et al.*, 1992; Kistler *et al.*, 1991; McDonald and Martinez, 1991; Milgroom *et al.*, 1992; Rodriguez and Yoder, 1991). In some species such as *Cladosporium* (McHale *et al.*, 1992), *Fulvia* (McHale *et al.*, 1989), *Fusarium* (Daboussi *et al.*, 1992; Julien *et al.*, 1992), *Magnaporthe* (Dobinson *et al.*, 1992), *Neurospora* (Kinsey and Helber, 1989), *Saccharomyces* (Boeke, 1989) and *Schizosaccharomyces* (Levin *et al.*, 1990), these repeated DNA sequences have been identified as transposable elements, while in other speccies ribosomal RNA genes (rDNA) represent tandem repeats that can be present in multiple copies (Russell and Rodland, 1986).

Other factors that have been proposed to explain polymorphism in chromosome number (aneuploidy) are dispensable chromosomes (also known as *B* chromosomes) which often display non-Mendelian patterns of inheritance. Such supernumerary chromosomes have been reported in *Cochliobolus heterostrophus* (Tzeng *et al.*, 1992), *Colletotrichum gloesporiodes* (Masel *et al.*, 1993) and *Magnaporthe grisea* (Valent and Chumely, 1991), but only the pathogen *Nectria haematococca* is known to carry a pathogenicity gene on such a chromosome (Miao *et al.*, 1991).

Although, aneuploidy and chromosome-length-polymorphism are common features of many fungal electrophoretic karyotypes, very little is known about the mechanism responsible for these events and their impact on pathogenicity. It is, however, hypothesized that the observed genomic plasticity of these fungi contributes to the ability of many pathogens to adapt to new and more challenging environments.

Mating, sexuality and fungal dimorphism

Many fungi are able to change morphology in response to mating interactions or environmental conditions. The ability to switch between two phenotypes appears to be prevalent among numerous zoo- and phytopathogenic fungi and has been postulated to contribute to pathogenicity and/or virulence (Scherer and Magee, 1990)

The term dimorphism was first used in medical mycology to specify phenotypic duality of form in which a fungus exhibits distinct saprophytic and

parasitic phases (Ainsworth, 1955). The term was utilized in a manner that implied that the saprophytic phase is hyphal whereas the parasitic phase is a yeast (Scherr and Weaver, 1953). However, fungal species such as *Mucor* and *Ustilago* have saprophytic yeast phases and parasitic filamentous phases, and the term dimorphism is, currently, used solely to describe alternate growth morphologies. Furthermore, the word "dimorphism" evokes the existence of two exclusive states. This is not exactly the case, because virtually all dimorphic fungi have more than two different cell types (San-Blas and San-Blas, 1983). For example, three cell types have been observed in the life cycles of the smut fungi: yeast-like haploid cells, dikaryotic cells resulting from mating between haploid cells and diploid teliospores formed by sporulation of the dikaryon within the plant (Day and Anagnostakis, 1971).

In *U. hordei,* as well as in many other smut fungi, the switch from the sporidial to the filamentous phenotype is primarily induced through mating interactions. *U. hordei* possesses a bipolar mating system in which a single mating-type locus with two alternate forms, *MAT-1* and *MAT-2* (also known as *a* and *A*) controls dikaryon formation (Thomas, 1991). Other smut fungi have a tetrapolar mating system in which two different, unlinked genetic loci are involved in establishing the infectious dikaryon. The tetrapolar mating system of *U. maydis*, the causal agent of corn (*Zea mays*) smut, has been studied in detail, and haploid cell fusion in this pathogen is controlled by the mating-type locus *a* (or *MAT*), which has two alternative forms *a1* and *a2*. The *a1* and *a2* sequences have recently been shown to be idiomorphs (Froeliger and Leong, 1991) and sequence analysis suggests that they encode pheromones and pheromones receptors (Bolker *et al.*, 1992). The *b*

locus, which appears to control the establishment of the infectious dikaryon, has 25 different known forms and encodes at least two products, *bE* and *bW*. It is believed that the interaction of the *bE* product from one form of the *b* locus with the *bW* product from another produces a novel regulatory protein that causes dikaryon formation (Gillisen *et al.*, 1992). The alignment of the predicted amino-acid sequences of several alleles of the *bE* and *bW* genes revealed that each contains a variable N-terminal domain, a central homeodomain-like motif and a conserved C-terminal region (Kronstad and Leong, 1990; Schultz *et al.*, 1990; Gillissen *et al.*, 1992). Recently, a 30-48 amino-acid region has been identified in the variable region of the *bE* gene, which is believed to determine the specificiyt of interaction between the *bE* and *bW* gene products (Yee and Kronstad, 1993). In addition, hybridization studies with the cloned *a* and *b* sequences of *U. maydis* revealed that these genes are not only present in other tetrapolar *Ustilago* species, but also in smut fungi with a bipolar mating system (Bakkeren et al. 1992). The mating type gene of *U. hordei* was cloned by homology with the *b* locus of *U. maydis* (Bakkeren and Kronstad, 1994) and molecular analyses revealed that smut fungi with a bipolar mating systems, do indeed have *b* genes. Experiments, in which *U. hordei b* alleles were introduced into *U. maydis* and vice versa, yielded pathogenic transformants capable of producing the filamentous dikaryon, indicating that the sequences between these two smut fungi are functionally conserved. It is hypothesized that the *b* locus has not been genetically defined in the smut fungi with a bipolar mating type system, because the *b* sequences are too closely linked to the genetically defined *MAT* locus, in order to be distinguished.

The dimorphic switch is also known to be induced by cultural and environmental conditions. This type of dimorphic switch has been well studied and documented in a variety of fungi. Most studies of fungal morphogenesis have dealt with the biochemical changes that occur during the dimorphic switch and various factors have been studied such as respiratory activity, carbon and nitrogen metabolism, intracellular levels of cyclic nucleotides and polyamine levels. Fungal morphology also appears to depend on a complicated set of environmental factors. Among these are temperature, pH, nutrients availability, oxidation-reduction potential, serum factors, and cell interactions. However, the understanding of the phenomena that underline dimorphism is not clear and the identification of genes involved in the regulation of morphological transition has been hampered by the lack of morphological mutants with a well-defined background.

Dimorphism and cyclic AMP

Cyclic AMP (cAMP), a cyclic nucleotide, is thought to play a central role in the control of cellular processes. Evidence has been obtained which links cAMP to the control of a variety of functions in fungi, including utilization of endogenous and exogenous carbon sources, conidiation (Rosenberg and Pall, 1979), changes of enzyme activities (Herman *et al.*, 1990; Terenzi *et al.*, 1992), altered colony morphology (Robson *et al.*, 1991; Pereyra *et al.*, 1992), restricted growth of mycelia (Yarden *et al.*, 1992), appressorium formation (Lee and Dean, 1993), dimorphism, and prototropism (Orlowski, 1981). In almost all cases studied, the action of this small molecule is confined to the intracellular space, where it functions as a

secondary messenger to signals that are received at the cell surface (San Blas and San Blas, 1993). However, cAMP can also play a role in intercellular communication acting as a primary messenger. This function is well established in the differentiation process of the slime mold *Dictyostelium discoideum*

The involvement of cAMP in dimorphism has been explored in various fungi and several lines of evidence indicate that intracellular levels of cAMP are correlated with morphology (Orlowski, 1981). Larsen and Sypherd (1974) as well as Paveto *et al.* (1975) showed that high intracellular levels are associated with yeast-like cells while low levels are characteristic of hyphal cells. The opposite has been observed in *Histoplasma capsulatum*, the causative agent of human histoplasmosis, where the intracellular level of cAMP is about five times higher in the mycelial phase than in the yeast phase (Maresca *et al.*, 1977; Medoff *et al.*, 1980). In related studies, cAMP levels have been reported to increase during germ tube formation and subsequent hyphal development in the pathogenic fungi *H. capsulatum* (Maresca *et al.*, 1977; Medoff *et al.*, 1980), *C. albicans* (Niimi *et al.* 1980) and *Blastomyces dermatidis*, the causal agent of Gilchrist's disease (blastomycosis) (Paris and Garrison, 1983). However, in other studies, an increase in cAMP levels during sporangiospore germination followed by a decline to very low levels when germ tubes emerge, has been documented in *M. rouxii* and *M. racemosus* (Paznokas and Sypherd, 1975; Paveto *et al.* 1975). In similar studies, Orlowski (1980) reported that exogenous cAMP levels in *M. racemosus* prevents the germination of sporiangiospores, but not the swelling of spores.

It has been hypothesized that the fluctuating intracellular levels of cAMP levels are due to the activation or inactivation of adenylate cyclase rather than phosphodiesterase, and mutations in the adenylate cyclase gene produce a variety of phenotypes in fungi. For example, mutations in the *CYR1* gene of *S. cerevisiae* causes cells to arrest in the G1 phase of the cell cycle and exogenous cAMP restores growth (Casperson *et al.*, 1985; Kataoka *et al.*, 1985; Ishikawa *et al.*, 1988). Disruption in the gene encoding adenylate cyclase in *Schizosaccharomyces pombe* results in a reduced growth rate, compared with wild type cells, and a tendency to enter the sexual reproduction pathway in rich medium (Maeda *et al.*, 1990). In *N. crassa* mutants in the *cr-1* allele form small compact colonies that lack extensive hyphal development and display short aerial hyphae with tight clusters of dense conidia. Growth in the presence of cAMP restores colony morphology more like that of the wild type.

Recently, various mutants displaying a constitutively filamentous phenotype were described in *U. maydis* (Barret *et al.*, 1993). One of those mutants *uac1* (formerly called *rem1*) is defective in the adenylate cyclase gene and has a non-pathogenic phenotype. Subsequent work identified other morphological mutants such as *ubc1*, which encodes for the regulatory subunit of cAMP-dependent protein kinase and results in attenuated filamentous growth that normally occurs in response to mating and exposure to air (Gold *et al.*, 1994).

In *U. hordei* a similar, filamentous mutant *fil1-1* was identified after heat shock treatment (McCluskey *et al.*, 1994). CHEF PFGE analysis revealed that the mutant strain *fil1-1* has suffered a 50 kb deletion in a 940 kb chromosome. In a cross

of wild type (sporidial) X mutant (filamentous), the progeny of the six ordered tetrads segregated 2:2 for the mutant and wild type phenotypes and the deleted form of the 940 kb chromosome was consistently associated with the *fil1* mutation. Genetic and physical analyses place the *Fil1* locus and the deletion near one arm of the 940 kb chromosome.

General goals of the present study

Variation in chromosome length and number have been detected by CHEF PFGE in the strains representing the 14 races of *U. hordei* (McCluskey and Mills, 1990). To gain a better understanding of the biological significance of chromosomal polymorphism, this study was undertaken to examine karyotypic variation in more detail in *U. hordei* by 1) ascertaining the chromosome number for each race and 2) by establishing linkage groups among the 14 races.

The second objective of the present study was to investigate the molecular and genetic basis of dimorphism in *U. hordei.* It is anticipated that an understanding of the factors and signals leading to the dimorphic switch will provide insight into fungal pathogenesis on plants and into the regulation and mechanisms of dimorphic growth in fungi.

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Chapter II.

CHARACTERIZATION OF ELECTROPHORETIC KARYOTYPES OF STRAINS REPRESENTING THE 14 RACES OF *USTILAGO HORDEI* BY USING A TELOMERE-SPECIFIC REPEAT AND CHROMOSOME-SPECIFIC PROBES AS MARKERS

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Abstract

Variation in chromosome number and length has been detected by contour-clamped homogenous electric field (CHEF) pulsed-field gel electrophoresis (PFGE) in strains representing the fourteen races of *Ustilago hordei*, a fungus causing covered smut on barley *(Hordeum vulgare)*. Our objective was to determine the exact chromosome number for each race and to ascertain using two approaches whether some strains are aneuploid. Chromosome-specific libraries were constructed, and three single-copy probes from each library were hybridized onto Southern blots of CHEF gels containing chromosomes from strains of the 14 races. The homologous chromosomes identified were typically monosomic with length polymorphisms ranging to 100 kilobase (kb), but cases of disomy were also observed in some strains. The second method involved a telomere-specific repeat from *Fusarium oxysporum*, which was used as a hybridization probe onto Southern blots of restriction digests of individual chromosomes. Using this technique, 19 to 23 chromosomes were detected in 14 to 19 chromosome bands visualized by CHEF PFGE analysis in the strains representing the 14 races. The telomere-specific probe also generated specific restriction fragment length polymorphism (RFLP) patterns for each chromosome and allowed comparisons of homologous chromosomes. Twelve races of *U. hordei* showed a unique RFLP pattern for each of its chromosomes except for strains representing race 10 and 13. These strains have identical karyotypes and no RFLP differences.

Key words: electrophoretic karyotype, phytopathogen, chromosome polymorphism

INTRODUCTION

Fungal phytopathogens adapt rapidly to the introduction of resistant host genotypes in the field by producing new virulent races and pathotypes. These adaptations may be due to altered or lost genes that condition recognition by the plant's sensor system such as avirulence genes, or new virulence might be gained by acquiring host-defense suppressing genes, which enable pathogens to infect newly deployed resistant host genotypes. At present little is known about the mechanisms which generate genetic variation in the genome of fungal pathogens. Some mechanisms which have been proposed to mediate pathogen evolution are sexual and parasexual recombination, gene mutations, transposons and chromosomal rearrangements, such as duplications, deletions and translocations (Mills and McCluskey, 1990; Kistler and Miao, 1992; Masel *et al.*, 1993). Elucidating the molecular mechanisms that determinine genetic variation in pathogenic fungi would allow a better understanding of host-pathogen interactions.

The fungus *Ustilago hordei* causes covered smut on barley and is an attractive model system for host-pathogen interactions because of its race structure, small genome size, short generation time, large progeny and economic significance. It has been the subject of investigation regarding its genetic elements which determine pathogenicity and compatible/incompatible host-pathogen interactions since 1924 (Faris), but karyotypic studies at the molecular level were difficult because of the small size of its chromosomes. Recent developments in pulsed-field gel electrophoresis (PFGE) have made it possible to separate chromosomal DNAs by size in agarose gels, and in combination with Southern Hybridization of various cloned genes. Electrophoretic karyotypes (EK) of organisms which were not amenable to molecular genetic analyses (Schwartz and Cantor, 1984; Carle and Olson, 1985) Using the contour-clamped homogenous electric field (CHEF) technique (Chu *et al.*, 1986), McCluskey and Mills (1990) were able to compare the

electrophoretic banding patterns of different strains representing the 14 races of *U. hordei*.

McCluskey and Mills (1990) showed that each race had a unique karyotype and that these strains varied considerably in both the numbers and the sizes of bands that could be resolved. While karyotypic differences between various strains were apparent, it was not obvious what similarities existed and how many chromosomes were present in each race. To address this question, McCluskey *et al.* (1994) attempted to identify linkage groups in PFGE-separated chromosomes among the 14 races with random and chromosome-specific probes. The hybridization data, however, were limited because not enough probes were used. Insufficient information was generated to suggest the mechanisms by which the karyotype variation might have arisen.

To investigate whether gross genome rearrangements, such as duplications, deletions, insertions or translocations contribute to karyotypic polymorphism, linkage groups were established with chromosome-specific DNA probes of blots containing strains representing the 14 races of *U. hordei*. To establish the basic haploid chromosome number and to ascertain the number of linkage groups, a telomere-specific repeat from *Fusarium oxysporum* was used as a probe and hybridized onto Southern-blotted restriction digests of individual chromosomes. In this paper, a detailed and comparative study of the electrophoretic karyotype of the strains representing the 14 races of *U. hordei* is reported and mechanisms believed to cause karyotypic variability in fungi are discussed.

Materials and Methods

Strains and culture conditions.

The strains representing 14 physiologic races of *U. hordei* and the culture conditions have been previously described (McCluskey and Mills, 1990).

Saccaromyces cerevisiae, Schizosaccharomyces pombe and *Hansenula wingei* chromosomes were used for size markers and were purchased from Bio-Rad (Richmond, Ca).

Plasmids and source of DNA probes.

Chromosome-specific DNA was cloned into the high-copy cloning vector pUC19 (Yanich-Perron *et al.*, 1985) and the inserts used as probes are listed in table II.1. Transformation of plasmid DNA into *E.* coli DH5α and plasmid extraction were done following standard procedures (Maniatis *et al.*, 1982)

Pulsed-field gel electrophoresis.

DNA samples were prepared by embedding *U. hordei* cells in agarose plugs as previously described (McCluskey and Mills, 1990). CHEF PFGE electrophoresis was performed with the CHEF-DR II apparatus (Bio-Rad) and chromosomal bands in the 100 to 1,500 kilobase (kb) range were separated as reported by McCluskey and Mills (1990). The resolution of bands in the 1,500-3,500 kb range was achieved by running a 1% agarose (Sigma type II, medium EEO) gel at 10^{o} C in 0.5X TBE (Maniatis *et al.* 1982) for 96 h at 75 V with a pulse time of 3600 s, followed by a ramped switching interval of 480 to 900 s for an additional 72 h.

Table II.1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli DH5α	FØ80d*lacZ*ΔM15Δ(*lacZ*YA-argF)	Focus 8(2):9 (1986)[a]
pUC19	Ap^r cloning and sequencing vector	Yanich-Perron *et al.* 1985
pBH100	1.5 kb *U. hordei Bam*HI fragment from mating type gene bE.	Bakkeren and Kronstad, 1993
pNLA17	$[TTAGGG]_{18}$ telomere repeat from *Fusarium oxysporum* cloned into pUC119	Powell and Kistler 1990
pOSU220-1	0.4 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU220-2	1.2 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU220-3	2.4 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU460-1	0.9 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU460-2	3.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU460-3	0.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU570-1	0.6 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU570-2	0.8 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU570-3	0.4 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU650-1	2.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU650-2	1.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU650-3	0.8 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU700-1	0.6 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU700-2	1.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU700-3	3.2 kb *U. hordei Bam*HI fragment in pUC18	This study

Table II.1 (continued)

pOSU700-4	2.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU700-5	0.4 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU700-6	0.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-1	1.9 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-2	1.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-3	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-4	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-5	1.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-6	1.8 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-7	4.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-8	3.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU760-9	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-1	1.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-2	1.9 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-3	3.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-4	3.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-5	0.9 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU940-6	2.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-1	2.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-2	1.8 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-3	1.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-4	1.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-5	4.2 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-6	6.1 kb *U. hordei Bam*HI fragment in pUC18	This study

Table II.1 (continued)

pOSU960-7	0.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-8	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU960-9	3.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1100-1	0.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1100-2	2.2 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1100-3	0.7 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1190-1	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1190-2	3.2 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1190-3	4.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1290-1	0.8 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1290-2	1.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU1290-3	0.4 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2200-1	1.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2200-2	3.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2200-3	0.5 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2500-1	2.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2500-2	1.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2500-3	3.0 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2830-1	4.1 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2830-2	2.3 kb *U. hordei Bam*HI fragment in pUC18	This study
pOSU2830-3	0.2 kb *U. hordei Bam*HI fragment in pUC18	This study

[a]Published by Bethesda Research Labs., Gaithersburg, MD

Ap^r: ampicillin resistance

*Bal*31 digest

*Bal*31 nuclease (New England Biolabs, Beverly, MA) digests were performed in 200mM Tris pH 8.0, 120 mM $CaCl_2$, 120 mM $MgCl_2$ and 6M NaCl. Twenty units of enzymes were added to 10 μg DNA in a 200 μl starting volume. Aliquots were withdrawn at 5-min intervals. *Bal*31 was inhibited with 20mM EGTA, followed by heat inactivation at 70° C for 10 min. The time points were digested with *Bam*HI (Gibco BRL, Gaithersburg, MD) after the buffer had been adjusted according to the manufacturer's directions.

Isolation of chromosome-specific probes and Southern hybridizations.

Chromosome-specific DNA for chromosome-specific libraries and for telomere analysis was isolated from CHEF PFGE gels as described by Mills *et al.* (1995). Agarose gels to be blotted were denatured in a 0.4 N NaOH solution for 15 min and the DNA was transferred onto Zeta-Probe^R GT nylon membrane (Bio-Rad) by capillary action (Southern, 1975).

DNA was radiolabelled with ^{32}P by means of a random priming kit (Pharmacia Biotech, Piscataway, NJ). Hybridizations and washes were done in a Robbins Scientific Hybridization Oven (Robbins Scientific Corporation, Sunnyvale, Ca). Blots were hybridized for 10 min in 7% SDS , 250 mM Na_2PO_4 pH 7.2 prior to adding the probe, and then hybridized for 8 h. High-stringency conditions were used with final washes at 65° C with 5% SDS, 20mM Na_2PO_4 for one hour, followed by a second wash with 1% SDS, Na_2PO_4 for another hour. Membranes were exposed to Kodak X-Omat AR film for 4-48 h at -70° C.

Results

Electrophoretic karyotypes of *U. hordei*

McCluskey and Mills (1990) established the electrophoretic karyotypes of the strains representing the 14 races of *U. hordei.* We attempted to optimize the separation of chromosome bands larger than 2,000 kb by changing the pulsed-field gel electrophoresis conditions (Fig. II.1). The new parameters are described in the Materials and Methods and the size estimations of the chromosome bands are given in Table. II.2. The size of each band was calculated with the computer program Cricket Graph (Cricket Graphics, Inc., Philadelphia, PA). *S. cerevisiae*, *S. pombe* and *H. wingei* chromosomes were used as size standards.

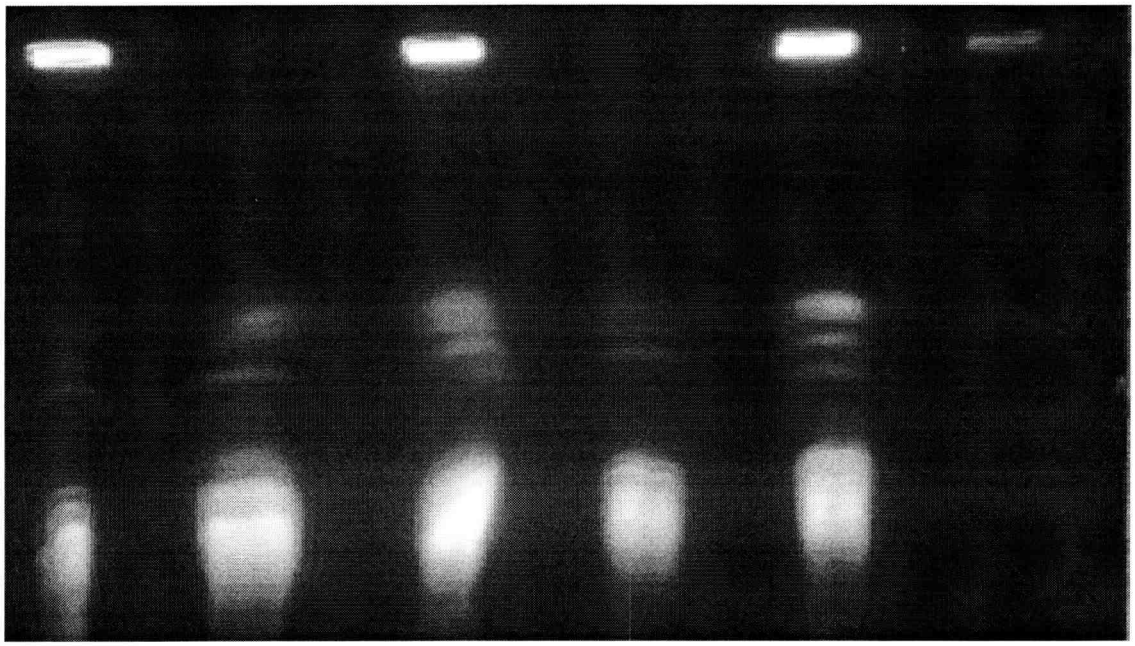
Although the same parameters for the separation of chromosome bands smaller than 2,000 kb were used, the number of chromosome-sized bands resolved by PFGE slightly differed from those reported by McCluskey and Mills (1990). For example, McCluskey and Mills (1990) report 18 chromosome bands for race 12, while our PFGE gels show only 14 chromosome bands. The contrasting results from these two investigations most probably reflect the difference in interpretation of data. In some instances for example, chromosome bands that are broad and wide can either be interpreted as one big chromosome band or as a multiple of smaller chromosome bands migrating close by. One objective of this study was to determine the chromosome number contained in each chromosome band using a telomere-specific probe. To facilitate these studies and to avoid confusion, each clearly resolved chromosome band by CHEF PFGE was counted as one, regardless its width or broadness.

Establishment of the basic haploid chromosome number in *U. hordei.*

The number of chromosomes contained within a band separated by CHEF PFGE was determined by hybridization of a telomere-specific repeat from *F. oxysporum* onto a

Strains representing races:

1.3 2.1 3.1 4.1 5.1 *H. wingei*



FigureII.1. CHEF PFGE resolution of large chromosome-sized DNA molecules of *Ustilago hordei* strains representing races 1-5. *H. wingei*, *S. cerevisae* and *S. pombe* (not shown) chromosomes were used as standards. Conditions of electrophoresis: 1% agarose gel at 10^{o} C in 0.5X TBE for 96 h at 75 V with a pulse time of 3600 s, followed by a ramped switching interval of 480 to 900 s for an additional 72 h.

Table II.2. Estimates (kb) of large chromosome-sized DNAs from the strains representing the 14 races of *Ustilago hordei* as determined by CHEF PFGE analysis

Strains	1.3	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	10.1	11.1	12.1	13.1	14.1
	2830^{a}_{1}	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1	2830_1
	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1	2500_1
	2200_1	2200_1	2250_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1	2200_1
	1290_1	1290_1	2100_1	1310_1	1290_1	1600_1	2100_1	1900_1	2110_1	1900_1	1900_1	1900_1	1900_1	1900_1
	1190_2	1190_2	1260_1	1230_1	1200_2	1300_1	1290_1	1260_1	1260_1	1260_1	1280_1	1260_1	1260_1	1260_1
	1100_1	1100_1	1210_1	1190_1	1040_1	1210_2	1230_2	1150_1	1180_2	1210_1	1180_3	1150_2	1210_1	1170_2
	1050_1	960_3	1200_1	1140_1	1000_3	1160_1	1140_1	1090_1	1100_1	1150_1	1130_1	1075_1	1150_1	1100_1
	910_1	940_2	1150_1	1060_1	950_1	1060_1	975_3	1040_1	980_3	1100_1	1075_1	1020_2	1100_1	1040_2
	900_2	760_3	1025_1	1040_1	910_1	990_2	900_2	980_2	850_2	1050_1	970_2	890_3	1050_1	920_3
	840_1	700_2	975_1	940_2	800_2	960_3	850_2	920_3	780_1	940_1	920_1	760_2	940_1	830_1
	770_2	650_1	940_2	860_1	700_2	875_2	800_2	850_2	720_3	890_3	830_1	720_3	890_3	760_2
	700_2	570_1	890_1	790_3	650_1	810_3	710_2	720_2	620_2	735_2	770_3	640_2	735_2	720_2
	570_1	460_1	840_1	720_2	585_1	700_1	650_2	650_2	470_1	710_3	700_2	490_1	710_3	630_1
	430_1	220_1	720_3	650_1	500_1	610_1	510_1	590_1	200_1	640_1	630_1	190_1	640_1	590_1
	170_1		620_2	585_1	245_1	510_1	245_1	450_1		590_1	580_1		590_1	460_1
			450_1	460_1		245_1		220_1		450_1	460_1		450_1	190_1
			220_1	210_1						170_1	200_1		170_1	
b	15	14	17	17	15	16	15	16	14	17	17	14	17	16
c	19	21	21	21	20	23	23	22	21	22	23	22	22	22
Σ^{d}	20.11	22.64	25.44	24.80	22.10	26.79	26.02	24.37	23.84	24.26	26.85	24.42	24.26	24.63

number in subscript represent the number of chromosomes identified in each chromosome band by the telomere-specific probe

[a]bands sizes are expressed in kilobase pairs (kb)

[b]estimated number of chromosome band resolved by CHEF PFGE in each strain

[c]number of chromosomes detected by the telomere-specific repeat in each chromosome band

[d]estimated genome size (in MB) for each strain

Southern blot of restriction digests of individual bands. Telomeric sequences are known to exist at the termini of each chromosome and two telomere restriction fragments from each chromosome were expected to hybridize to the probe. A multiple of two restriction fragments hybridized with the probe when two or more chromosomes were present in a band (Fig. II.2). The number of chromosomes contained within a band was therefore estimated to equal half the number of restriction fragments detected.

The telomere-specific probe detected between 19 to 23 chromosomes in the strains representing the 14 races of *U. hordei* (Table II.3). The majority of the chromosome bands contained single chromosomes, but cases of bands containing two to three chromosomes also were observed. The total genome size of *U. hordei* was estimated on the basis of the sizes of the chromosomal bands and the number of chromosomes detected by the telomere-specific probe. Strain 1.3 was found to have the smallest DNA content with 20.11 megabases (mb) and 19 chromosomes, whereas strain 6.1 had the highest DNA content with 26.79 mb and 23 chromosomes.

The telomere-specific probe also generated specific restriction-fragment length polymorphism (RFLP) patterns for each chromosome and allowed comparison of homologous chromosome. Twelve of the 14 races of *U. hordei* showed a unique RFLP pattern for each of its chromosomes. Strains representing races 10 and 13 had identical karyotypes and no RFLP differences (data not shown).

Because additional telomeric repeats near the centromere have been identified in tomato (Ganal *et al.*, 1991) and maize (Burr *et al.*, 1992), it was important to verify if such additional sequences exist in the *U. hordei* genome. Their presence would lead to an overestimation of the chromosome number. Total genomic DNA was digested with the exonuclease Bal*31*, which degrades both 3' and 5' termini of double stranded DNA. Subsequent complete digestion by *Bam*H1 produced fragments for Southern analysis with the telomeric repeat. After one minute of *Bal*31 treatment, no telomeric repeat was

Lanes: 1 2 3 4 5 6 7 8

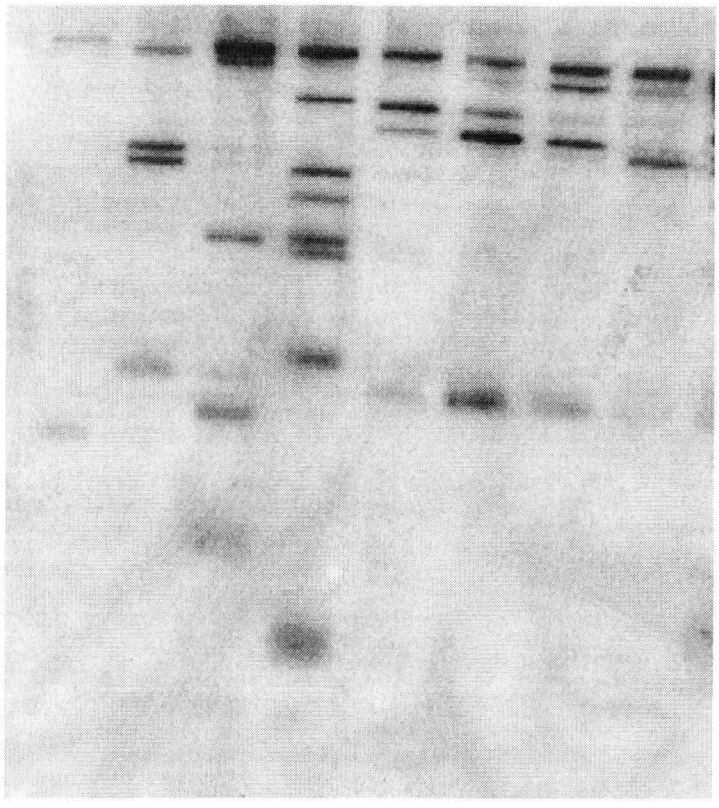


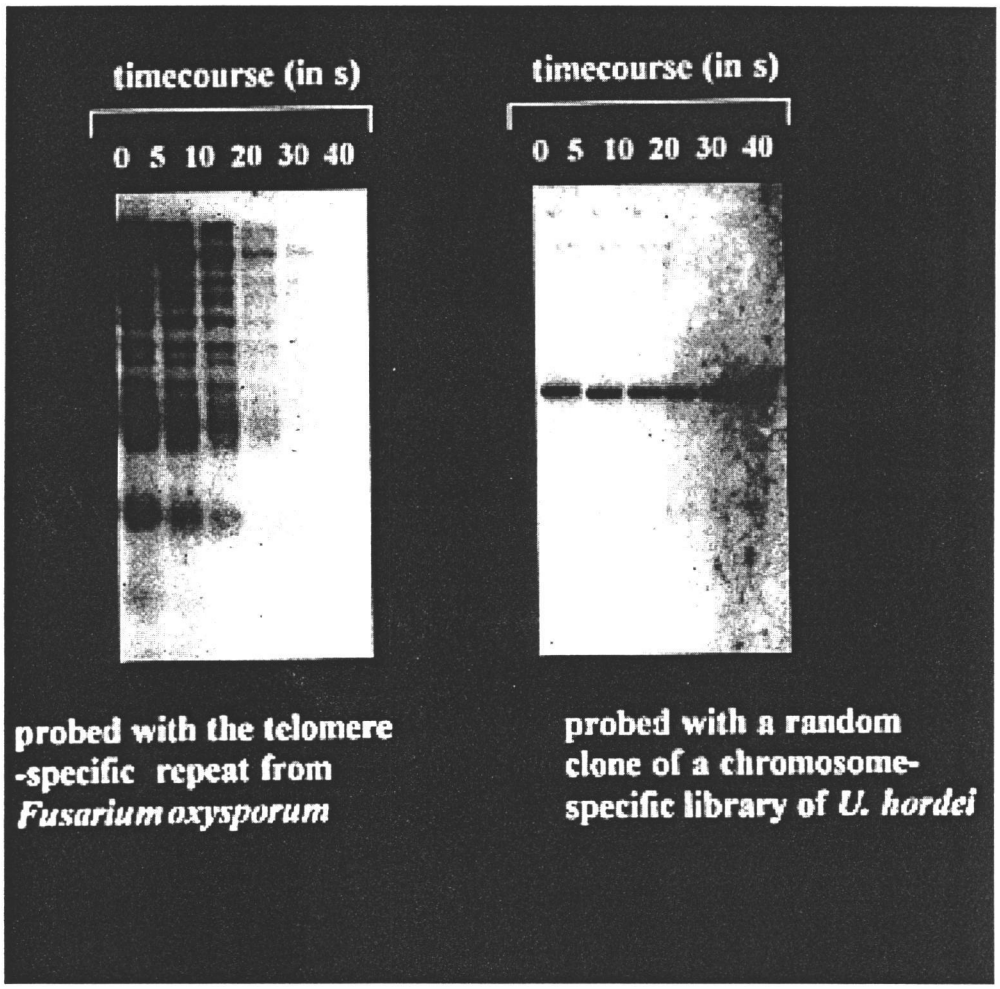
Figure II.2. Southern hybridization of a telomere-specific probe to determine the chromosome number in individual chromosome bands of *Ustilago hordei*. *U. hordei* chromosomes resolved by CHEF PFGE were excised, digested with *Bam*HI, Southern-blotted and probed with a telomere-specific repeat $(TTAGGG)_{18}$ from *Fusarium oxysporum*.
Lanes 1-8 contain chromosome bands IV-XI from a strain representing race 2.1. Most bands contain more than one chromosome. Note that contaminating chromosomal DNA is present in several lanes.

Table II.3. Chromosome number and genome size of the strains representing the 14 races of *Ustilago hordei*

U. hordei strain	Number of chromosome bands resolved by PFGE	Chromosome number detected by the telomere probe	Estimated genome size (in MB) [a]
1.3	15	19	20.11
2.1	14	21	22.64
3.1	17	21	25.44
4.1	17	21	24.80
5.1	15	20	22.10
6.1	16	23	26.79
7.1	15	23	26.02
8.1	16	23	24.37
9.1	14	21	23.84
10.1	17	23	24.26
11.1	17	21	25.73
12.1	14	22	24.42
13.1	17	23	24.26
14.1	16	22	24.63
Average	16.07	21.64	24.32

[a]genome size was estimated based on the number of chromosomes detected by the telomere-specific probe and the size of each chromosome band

Figure.II.3. *Bal31* digest of total genomic DNA of *Ustilago hordei*



detected, whereas a fragment that hybridized with a random probe showed no alteration (Fig. II.3). These results indicate that the fragments identified by the telomere-specific probe are not present within any *Bam*HI fragments, but are solely present at the termini of *U. hordei* chromosomes.

Assignment of chromosome-specific probes to resolved chromosomes

The karyotypes of the strains representing the 14 races of *U. hordei* described by McCluskey and Mills (1990) showed polymorphism in chromosome length and number. To correlate ethidium bromide stained bands with homologous pairs and to compare the genomic organization of the 14 *U. hordei* strains, pulsed-field gels were probed with cloned DNA sequences in order to allocate them to chromosomal bands. Three chromosome-specific, non homologous DNA clones were hybridized to CHEF blots. The chromosome bands of strain 2.1 chromosome bands were used as source for chromosome specific libraries, because it was believed to be the most representative strain with the haploid complement of chromosomes for *U. hordei* (McCluskey and Mills, 1990). The chromosome-specific libraries yielded single-copy fragments and a moderate amount of repetitive DNA. The frequency, however, of repetitive DNA fragments increased dramatically when the chromosome-specific library of the smallest chromosome was screened, and no single-copy fragment was recovered from this library.

Typical results of the probing data are shown in Fig.II.4 and a summary of all hybridization data and the chromosome assignments are shown in Fig.II.5. The hybridization profiles revealed that homologous chromosomes, identified by the chromosome-specific probes, were typically monosomic and similar or identical in size. Indeed, with the exception of the rDNA probe which depicted 38 percent variability in 14 strains (McCluskey and Mills,1990) the maximum chromosome length polymorphism observed in the 14 strains of *U. hordei*, did not exceed 100 kb, and represent less then 15 percent variability. However, for several probes, such as pOSU570-3 and pBH100, a

Strains representing races:

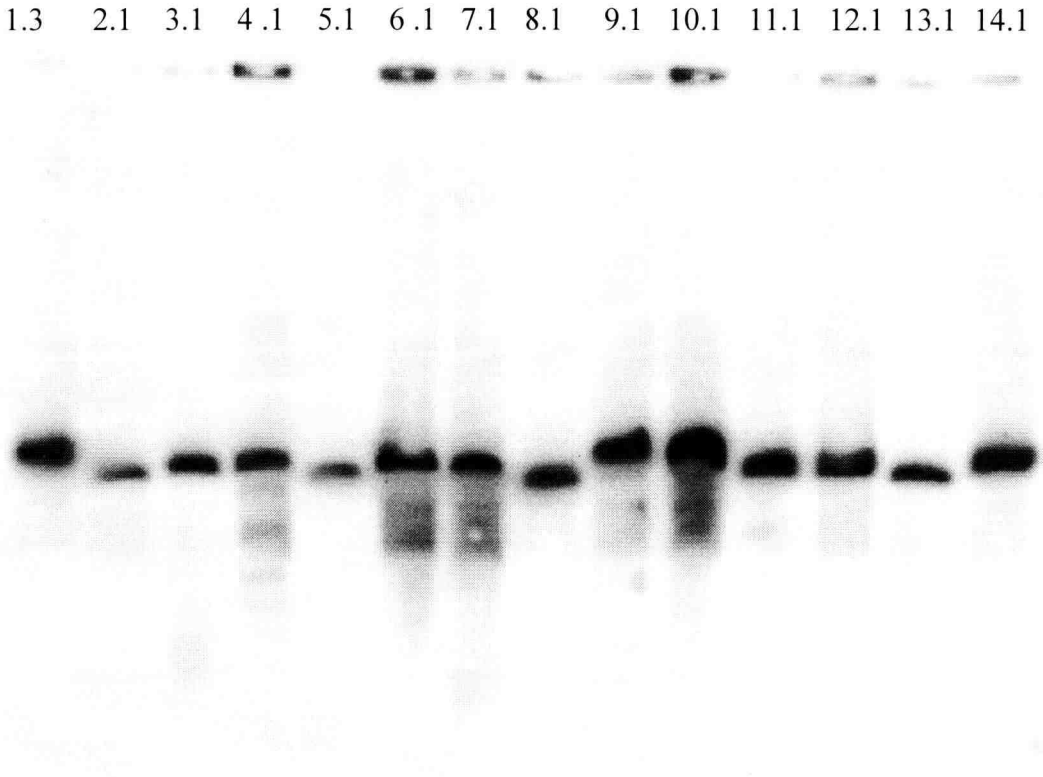
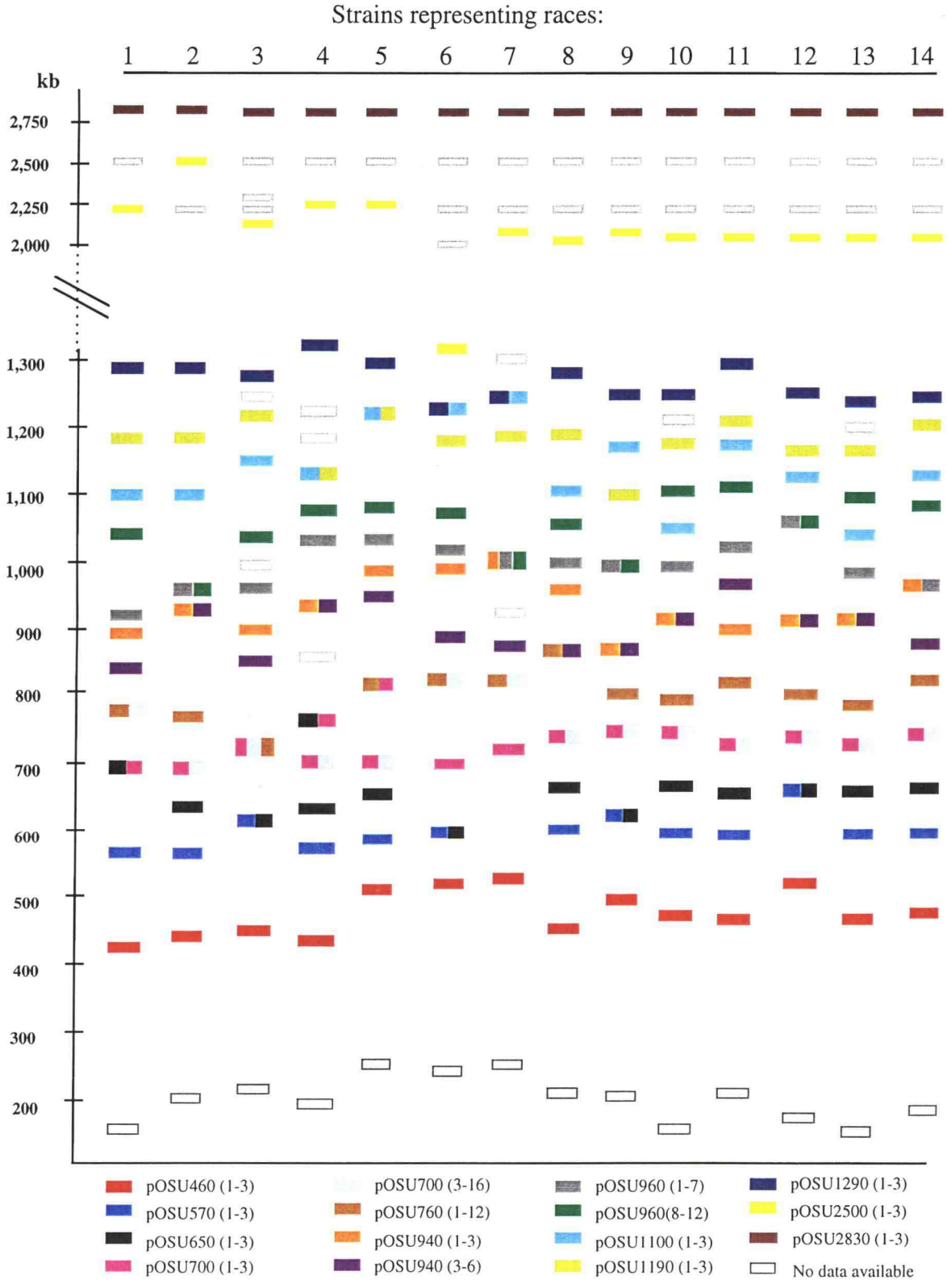


Figure II.4 A Southern blot of CHEF PFGE-fractionated DNA molecules of *Ustilago hordei* probed with a 0.4 kb *Bam*HI fragment (pOSU700-5). Lanes 1 through 14 contain the chromosomal DNA from the strains representing the 14 races of *U. hordei*

Figure II.5. Assignment of DNA fragments and heterologous genes to linkage groups of *Ustilago hordei.*



plasmid containing the mating type gene from *U. maydis*, no polymorphism was observed and the probes hybridized to the third largest chromosome in all races, respectively. Southern analysis of CHEF blots also revealed that chromosomes of identical size are not necessary homologs. For example, pOSU960-2 identified a 1,100 sized chromosome band in races1, 2, 8 and 14. In race 9, however, the same probe hybridized to a 1,180 kb chromosome band, although a 1,100 kb band was present in that same strain which does not hybridize with pOSU960-2. These results show that chromosome length polymorphism can change the relative position of some putative chromosomes in the CHEF gel with respect to other non homologous chromosomes of similar size. It is, however, important to note that the relative position of a chromosome compared to its homolog in another race, did not shift more than two positions above or below that homolog.

Cases of putative disomy were also observed. Probes pOSU760-6, pOSU760-7 pOSU790-8 hybridized to two chromosomes simultaneously in strains representing races 4 and 5, while in the remaining strains they hybridized to a single chromosome band. These findings suggest that disomy may be one of the mechanism generating polymorphism in chromosome number.

Discussion

A telomere-specific probe from *F. oxysporum* was used to determine the haploid chromosome number for the strains representing the 14 races in *U. hordei.* In each strain two, or a multiple of two, telomere fragments were detected for each chromosome band, confirming the hypothesis (Carle and Olsen, 1985; Morales *et al.* 1999; McCluskey and Mills, 1990) that chromosome bands separated by PFGE contain intact chromosomes.

Occasionally, chromosome bands of nearly identical size are impossible to excise without contamination from adjacent chromosome bands (Fig. II.2). Frequently, faint bands of telomere-containing restriction fragments were detected from adjacent chromosomes (Fig. II.2). Well resolved chromosome bands were required for application of this technique and care must be exercised when chromosome bands are extracted from PFGE gels, especially when other chromosome bands migrate nearby. To confirm chromosome numbers for each band, this probing procedure was repeated with at least three different restriction enzymes (eg. *Bam*HI, *Bgl*II, *Eco*RI). The number of fragments detected by the telomere-specific probe was identical regardless of the enzyme used to fractionate the chromosome-specific DNA, except when fragments co-migrate in the gel.

The telomere-specific repeat also allowed the detection of previously unidentified chromosomes in *U. hordei.* For example, strain 2.1 was thought to have the smallest chromosome number with 15 bands resolved by CHEF PFGE, whereas strains 10.1 and 11.1 were believed to have the largest chromosome number with 19 chromosomal bands resolved for each strain (McCluskey and Mills, 1990). However, using the telomere-specific repeat as a probe, 6 additional chromosomes were detected in strain 2.1, while 6 and 4 additional chromosomes were identified in races 10 and 11, bringing the chromosome number up to 21 and 23, respectively. This phenomenon was observed for all of the 14 strains. The number of chromosomes detected with the telomere-specific probe was six more than the number of bands per strain. Race 1 is currently considered to have the least number of chromosomes, while races 5, 6, 10 and 13 are considered to have the largest chromosome number. These results indicate that CHEF PFGE analysis alone cannot provide an accurate number of the haploid chromosome number and that additional analyses (e.g. probing with the telomere-specific probe) are needed to get a more accurate estimate of the actual chromosome number and genome size in *U. hordei.* This estimate is, however, constrained by the fact that not all the chromosomes are detected by the telomere-specific probe and should be treated as a minimum value

because additional chromosomes may be present in the *U. hordei* genome. When more than one chromosome is contained in a chromosome band, the telomere-specific probe can only distinguish chromosomes with different-sized restriction fragments in size. Disomic chromosomes with identical telomere termini, or nonhomologous chromosomes with similar-sized telomere restriction-fragments will be recognized as only one, single chromosome. Therefore, the chromosome number, we detected by the telomere-specific repeat probably only represents a minimum value of the actual haploid chromosome number in *U. hordei*.

It has been reported that shortening or lengthening of telomeres occurs during development and aging in higher organisms (Cooke and Smith, 1986; Kippling and Cooke, 1990; Harley *et al.*, 1990). In fungi, however, very little is known about telomere length variation during their life cycles. To investigate whether telomere length varies with age in *U. hordei*, telomere RFLP patterns were compared from one day and three-day old cultures. The telomere RFLP patterns obtained were identical for both ages (data not shown). These results support previous studies by D'Mello and Jazwinski (1991) who showed that in the yeast, *S. cerevisae*, no change in the length of the telomeres was observed in cells that had completed up to 83 percent of the mean life span.

Variation in chromosome numbers and size has been reported for isolates of plant pathogenic fungi such as *Gibberella fujikuroi* (Xu *et al.*, 1995), *Fusarium oxysporum* f.sp. cubense (Boehm *et al.* 1994), *Tilletia caries* and *T. controversa* (Russell and Mills, 1993, 1994). In the rice blast fungus, *Magnaporthe grisea* electrophoretic karyotypes were reported to change after prolonged serial transfer in culture, without affecting the isolate's pathotype (Talbot *et al.*, 1993). This was not observed for *U. hordei* strains and karyotypic variability only has been reported for strains representing the 14 races (McCluskey and Mills, 1990). In many fungi, electrophoretic karyotype variation has shown to be linked to gross chromosomal rearrangements such as deletions (Masel *et al.* 1993, Agnan and Mills, 1994), translocations (Tzeng et al. 1993; Trash-Bingham and

Gorman 1992), aneuploidy (Bakalinsky and Snow, 1990) and *B* chromosomes (Miao *et al.*, 1991; Kistler and Benny, 1992). The significance of this variation in relation to pathogenic specialization is unclear but gross chromosomal changes is believed to be a possible mechanism in the adaptation of a fungal pathogen to its host in the field (Masel *et al.*, 1993).

Our goal in undertaking this study was to determine the extent of the karyotypic variability and the mechanisms leading to the polymorphism in chromosome length and number in the strains representing the 14 races of *U. hordei*. Three random, chromosome-specific probes were used to detect homologous chromosomes and Southern blot analyses revealed that the homologs identified were typically monosomic with chromosome length polymorphism varying between 9 to 15 percent. These findings confirm other analyses of fungal chromosome-length polymorphism in which the variation in length never exceeded 15 percent. (McDonald and Martinez, 1990; Russell and Mills, 1993). It could be argued that three probes cannot represent an entire chromosome, and that the DNA molecules identified by these probes represent translocated or transposed segments on non-homologous chromosomes rather than homologous chromosomes.

Although it is not known where the chromosome-specific probes map on a given chromosome, three nonhomologous probes were used to ensure that more than one region of a chromosome would be detected by these probes. Moreover, all translocations identified and reported in fungi involve at least 500 kb DNA segments (Talbot *et al.*, 1990 Tzeng *et al.*, 1992; Trash-Bingham and Gorman, 1992), whereas the variability of the linkage groups in *U. hordei* did not exceed 100 kb and was consistent for all chromosomes analyzed. Although the probes cannot unequivocally exclude deletion of translocated chromosomes, any translocation that might be present involved chromosomes of nearly identical size in all strains.

For the majority of the chromosome bands studied, the number of chromosomes detected by the telomere-specific probe corroborated the number of linkage groups identified by the chromosome-specific probes. In some instances, however, the number of expected linkage groups based on the telomere-specific probe was not confirmed by the chromosome-specific probes. For example, in chromosome band 970 in race 2, three chromosomes were detected using the telomere-specific probe. However, when chromosome-specific DNA was used to probe the chromosomes, only two linkage groups were detected in that band, even though a total of 13 different probes from the 970 kb chromosome-specific library was used in this study. These results suggest at least two interpretations. It is possible that too few probes were used to distinguish multiple chromosomes contained within one chromosome band. Even though, 13 chromosome-specific probes were used, it is not obvious to which chromosome these probes hybridized in the chromosome cluster. If the probes fortuitously hybridized only to two of the three chromosomes, additional probes would be required to elucidate the nature of the third chromosome. If, however, the 970 kb chromosome bands contains three chromosomes of which two are disomic, only two linkage groups would be detected, regardless of the number of probes used. Evidence for disomy was obtained in races 4 and 5 and may indeed represent a mechanism for generating polymorphism in chromosome number (aneuploidy). Dispensable or *B* chromosomes were not detected in *U. hordei*, but not all chromosomes have been accounted for when the chromosome-specific probes were used (Fig. II.5). Putative *B* chromosomes may also be present in some chromosome clusters.

This investigation was the first extensive attempt to establish complete linkage groups of the genomes of the strains representing the 14 races of *U. hordei*. Although, PFGE represents an important tool for resoling entire chromosomes, additional analyses are needed to obtain a better understanding of the fungal genome structure. With the telomeric-specific repeat, a better estimate of the haploid chromosome number was

obtained in *U. hordei*. Establishment of specific linkage groups in each strain allowed a better understanding of the extent of chromosome length polymorphism and aneuploidy. However, the mechanism responsible for generating chromosome-length polymorphism remains obscure and will require fine-structural physical mapping of chromosomes. Electrophoretic karyotypes, coupled with Southern analyses with telomere-specific and random probes present an attractive approach for studying fungal genomes, especially for fungal pathogens with limited numbers of chromosomes.

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Chapter III.

CYCLIC AMP REGULATES MORPHOGENESIS IN THE FUNGAL PATHOGEN *USTLAGO HORDEI*

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Abstract

Ustilago hordei is the causal agent of covered smut on barley (*Hordeum vulgare*). This dimorphic fungus produces haploid cells that are yeast-like and nonpathogenic, while dikaryons produced upon mating of compatible strains are filamentous and pathogenic. A filamentous, haploid strain was isolated following heat shock treatment, and the mutation conferring this phenotype was designated *fil1*. Pulsed field gel electrophoresis (PFGE) and restriction analyses revealed that the mutant strain *fil1-1* has a 50 kilobase (kb) deletion in a chromosome that was 940 kb in size. The present work examines the biochemical and genetical properties of the *fil1* mutation. The mutant filamentous phenotype can be reversed to sporidial cell characteristic of wild type strains by addition of cyclic AMP to the growth medium. The same effect was observed with stimulators of adenylate cyclase, various analogs of cyclic AMP and inhibitors of cAMP phosphodiesterase. Furthermore, the levels of intracellular cyclic AMP were three-fold lower in mutant strains than in sporidia. These results suggest that cyclic AMP plays an important role in the regulation of dimorphic growth in *U. hordei,* and molecular characterization of the *fil1* mutation should contribute to a better understanding of the intracellular mechanisms and signal transduction pathways that control filamentous morphology and pathogenicity in *U. hordei.*

Key words: fungal dimorphism, signal transduction

Introdution

While numerous fungi exhibit yeast-like or mycelial morphologies, some fungi have the ability to grow in either form, in response to mating interactions or environmental conditions. Dimorphism, or the capacity to switch between two phenotypes, appears to be prevalent among numerous zoo-and phytopathogenic fungi and has been postulated to contribute to pathogenicity and/or virulence (Scherer and Magee, 1990). For example, it is known that the most severe mycososes in man are caused by dimorphic fungi, although it is not clear to what extent dimorphism contributes to pathogenicity (Cihlar, 1985). In many dimorphic fungi, such as the human pathogens *Mucor racemosus*, *Histoplasma capsulatum* and *Candida albicans,* the dimorphic switch is induced by cultural conditions (Herrera, 1985; Maresca *et al.*,1977; Soll, 1985). The morphology that persists depends on a complicated set of environmental factors. Among these are temperature, pH, available nutrients, oxidation-reduction potential, serum factors and cell interactions (San Blas and San Blas, 1983).

In the basidiomycete *Ustilago hordei* Pers. (Lagerh.), the causal agent of the covered smut disease of barley (*Hordeum vulgare* L.), the switch from sporidial to filamentous phenotype is primarily induced through mating interactions. *U. hordei* is a heterothallic biotroph with a life cycle consisting of a saprophytic and a parasitic phase. The saprophytic phase is characterized by haploid, secondary sporidia derived from basidiospores, that are nonpathogenic, while the parasitic phase is characterized by dikaryotic infection hyphae produced upon mating of compatible haploid strains (Fischer and Holton, 1957). Dikaryon formation and the dimorphic switch are believed to be

controlled by the mating type genes (Kronstad *et al.*, 1992). Little, however, is known about the signals regulating the dimorphic switch in fungi and a detailed understanding of the intracellular mechanism regulating filamentous morphology has been hampered by the lack of mutants in a defined genetically background (Szaniszlo, 1985)

Recently, a constitutively filamentous mutant strain designated *fil1-1* of *U. hordei* has been isolated following heat shock treatment (McCluskey *et al.*, 1994; Agnan and Mills, 1994). Pulsed field gel electrophoresis (PFGE) analysis of the *fil1*-1 mutant strain revealed a karyotype which contained an 890 kb chromosome derived from a 940 kb chromosome by a 50 kb deletion. In a cross of wild type (sporidial) X mutant (filamentous), the progeny of six ordered tetrads segregated 2:2 for the mutant and wild type phenotypes and the deleted form of the 940 kb chromosome was consistently associated with the *fil1* mutation.

The present study investigates the genetical and biochemical properties of the *fil1* mutation. It has been demonstrated that the intracellular levels of cyclic 3',5' adenosine monophosphate (cyclic AMP) are crucial in influencing cell morphology in many fungi (Cihlar, 1985). Cells expressing the mutant phenotype can be readily reverted to wild type sporidial cells in the presence of cyclic AMP. The intracellular pools of cyclic AMP in the wild type and *fil1-1* mutant strains were measured and changes in intracellular cAMP levels were correlated with changes in morphology. Results corroborate results of previous studies (Medoff *et al.*,1981; Orlowski and Ross, 1981) which demonstrated that shifts in intracellular levels of cyclic AMP may be important determinant of the morphological phase of a pathogen, and therefore of its disease producing potential. Molecular characterization of the *fil1* mutation should provide a better understanding of

the intracellular mechanisms and signal transduction pathways that control filamentous morphology and pathogenicity in *U. hordei*.

Materials and Methods

Fungal and bacterial strains and culture conditions.

The *U. hordei* strains, bacterial strains and plasmids used in this study are listed in Table III.1. Growth conditions for *U. hordei* have been previously described (McCluskey and Mills, 1990). Cyclic AMP analogs, inhibitors and stimulators (Sigma, St. Louis, Mo) were added to solid and liquid potato dextrose medium (PDA, PDB, Difco) at various concentrations.

Cyclic AMP measurements

U. hordei cells in the logarithmic phase of growth (1 day old cultures) were collected by centrifugation , washed briefly in water and suspended in 0.1N HCL. After 1 h at room temperature the cell suspensions were centrifuged at 20,000 x g at 4°C. The supernatants were assayed for cyclic AMP using a protein binding assay kit manufactured by Amersham Corp. (Arlington Heights, IL, USA).

DNA manipulations and microscopy.

Procedures for DNA isolation, restriction mapping and cloning were described previously (Mills *et al.* 1995). Fluorescence microscopy was carried out with a Zeiss

Table III.1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	FØ80d*lac*ZΔM15 Δ (*lac*ZYA-argF) U169*deo*R*rec*A1*end*A1hsdR17(r_K^-, m_{K+})*sup*E44λ-*thi*- 1*gyr*A96*rel*A1	Bethesda Research Labs. Gathersburg, MD
Ustilago hordei		
10.1a	21 chromosome bands, *Mat*-1	McCluskey and Mills, 1990
10.1a-1	(*fil1-1*) filamentous morphology mutant derived from 10.1a	McCluskey et al., 1994
Plasmids		
pUC19	Ap^r cloning and sequencing vector	Yanich-Perron *et al.* 1985
pBH100	1.5 kb *U. hordei Bam*HI fragment from mating type gene *b*E.	Bakkeren and Kronstad, 1993
pNLA17	$[TTAGGG]_{18}$ telomere repeat from *Fusarium oxysporum* cloned into pUC119	Powell and Kistler 1990

Ap^r, ampicillin resistance
Mat-1 and *Mat-2* are alternate genes (idiomorphs) at the A mating type locus

standard microscope using epifluorescent illumination. Cells were stained as described by McCluskey *et al.* (1994).

Results

Cell morphology of the wild type and the mutant strains

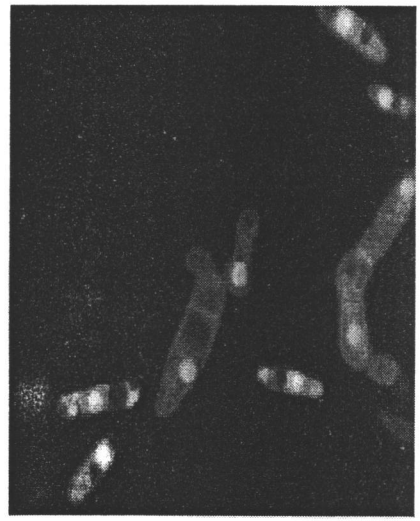
On solid and liquid medium, mutant strain *fil1-1,* as well as progeny carrying *fil1*, exhibited a striking filamentous phenotype (Fig.III.1). To investigate this phenomenon more closely, the cellular morphologies of the mutant strains were compared to its parental sporidial strains. Fluorescence microscopy demonstrated that the *fil1* mutation produces star-like branching pattern, eliminating the budding pattern of growth seen for sporidial haploid strains of *U. hordei.* Similar results have recently been reported by Kronstad *et al.*, (1993) for several filamentous mutants of the corn smut *U. maydis.*

Reversion of the filamentous phenotype to the sporidial wild type by cyclic AMP and other drugs

Cyclic AMP is known to play an important role in the morphogenesis of many zoo- and phytopathogenic fungi (Kronstad *et al.*, 1992; Gold *et al.*, 1994, Orlowski and Ross, 1981). When added to the growth medium, cAMP reversed the filamentous phenotype of the mutant *fil1-1* to the sporidial wild type (Fig. III.2). To ascertain which enzymes in the formation of cyclic AMP were affected, various drugs known to influence the intracellular levels of cyclic AMP were tested and their effect on the reversion of the mutant filamentous phenotype was measured. The results, summarized in Table III.2, show that the cyclic AMP analog 8-bromo cAMP was most effective for restoring the sporidial phenotype, followed by N^6 monobutyryl and cyclic AMP. N^6, $O^{2'}$-dibutyryl cAMP was not effective, nor were cyclic GMP, AMP and ATP.

Fig. III.1 Morphologies of the *fil1-1* mutant and wild type cells

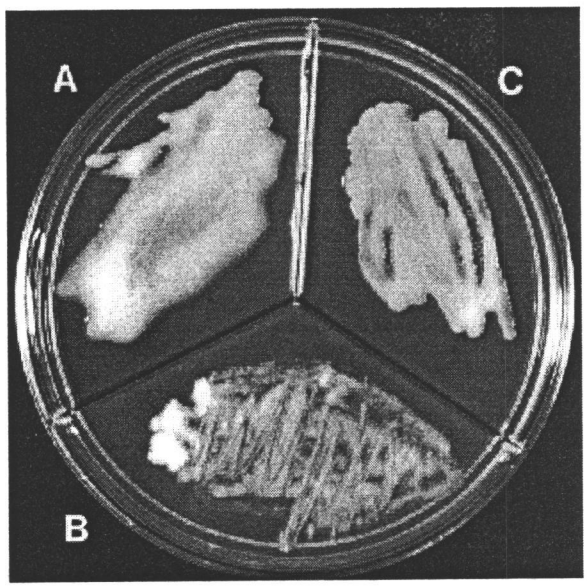
A. *fil1-1* mutant strain.



B. wildtype sporidial strain

Figure III 2 Reversion of the filamentous phenotype to the sporidial wild type by cyclic AMP

1. Colony morphology of *U. hordei*

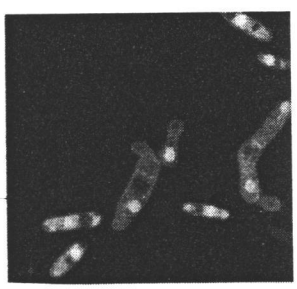


A: sporidial wildtype on PDA

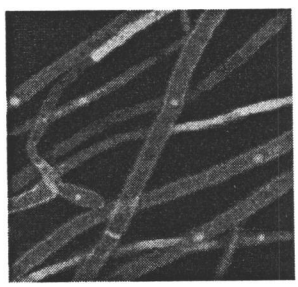
B: filamentous mutant on PDA

C: reverted mutant on PDA wtih 25mM cyclic AMP

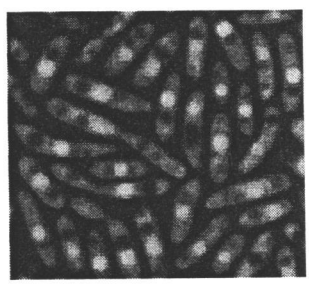
2. Cell morphology of *U. hordei*



A. wildtype sporidial cells



B. filamentous mutant cells



C. reverted cells of the filamentous mutant

Table III.2. Effect of various cAMP related drugs on the *fil*1 mutant phenotype

Treatment	Results[1]	Concentration[2]
cyclic AMP analogs		
cAMP	+	1.0 mg/ml
ATP	--[3]	
Adenosine	--[3]	
cGMP	--[3]	
GTP	--[3]	
N^6,$O^{2'}$-Dibutyryl cAMP	--[3]	
8-Bromo cAMP	+	0.8 mg/ml
N^6-Monobutyryl cAMP	+	0.9 mg/ml
stimulators of adenylate cyclase		
Ca^{2+}	--[3]	
NaF	lethal[3]	
2-deoxyadenosine	--[3]	
3-deoxyadenosine (cordycepin)	+	1.2 mg/ml
forskolin	+	1.5 mg/ml
Calcium antagonist		
LiCl	lethal[3]	
Inhibitor of phosphodiesterase		
3-isobutyl-1-methylxanthine (IBMX)	+	1.5 mg/ml

[1]minimum concentration for reversion of filamentous phenotype
[2]+:reversion to sporidial wild type phenotype;
--: no effect

[3]concentration of drugs: 2mg/ml

As with other systems, cyclic AMP levels in *U. hordei* appear to be regulated by the enzymes adenylate cyclase and phosphodiesterase, responsible for the synthesis and degradation of cyclic AMP, respectively. We next examined whether intracellular levels of cAMP could be raised in the filamentous mutant by either stimulating adenylate cyclase or blocking the degradation of cAMP by inhibiting phosphodiessterase. Cells were grown in the presence of 3-isobutyryl-1 methylxanthine (IBMX) an inhibitor of phosphodiesterase, and cordycepin, forskolin, 2-deoxyadenosine and NaF, known stimulators of adenylate cyclase in other eukaryotes. The drugs IBMX, forskolin and cordycepin restored the sporidial wild type while, NaF and 2-deoxyadenosine had no effect on the filamentous phenotype (Table III.2).

Intracellular levels of cAMP

The endogenous cyclic AMP concentration in wild type and mutant cells was measured in three independent experiments. Although the absolute levels of cyclic AMP detected varied due to differences in experimental conditions, all three experiments showed that high intracellular levels of cyclic AMP are associated with yeast-like cells and low levels are characteristic of hyphal cells (Fig. III.3).The level of intracellular cyclic AMP was found to be approximately three-times higher in the sporidial phase than in the mycelial phase.

Mapping of the *fil1* locus

Genetical analyses of the cross *fil1-1* X *Fil1-1* indicated that the deletion was distantly linked to the centromere (McCluskey *et al.*, 1993). Restriction digests with *Bam*HI and *Bgl*II followed by Southern analysis with a telomere-specific repeat confirmed that the *fil1* mutation is located near the terminus of one arm of the 940 kb chromosome On the basis of these restriction data, a model of the genesis of the *fil1* mutation has been proposed (Fig. III.4).

Figure III.3. Intracellular levels of cyclic AMP in the wildtype and mutant cells

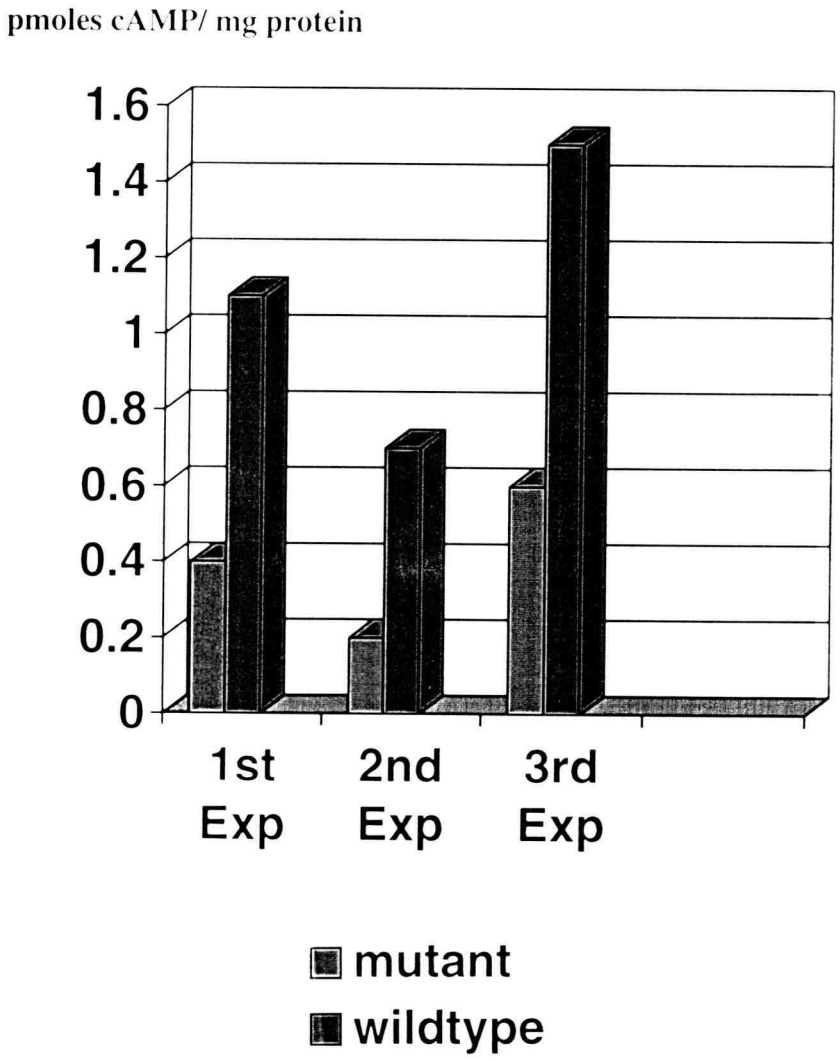


Fig.III.4. Restriction map and proposed model of the *fil1* mutation

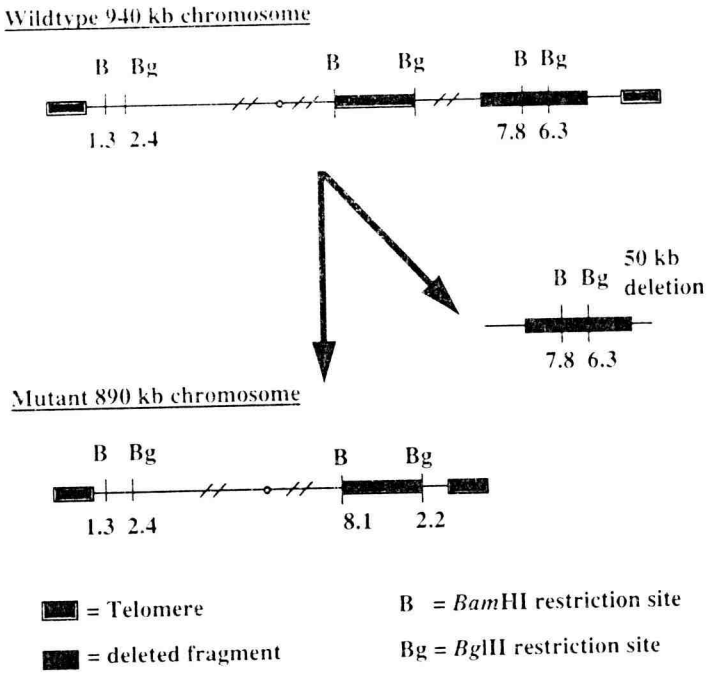
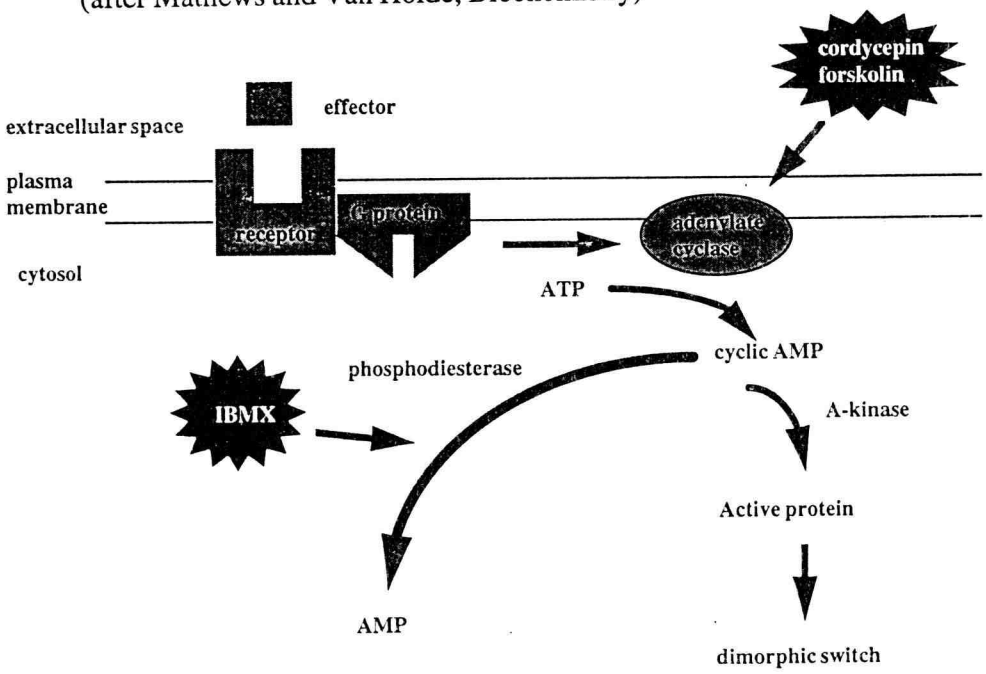


Figure III.5 Signal transduction pathway involving G protein and adenylate cyclase
Forskolin and cordycepin act as an adenylate cyclase activator.
The hydrolytic catabolism of AMP is inhibitd by IBMX
(after Mathews and Van Holde, Biochemistry)



Discussion

Cyclic AMP has been established as one of the most important biochemical mediators of dimorphism in fungi. Since its discovery 30 years ago, this cyclic nucleotide has been linked to a variety of functions such as morphogenesis, cell division, enzyme production, infection structure formation and cell differentiation. Smith *et al.* (1990) report that the levels of cyclic AMP fluctuates during the cell division cycle of the budding yeast *Saccharomyces cerevisiae,* and that the intracellular concentration of cyclic AMP is at its highest during the division cycle and at its lowest immediately prior to and just after cell separation. In the slime mold, *Dicyostelium discoideum*, cyclic AMP affects the differentiation pathways of prespore and prestalk cells (Yamada and Okamoto, 1992), while in the white-rot basidiomycete *Phanerochaete crysosporium*, cyclic AMP is involved in the regulation of the lignin-degrading enzyme system (Boominathan and Reddy, 1992). In the rice blast fungus, *Magnaporthe grisea*, cyclic AMP regulates infection structure formation by inducing the formation of appresesoria.

In *U. hordei*, cyclic AMP is involved in the signal transduction pathway regulating the filamentous phenotype and the dimorphic switch. Higher levels of cyclic AMP have been linked to the yeast-like phase, while low levels of cyclic AMP are characteristic of hyphal growth. Similar results were obtained by Larsen and Sypherd (1974) and Paveto *et al.*(1975) who reported that cyclic AMP concentrations are four fold higher in yeast cells than in hyphal cells in *M. racemosus* and *M. rouxii.* In *H. capsulatum,* however, the opposite has been observed, and the cyclic AMP

levels are five-fold higher in the mycelial phase than in the yeast phase (Medoff *et al.*, 1980). The mechanism, however, by which cyclic AMP regulates the switch from the filamentous morphology to the sporidial wild type remains unclear. In *M. rouxii*, the lower intracellular level of cyclic AMP has been attributed to an increase in the activity of the phosphodiesterase enzyme. Orlowski (1980), however, investigated cyclic AMP metabolism during hyphal development from sporangiospores of *M. genevensis* and *M. mucedo*, and observed that intracellular cyclic AMP levels increased during the stages of spore germination, and followed by a sharp drop prior to the appearance of germ tubes. In *M. genevensis* and *M. mucedo*, changes in adenylate cyclase level, rather than phosphodiesterase, were believed to regulate endogenous cyclic AMP levels. The contrasting results from these two investigations are perplexing and most probably reflect the difference in experimental conditions and the difficulty of interpretation of data (Cihlar, 1985).

In regard to *U. hordei*, the data presented suggest that the *fil1* mutation does not affect the enzymes responsible for the regulation of cyclic AMP. Stimulators of adenylate cyclase (forskolin, cordycepin, 2-deoxyadenosine) restored the sporidial wild type phenotype, indicating that the adenylate cyclase enzyme is functional. This result is suggestive that the *fil1* mutation probably affects gene located upstream of the adenylate cyclase enzyme in the signal transduction pathway. In most signal transduction pathways studied, a surface receptor transmits the environmental signal via a G protein, which in turn activates a protein phosphorylation cascade (Janssens and Van Haastert, 1987) (Figure III.5). Our data indicate that the *fil1* mutation probably affects a gene encoding a G protein, a cell

surface receptor, or a ligand. Cloned genes, that are known to affect cell morphology have been reported to show homology to genes encoding a protein kinase or adenylate cyclase (Kronstad *et al.* 1993; Gold *et al*, 1994). Our data indicate that the *fil1* mutation does not affect these genes. The cloning of the *Fil1* mutation should provide new insights into the role of cyclic AMP in the signal transduction pathway in *U. hordei.*

Molecular and genetical analyses allowed the mapping of the *fil*1 locus. Southern analyses with the telomere-specific probe revealed that two of four terminal restriction sites of *Bam*HI and *BglII* show different sizes in the deleted form of the 940 kb chromosome. Our model (Fig. III.4) is consistent with two sites at one end of the 940 chromosome being unaffected and those at the other end being changed. The original *Bam*HI and *Bgl*II restriction sites are assumed to be lost by deletion, and the new *BglII* and *Bam HI* sites proximal to the deletion, have been brought closer to the terminus of one of the arm of the 940 chromosome. The result is an 890 kb chromosome with new restriction sites at one terminus.

Overall biochemical and genetical analyses indicate an important role for cyclic AMP during fungal dimorphic switching. In *U. hordei*, sporidial cells are found only in soil and never in infected plant tissue (Kozar, 1969). These findings imply that each growth phase is an adaptation to two critically different environments.. The morphological phase that persists may conceivably be simply determined by cell interactions. The relative ease with which the phase transitions can be reversibly accomplished in culture implies that the regulatory mechanisms of

morphogenesis can be studied and should provide insight into the relationship between cyclic AMP and pathogenicity.

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Chapter IV.

FUTURE RESEARCH PERSPECTIVES

Recent developments in pulsed-field gel electrophoresis (PFGE) have resulted in studies of the chromosomal organization of an impressive number of fungi, for which classical, and cytological analyses were difficult, either because of the small size of their chromosomes or because of a lack of a sexual stage. An example of such a fungus is *U. hordei* which has chromosomes that are too small to be studied by light microscopy. Although, four chromosomes were detected in *U. hordei* strains in 1942 by Kharbush, genetic evidence indicated a higher chromosome number in this fungus. It was as recent as 1990 that electrophoretic karyotypes of the strains representing the 14 races were established for *U. hordei* by McCluskey and Mills (1990) and an extensive amount of polymorphism in chromosome length and number was observed..

One goal of this study was to establish the basic haploid chromosome number in *U. hordei* and to investigate the extent of chromosome polymorphism in length and in number among the strains representing the 14 races. A telomere-specific probe from *F. oxysporum* was used to determine the chromosome number contained in each chromosome band. Telomeres have DNA sequences that are highly conserved among many eukaryotes and are organized in tandem repeats at the end of each chromosome (Burr and Burr, 1992). The telomere-specific probe not only allowed the identification of previously undetected chromosomes in *U. hordei,* but also generated specific telomere

restriction fragment length polymorphism (RFLP) markers for each chromosome of each strain studied. Each chromosome and each strain analyzed with the exception of races 10 and 13, showed unique telomere RFLP patterns, indicating that the telomere-specific probe represents a powerful tool for the identification, delineation and study of many eukaryotic organisms. Furthermore, the telomere- RFLP patterns obtained with the telomere-specific probe were stable after one generation of inbreeding (unpublished data). It would, however, be of interest to study the segregation patterns of the telomere RFLP fragments of progeny from a cross involving strains representing two different races over several generations. We hypothesize that any chromosome in the progeny will display telomere RFLP patterns from one or the other parents when no recombination has occurred, but will contain terminal fragments from homologs of both parents in recombinant chromosomes.

To ascertain the chromosome number with the telomere-specific probe, linkage groups were first established using chromosome-specific DNA as probes onto Southern blots containing chromosomes of the 14 races of *U. hordei*. Ideally, every chromosome should have been tagged by a chromosome-specific probe. Unfortunately, *U. hordei* has a very complicated karyotype with a large number of chromosomes, many of which cosegregate in chromosome clusters. However, we were not able to account for all the chromosomes detected by the telomere-specific probe, because of the large number of chromosomes present in the strains representing the 14 races of *U. hordei*. Nevertheless, for fungi with relatively few chromosomes (e.g. 10 or fewer), such as *Neurospora crassa*, *Aspergillus nidulans* and *Candida albicans*, the telomere-specific probe combined with

the establishment of linkage groups, would represent an excellent approach for analyzing fungal karyotypes.

Disomic chromosomes were detected with the chromosome-specific probes. Translocations, insertions and *B* chromosomes have been reported to contribute to chromosome polymorphism, although none of these events was observed in *U. hordei*. However, recently a filamentous mutant of *U. hordei*, designated *fil1*, was determined to have a 50 kb deletion following heat-shock treatment. These findings suggest that deletions of that magnitude may be tolerated and suggest a mechanism by which chromosome-length polymorphism is generated in *U. hordei*. The filamentous phenotype is of interest because *U. hordei* is a dimorphic fungus that must switch morphology to form the filamentous infection hyphae in order to be pathogenic on barley plants (Fischer and Holton, 1985). The filamentous phenotype can be reverted to the sporidial wild type phenotype in the presence of cyclic AMP, supporting previous studies that cyclic AMP is an important morphogenetic determinant in many dimorphic fungi (Medoff *et al.* 1981). In *U. hordei*, high intracellular levels of cyclic AMP were associated with the yeast-like phase, whereas lower intracellular levels were characteristic of the filamentous phase. These results suggest that in order for mating to occur, endogenous cyclic AMP levels of the two compatible basidiospores, have to be down regulated to allow formation of the filamentous infectious hyphae. We investigated whether the mating reactions could be inhibited by cyclic AMP. Preliminary studies indicated that cyclic AMP had no effect on the formation of infection hyphae during mating. However, more detailed studies with the more lipophilic drug 8 bromo cAMP, have to be performed for more reliable results. Moreover, it would be of interest to measure the intracellular level of cyclic AMP in the

infection hyphae. Based on our studies, we would anticipate a low level of intracellular cyclic AMP in the infection hyphae.

In related studies, we examined whether the ability to cause disease on barley plants was affected by the *fil1* mutation. Crosses of sexually compatible *fil1-1* mutants were made, and eight barley differentials were inoculated. No disease symptoms were observed for the mutant cross, whereas disease symptoms developed on all differentials except "Excelsior" when inoculated with the wild type. Race 10, from which the *fil1-1* mutant was obtained, is incompatible on "Excelsior." In *U. maydis*, a similar mutant was described that is filamentous and nonpathogenic (Kronstad *et al.*; 1993). The mutant is defective in the *uac1* gene, which encodes the adenylate cyclase enzyme. It was able to infect corn plants, but did not generate any disease symptoms on corn. Therefore, additional studies are needed to investigate whether the non pathogenicity of the *fil1-1* mutant is due to inability to mate or inability to infect susceptible cultivars.

A number of investigators have used the technique of differential display to study genes that are expressed during the infection stage. This technique would also be of interest in continued studies. The wild type strain 10.1a and mutant strain *fil1-1* are isogenic except for the *fil1* mutation. Cloning the genes expressed during the filamentous stage may provide new insight into the intracellular mechanism of pathogenicity genes.

Also in progress is the cloning of the *Fil1* locus. The Amplified Fragment Length Polymorphism (AFLP) technique was used to identify DNA fragments that are present in the wild type strain but absent from the mutant. Several fragments have been identified and isolated , and they are presently being screened for homology with the region that was deleted from the 940 kb chromosome.

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APPENDIX

CHARACTERIZATION OF GENOME PLASTICITY IN *USTILAGO HORDEI*

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Abstract

Southern blot hybridization analysis was used to identify and quantify chromosome-length polymorphisms for 10 linkage groups of 14 races of *Ustilago hordei*. The bands identified by the probes were shown to vary as much as hundreds of kilobasepairs, but the magnitude of the variability was typically 5 to 15 % of the average size of all bands to which a particular probe hybridized. A filamentous morphology mutant, recovered following heat shock treatment of a strain with the greatest number of chromosome bands, was shown to have suffered a 50 kb deletion in a 940 kb chromosome. The mutation to filamentous morphology, designated *fil1-1* and the deletion, were shown to invariably cosegregate 2:2 with the wild type (sporidial) morphology in an ordered tetrad. Genetic and physical analyses place the *Fil1* locus and the deletion near the terminus of one arm of the 940 kb chromosome. These results suggest that deletions of this type may be one of the causes of chromosome-length polymorphisms observed in field isolates of *U. hordei*.

Key Words: PFGE, Electrophoretic karyotypes, deletion

Introduction

Fungal genetics has been revolutionized by the development of pulsed-field gel electrophoresis (PFGE) technology and its application to questions of genome size, organization and stability (for recent reviews, see Mills and McCluskey; 1990; Skinner **et** *al,*. 1991; Mills *et al.*, 1993). A large amount of karyotypic variability has been detected among strains of phytopathogenic fungi, as predicted by Tolmsoff (1983). In general, however, the variability is described only as the number of chromosomes and their sizes. For some fungi, a few of which are phytopathogens, there has been a better description of the identity of the individual bands. Physical and genetic maps are correlated for *Neurospora crassa* (Orbach et al,. 1988), *Saccharomyces cerevisiae* (Carle and Olson, 1985), *Cochliobolus heterostrophus* (Tzeng et al, 1992), *Magnaporthe grisea* (Talbot *et al,*. 1993), and for some linkage groups of *Leptosphaeria maculans* (Morales *et al,*. 1993), and this approach is being increasingly pursued. For fungi whose genetics have not been extensively described, as is the case for *Ustilago hordei*, which causes covered smut on barley (*Hordeum vulgare* L.), and fungi for which sexual crosses are either difficult or impossible to perform, this approach is precluded and the chromosomes resolved by PFGE typically remain anonymous.

The karyotypes of representative strains of the 14 races of *U. hordei* have been shown to be variable with respect to the number of chromosome bands and lengths of putative homologous chromosomes (McCluskey and Mills, 1990). However, the amount of variability among homologous chromosomes could not be determined merely by visual

inspection of electrophoretic karyotypes because some nonhomologous chromosomes appear similar in size and are not resolved. Additional karyotype variability may arise if some strains are aneuploid or harbor B chromosomes (Miao *et al.*, 1991).

To examine some plausible causes for the apparent genome plasticity among strains representing the 14 races of *U. hordei*, blots of whole chromosomes were probed with anonymous DNA fragments and conserved genes to potentially identify chromosome-length polymorphisms, multiple copies of chromosomes and B chromosomes. To determine whether any chromosomes are dispensable, the heat shock approach described by Hilton *et al.* (1985) for *Candida albicans* was adapted to a *U.. hordei* strain with the greatest number of chromosome bands. None of the chromosomes was shown to be dispensable, but a filamentous mutant recovered from the heat shock was shown to have a 50 kilobase (kb) deletion in one of its chromosomes. The deletion mutation and its associated filamentous phenotype, designated *fil1-1*, cosegregated in 6 ordered tetrads.

Materials and methods

Strains and culture conditions. The strains representing the 14 physiologic races of *U. hordei* have been previously described (McCluskey and Mills, 1990). *S. cerevisiae* chromosomes were obtained from Bio-Rad (Hercules, CA), and used as size markers. Bacterial strains and plasmids used for DNA manipulations are described in Table 1.

Contour-clamped homogenous field pulsed-field gel electrophoresis (CHEF PFGE). Electrophoresis was conducted in a Bio-Rad CHEF Dr-II electrophoresis system (Richmond, Ca). Samples for PFGE were prepared and electrophoresed as described by McCluskey *et al.* (1990) .

Sources of DNA probes. Probes were made from chromosome-specific libraries or total genomic DNA. Chromosome specific DNA was extracted from preparative low gelling temperature agarose (FMC, Rockland, ME) CHEF gels using the freeze-thaw method. Bands were excised under UV illumination, taking care to minimize UV exposure, and transferred to 15 ml polypropylene tubes. The agarose was melted at 68°C for 10 to 20 min in 5 ml of buffered solution (25 mM Tris pH 7.5, 1 mM EDTA, 1 M NaCl), gently mixed, and then frozen at -20°C for 1 to 16 h. The samples were incubated for 30 to 60 min at 37°C, mixed gently and the freezing and warming cycle was repeated once. After centrifuging at 10,000 X g for 30 min at 5°C, the supernatants were transferred to clean tubes, mixed with equal volumes of isopropyl alcohol, and incubated at -20°C to precipitate the DNA. The DNA was collected by centrifugation for 30 min at 10,000 X g, and the pellets were suspended in 400 ml of 10 mM Tris HCl, pH 7.5, 1 mM EDTA (TE). This solution was transferred to a 1.5 ml microfuge tube, extracted twice with TE-saturated phenol followed by chloroform extraction, and then precipitated with ethanol. This DNA was digested with *Bam*HI or *Eco*RI and ligated to alkaline phosphatase-treated pUC19 or pCM54 using standard cloning techniques, and the entire ligation reaction

mixture was used to transform competent *Escherichia coli* DH5α cells (Sambrook et al,. 1989). Plasmid DNA was prepared by an alkaline lysis procedure (Birnboim and Doly, 1979), and radiolabelled DNA was prepared using the random priming technique of Feinberg and Vogelstein (1983). Total genomic DNA was isolated from strain 8. la (Table 1) using the method of Specht *et al.* (1982). The DNA was digested to completion with *Eco*RI and cloned into pUC18 as previously described.

Chromosome number. To ascertain the number of chromosomes present in an individual band, an agarose slice containing the band of interest was excised from a CHEF gel, and the DNA was extracted by the freeze-thaw method and digested with *Bam*HI restriction enzyme (Gibco-BRL, Grand Island, NY) following the manufacturer's instructions. The *Bam*HI fragments were separated by electrophoresis and Southern-blotted (Southern 1975) to nylon membranes using standard techniques (Sambrook et al. 1989). A radiolabelled DNA probe was prepared from a gel-purified telomere sequence from *Fusarium oxysporum* (Table 1: Powell and Kistler, 1990) using the random priming technique (Feinberg and Vogelstein, 1983). The number of chromosomes in any band was determined to be equal to one-half the number of restriction fragments that hybridized to the telomere probe.

DNA hybridizations. The DNA in CHEF gels was denatured in 0.4 N NaOH and transferred in that solution to Genetran nylon membranes (Plasco, Woburn, MA)

(Sambrook *et al.*, 1989). Hybridization reactions were carried out as described by McCluskey and Mills (1990).

Heat shock treatment of *U. hordei*. A 3 ml aliquot of strain 10.1 a which had been cultured in Potato Dextrose Broth (PDB, Difco Laboratories, Detroit, MI) to mid log phase (ca. 106 cells per ml) was transferred to a 5 ml polypropylene tube and incubated at 52°C for 2 to 10 min. Heat shock was terminated by transferring 100 ml of the heat-treated sample to 900 ml PDB at room temperature, and dilutions were spread onto Potato Dextrose Agar (PDA) medium and incubated at room temperature for 2 to 5 days until colonies were visible. An aliquot of the starting material was diluted and plated onto PDA to determine the concentration of the starting culture, and this value was compared to the average of several platings of the heat-treated sample to determine the percentage of surviving cells.

Microscopy. Fluorescence microscopy was carried out with a Zeiss standard microscope using epifluorescent illumination. Cells were either stained for 5 min at room temperature with ethidium bromide (1 mg/ml) and immediately visualized, or stained for 1 h at room temperature in 0.0025% calcofluor (Sigma, St Louis, MO) and 20 μg/ml Hoechst 33258 (Sigma) in TE.

Results

Identification of homologous chromosomes

The electrophoretic karyotypes of the strains representing the 14 physiologic races of *U. hordei* are presented in Figure 1A. The varying numbers of bands and clusters of chromosomes precluded using the karyotypes alone to identify homologous chromosomes in these strains. However, by using Southern blot analysis with a variety of DNA probes (Table 1), 10 putative homologous chromosomes have been identified among the 14 strains studied (Table 2). The hybridization profiles revealed that for some putative chromosomes, length polymorphisms were sufficient to change their relative position in the CHEF gel with respect to other nonhomologous chromosomes of similar size. Moreover, the chromosomes at the top and bottom of a cluster could be distinguished by hybridization with different probes. Thus, Southern analysis of CHEF blots frequently revealed that chromosomes of identical size may not necessary be homologous.

A typical example of the heterogeneity observed for 8 of the 10 chromosomes is seen in the Southern analysis presented in Figure 1B. The amount of variability among these 8 chromosomes was typically 9 to 15% of the average band size for each probe (Table 2). Probes pOSU4088, pOSU4182, pOSU4062, pOSU4611 pOSU4172 and pOSU4012 identified maximum chromosome-length polymorphisms of at least 100 kb. Moreover, probes pOSU4088 and pOSU4062 clearly distinguished different chromosomes at the top and bottom of a 975 kb unresolved cluster of chromosomes in the karyotype of strain 7.1 (Table 2). Probe pOSU4843 displayed an interesting hybridization

pattern. It hybridized only with a 650 kb chromosome band in strain 8. la, but not with a 650 kb chromosome that was present in the karyotypes of strains 1.3, 2.1a, 3.1, 4.1 and 5.1. Moreover, this probe hybridized with the fourth smallest chromosome in 3 strains, the fifth smallest in 10 strains and the sixth smallest in one strain. The actin gene probe (pSF8) from *Aspergillus nidulans* (Fidel *et al,*. 1988) hybridized to the third smallest chromosome in all strains, which ranged in size from 570 to 650 kb (Table 2).

Two probes identified putative chromosomes that showed either no length-polymorphisms or substantially greater variability than observed for other chromosomes. The probe made from pOSU4811 hybridized to the 3,150 kb band in all 14 strains. This apparent lack of variability may result from the inability to resolve chromosomes with length polymorphisms in this size range. Conversely, the rDNA probe, pOSU1011, detected the greatest amount of chromosome variability with a range of 86% of the average for this chromosome (Table 2).

Heat shock treatment and characterization of mutant strain 10. la-1

Heat shock at 52°C produced approximately 99.9% lethality after 7 minutes for strain 10. la. Strain 10.l a typically produces mucoid, convex colonies comprised of sporidia, while several of the colonies that arose following heat-shock had altered morphologies. One strain, 10.1 a-1, has a dry, mycelial colony with an overall appearance of being crusty and the cells, in contrast to the wild type are filamentous rather than sporidial (Fig. 2). Mutations conferring this phenotype have been designated *fil*, and the mutation in 10.1 a-1 has been designated *fil1-1*. Strain 10.1a-1 (*fill-1*) exhibits slower

growth, both in liquid and on agar-solidified medium, than the wild type progenitor strain (Fig. 2B).

Of 25 various colony morphology mutants, mutant strain 10.1 a-1 (*fil1-1*) was determined to have a chromosome band of 940 kb missing from its karyotype (Fig. 3A). Both the colony morphology and the altered karyotype were stable through repeated sub-culturing. The higher molecular weight bands not resolved in the gel shown in Figure 3 were resolved by longer-term electrophoresis, and determined to be unchanged in 10.1 a-1 (*fil1-1*) (data not shown).

Southern-blot analyses with DNA probes made from restriction fragments from the 940 kb chromosome of strain 10.1 a, and a telomere probe from *F. oxysporum* (Powell and Kistler, 1990), have revealed the fate of the missing 940 kb chromosome in the mutant. Hybridization probes made from the inserts in pOSU4126, pOSU4132 and pOSU4135, which were determined by Southern hybridization to be nonhomologous (data not shown), hybridized only to the 940 kb chromosome in strain 10.1 a, and specifically to the 890 kb chromosome cluster in the mutant strain 10.1a-1 (*fil1-1*) (Fig. 3B). These results suggested that approximately 50 kb of DNA was deleted from the 940 kb chromosome in the genesis of an 890 kb chromosome. To corroborate these results, a telomere-specific DNA probe was hybridized with *Bam*HI restriction fragments from the 940 kb chromosome from 10.1 a, and the 890 kb cluster of chromosomes from 10.1a in 10.1 a-1 (*fil1-1*). Two *Bam*HI fragments from the 940 kb chromosome hybridized with this probe indicating that this band contains a single chromosome with *Bam*HI sites 1.3 and 7.8 kb from its termini. Six fragments from the 890 kb cluster of strain 10.1 a hybridized with this probe, indicating that this cluster from the wild type contains 3

chromosomes (Fig. 4). However, the probe hybridized to 8 *Bam*HI fragments from the 890 kb cluster of the mutant 10.1 a-1 (*fil1-1*), six of which were identical to those found in the 890 kb cluster of the wild type progenitor strain. Two additional fragments of 1.3 kb and 8.1 kb hybridized with the probe, indicating that one end of the deletion chromosome was unaffected, whereas the other end had a new *Bam*HI site (Fig. 4). To verify that the new *Bam*HI did not result froma translocation event, individual chromosome bands from the mutant and wild type strains were excised, digested with *Bam*HI, and Southern analysis was performed with the telomere probe. The terminal *Bam*HI fragments of all of the chromosomes bands were unchanged except for one terminal fragment of the deletion chromosome (data not shown).

Genetic analysis of the *fill-1* mutation

The mutant strain 10.1a-1 (*fil1-1*) was crossed with a sexually compatible strain isolated from the teliospore collection that gave rise to 10.1 a, and the dikaryon was inoculated onto the universally susceptible barley cultivars, Odessa and Junior, using the technique of Gaudet and Kiesling (1991). In 6 ordered tetrads analyzed from this cross, the filamentous and sporidial morphologies segregated 2:2. Moreover, first and second division segregation patterns occurred with equal frequencies, indicating that *fil1-1* is distantly linked to the centromere, a result that corroborates the data obtained by hybridization with the telomere-specific probe. The karyotypes of progeny from the 6 tetrads revealed that the 940 kb chromosome always cosegregated with the wild type sporidial cell morphology and that all of the mutant progeny lacked a 940 kb chromosome (Fig. 5A). In Southern analyses of CHEF blots, one of which is presented in

Figure 5B, all of the mutant progeny in the 6 tetrads have an 890 kb chromosome that hybridizes with a probe that is specific for the 940 kb chromosome in the parental strain.

Discusssion

The hybridization of a particular DNA probe to chromosomal bands of different sizes among these 14 presumed unrelated strains of *U. hordei* can be interpreted in several ways. In this study and in numerous others (Carle and Olson, 1985; Morales *et al.*, 1993; Russell and Mills, 1993) the bands are interpreted to be chromosomes, and the length polymorphisms are presumed to be a manifestation of subtle differences among homologs because of deletions, duplications, translocations and possibly variation in the sizes of the telomeres. While all of these phenomena could have contributed to the chromosome-length polymorphisms apparent in these strains of *U. hordei*, some appear more plausible than others.

In the present study, some putative homologs were shown to differ by more than 100 kb in size. These length polymorphisms changed the relative ranking of some chromosomes with the respect to others of similar size. For example, the Southern analyses with pOSU4843 (Table 2) indicate that the fourth smallest chromosome in some strains is homologous with the fifth or sixth smallest chromosomes in other strains. The relative size of this chromosome in the karyotype of different strains was unchanged under constant conditions of electrophoresis, and therefore the variability could not be attributed to anomalous migration in the CHEF gel. These results indicate that its rank, or

the assignment of a number for this chromosome which is based on size, will vary depending upon the karyotype of the field isolate being investigated.

It appears very unlikely that chromosome-length polymorphisms in excess of 100 kb could be attributed to changes in the number of telomere repeats. In this study and in others (Mills *et al*, 1994; Agnan, Abdennadher and Mills, unpublished data), terminal chromosomal *Bam*HI fragments as small as 1.3 kb have not been observed to vary in size. It is also unlikely that chromosome-length polymorphisms of 9 to 15 % observed for 8 of the 10 putative chromosomes examined resulted from translocated segments from other chromosomes, or fragmented chromosomes arising from cleavage or breakage at specific sites during sample preparation. Translocations or the occurrence of fragile sites would be expected to segregate in progeny, leading to populations with chromosomes that differ greatly in size. Preliminary evidence supporting the supposition that the bands represent intact chromosomes rather than fragments was recently reported by Mills *et al.* (1994). The putative chromosome bands excised from a race 2 strain of *U. hordei* were shown to have 2, or multiples of 2, terminal *BamHI* fragments

The chromosome containing the rDNA repeat varied by 86% of its average length (Table 2), but it is unlikely that this variation resulted from reciprocal or non-reciprocal translocation. Unless all copies of the rDNA repeat were translocated, the rDNA probe would have hybridized to 2 chromosome bands. In the closely related bunt fungi, *Tilletia caries* and *T. controversa*, and their hybrid progeny, a rDNA probe hybridized with 1 to 3 chromosomes that differed in length by approximately 40% (Russell and Mills 1993). However, in none of the 14 strains of U. hordei were 2 bands observed to hybridize with the rDNA probe (Table 2, and data not shown). It appears

therefore, that duplications or deletions of the rDNA repeat, or combinations of these events contributed to the larger amount of variation observed for this chromosome.

The smaller amount of variability (9-15%) in the lengths of 8 of the 10 chromosomes is most likely the result of changes in the copy number of moderately repeated DNA, or the accumulation of small deletions or duplications which do not affect viability. The recovery of strain 10.1 a-l (*fil1*-l) following heat shock treatment supports the hypothesis that deletions alone may account for the magnitude of karyotypic variability observed in this study. A deletion of approximately 5 % of the 940 kb chromosome was not lethal, but was shown to always cosegregate with the *fill1* mutation. That first and second division segregation patterns for the *fil1*-l mutation and the deletion chromosome occur with equal frequency, and a terminal *Bam*HI restriction site 7.8 kb from I end of the mutant chromosome is lost, strongly suggest that the deletion will map near the terminus.

The *fil1-l* mutation of *U. hordei* is constitutive for filamentous growth and resembles other mutations that affect cell morphology in fungi (Harold, 1990). Strains carrying *fil1-l* superficially resemble a recently described mutant of *U. maydis* designated *rem1-l*, (repressor of mycelial phenotype, Barrett *et al.*, 1993). Both mutations block budding growth, although functions have not been described for either of these putative genes. In both species, crosses of mutant with wild type strains produce dikaryons that are infectious on their respective hosts. However, unlike *U. hordei* which showed 2:2 segregation for the mutant and wildtype phenotypes in 6 ordered tetrads analyzed, the segregation of the phenotype resulting from the *rem1-l* mutation in random sporidia of *U. maydis* varied from 1 to 47 % between experiments, possibly because of a reduced

growth rate for mutant cells. Mutant *fil1-1* cells are competent to mate with sexually compatible wild type strains, and the infectious dikaryon will ultimately produce teliospores that germinate to produce 4 primary sporidia. This result implies that the *fil1*-1 mutation is either recessive, or that the mechanism of primary sporidia (basidiospores) production is different from the mechanism of budding by secondary sporidia. The cloning and molecular characterization of the *Fill* locus are in progress.

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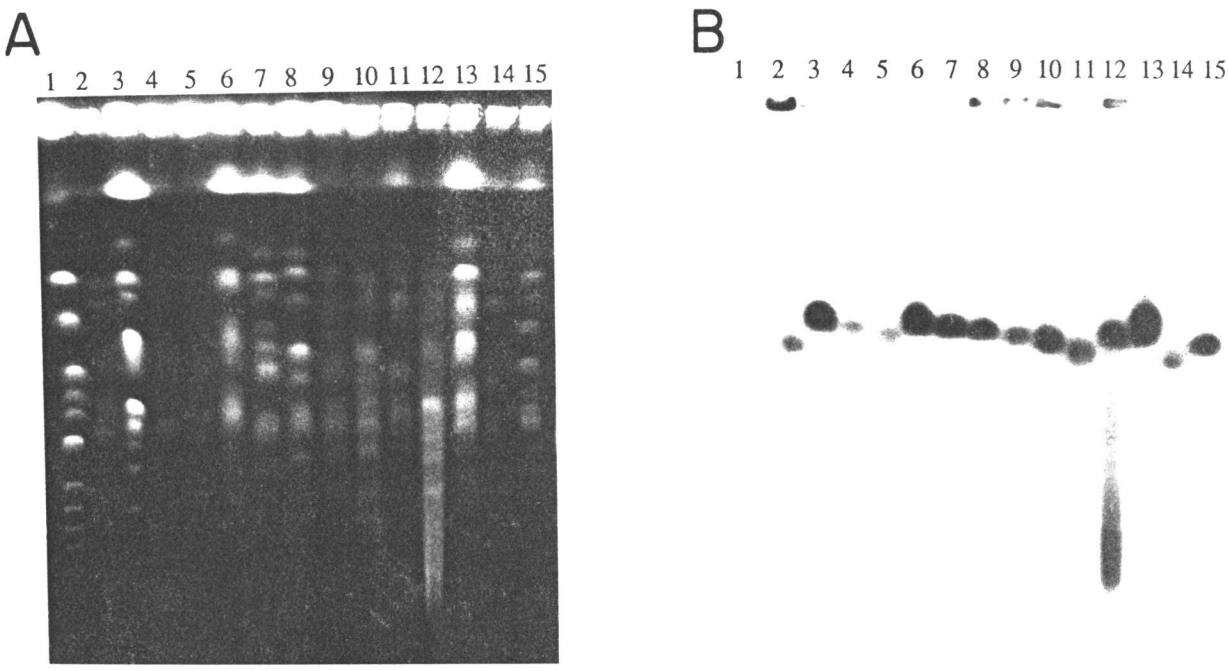

Figure. 1 A,B. Electrophoretic karyotypes of strains representing the 14 races of *U. hordei* and assignment of an anonymous fragment to a homolog in each strain. **A**) Pulsed-field gel showing chromosomes of strains of *U. hordei*. Lane 1, *S. cerevisiae* chromosomes; Lanes 2 through 15: *U. hordei* strains 1.3; 2.1a; 3.1; 4.1; 5.1; 6.2a; 7.1a;8.1a;9.1; 10.1a; 11.2a; 12.1; 13.1; 14.1a. **B**) Southern blot of chromosomes shown in panel A, probed with the insert from pOSU4182.

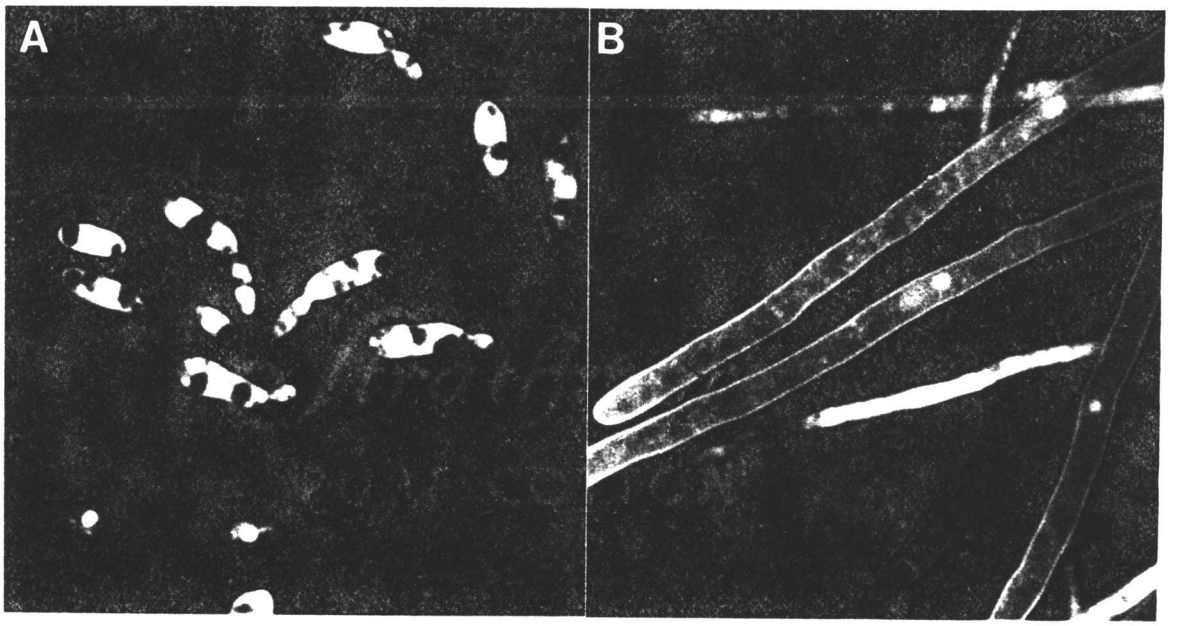

Figure. 2. Cell morphology of *U. hordei* strain 10. la-l (*fil1*-l). **A**) Wild type sporidial cells of parental strain 10. la. **B**) Filamentous mutant cells of strain 10. la-l (*fil1-1*). Cells were photographed following ethidium bromide staining.

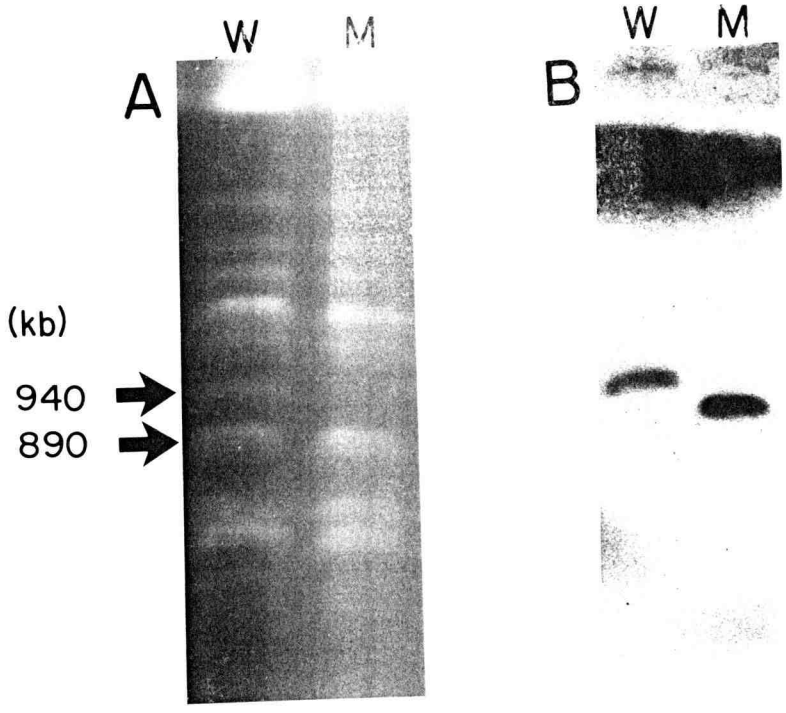

Figure. 3. Southern blot analysis revealing the fate of the 940 kb chromosome in strains having the *fil1-l* mutation. **A**) Electrophoretic karyotype of strains 10. la and 10. la-l *(fil1-l)*. **B**) Southern blot of the gel shown in panel A probed with a 940 kb chromosome-specific insert from pOSU4126.

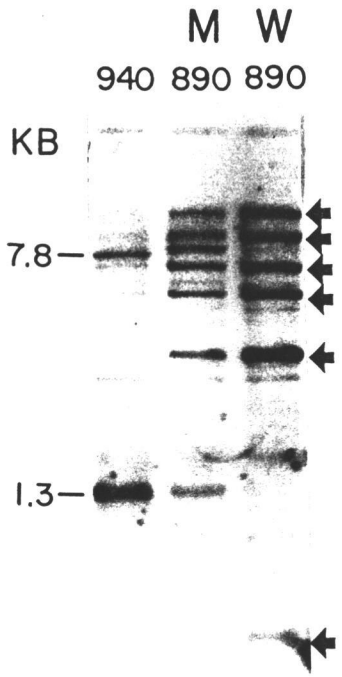

Figure. 4. Identification of telomere-containing *Bam*HI fragments from the 940 kb and 890 kb chromosome bands in strains 10. la and 10. la-l (*fil1-l*) by Southern blot analysis with a probe made from the telomere repeat of *F. oxysporum*. Lane 1, *Bam*HI fragments from the 940 kb chromosome from strain 10. la; Lanes 2 and 3, fragments from the 890 kb cluster of chromosomes from mutant (M) strain 10. la-l (*fil1-l*) and wild type (W) strain 10.1a, respectively. The positions of the 1.3 kb and 7.8 kb *Bam*HI fragments from the 940 kb chromosome that hybridized with the probe are indicated at the left margin. The fragments from the 890 kb cluster from the wild type 10. la strain are indicated by the arrows at the right margin. The mutant (M) 890 chromosome cluster (middle lane) has additional fragments of 1.3 kb and 8.1 kb. Additional faint bands detected by the probe are BamHI fragments from adjacent chromosomes which cannot be excluded when these chromosomes are removed from the gel.

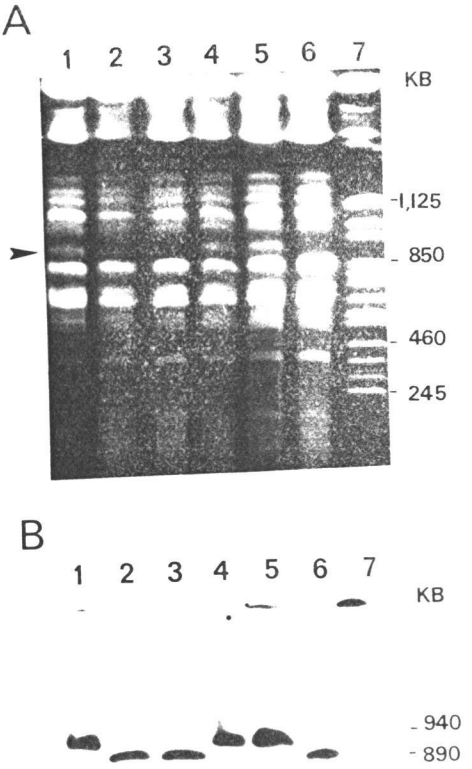

Figure. 5. Cosegregation of the 890 kb deletion chromosome and the filamentous phenotype in the progeny of an ordered tetrad. A sexually compatible wild type strain 10.2 was mated with 10.1a-1 (*fil1-1*) to obtain the tetrad. **A**) The electrophoretic karyotypes of parental strains and the four progeny strains. Lane 1, strain 10. la; Lanes 2-5, progeny strains; Lane 6, strain 10. la-l (*fil1-1*); Lane 7, Yeast chromosome size markers. **B**) The gel shown in panel A blotted and probed with the insert from pOSU4126.

Table 1. Strains and plasmids

Strains	Relevant Characteristics[a]	Reference/Source
Ustilago hordei		
8. la	19 chromosome bands, *Mat- 1*	McCluskey & Mills, 1990
10. la	21 chromosome bands, *Mat*-l mating type	McCluskey & Mills, 1990
10. la-l(*fil1-l*)	Filamentous colony morphology mutant derived from 10.l a following heatshock treatment	This study
10.2	Randomly isolated monosporidial strain, race 10 collection, *Mat-2* mating type	This study
Escherichia coli		
DH5α	F∅80d*lac*ZΔM15 Δ (*lac*ZYA-argF) U169*deo*R*rec*A1*end*A1hsdR17(r_K^-, m_K^+,)*sup*E44λ-*thi*- 1*gyr*A96*rel*A1	Bethesda Research Labs. Gathersburg, MD
Plasmids		
pCM54	Cloning vector, UARS, Ap^r Hyg^r	Tsukuda *et al,*. 1989
pUCl8, pUCl9	Sequencing and cloning vectors; Ap^r	Yanisch-Perron *et al,*. 1985
pOSU4012	1.2 kb *Eco*RI fragment from	McCluskey & Mills,1990

Table 1. (continued)

	genomic DNA of strain 8.la in pUC 18; Ap^r	
pOSU4843	1.5 kb *Bam*HI fragment from the 650 kb chromosome of strain 8. la in pCM54,Ap^r Hyg^r	This study
pOSU4062	4.4 kb *Eco*RI fragment from the 850 kb chromosme of strain 8.1a in pUCl9 Ap^r	This study
pOSU4088	5 kb *Eco*RI fragment from the 980 kb chromosome of strain 8.1a in pUCl9 Ap^r	This study
pOSU4126	2.0 kb *Bam*HI fragment from the 940 kb chromosome of strain 10.1 in pCM54; Ap^r Hyg^r	This study
pOSU4132	3.6 kb *Bam*HI fragment from the 940 kb chromosome of strain 10.1 in pCM54; Ap^r Hyg^r	This study
pOSU4135	4.0 kb *Bam*HI fragment from the 940 kb chromosome of strain 10.1 in pCM54; Ap^r Hyg^r	This study
pOSU4172	1.0 kb *Eco*RI fragment from the 200 kb chromosome of strain	This study

Table 1.(continued)

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[a]Symbols: *Mat-1* and *Mat-2* are alternate alleles at the *A* mating type locus; UARS, *Ustilago maydis* autonomous replicating sequence; Ap^r, ampicillin resistance; Hyg^r, hygromycin B resistance.

[b]pNLA17 was constructed by subcloning the $[TTAGGG]_{18}$ repeat on an *Nla*III fragment from pFOLTAR4 described in Powell and Kistler (1990).

Table 2. Assignment of anonymous DNA fragments and heterologous genes to chromosomes of Ustilago hordei.

	Strains[a]														Calculated Variability			
Probe	1.3	2.1a	3.1	4.1	5.1	6.2a	7.1a	8.1a	9.1	10.1a	11.2a	12.1	13.1	14.1a	Avg Size	SD (Kb)	SD (%)	Range (%)
pOSU4811	3150[b]	3150	3150	3150	3150	3150	3150	3150	3150	3150	3150	3150	3150	3150	3150	0	0	0
pOSU1011	1190	2850	2400	2700	3150	1300	2450	2200	2450	2200	ND[c]	2200	2200	2200	2268	540	23	86.4
pOSU4012	1290	1290	1260	1310	1290	1210	1230	1200	1260	1260	1280	1260	1260	1260	1261	31	2.4	8.75
pOSU4088	1110	960	1025	1060	1040	1060	975	1040	980	1100	ND	1075	1100	1040	1043	48	4.6	14.6
pOSU4182	1050	960	975	1060	1000	990	975	980	980	1050	ND	1075	1060	1040	1015	41	4.0	11.2
pOSU4062	910	960	940	1025	1000	990	975	920	925	890	890	890	890	920	937	45	4.8	14.4
pOSU4611	840	940	840	940	840	875	850	850	850	850	830	960	850	830	867	44	5.0	12.7
pOSU4843	700	700	720	710	700	700	710	650	720	710	700	760	710	720	707	22	3.1	15.4
pOSU4172	960	960	940	1060	1040	990	975	980	980	1050	920	1075	1060	1040	1002	50	4.9	15.5
pSF8	570	570	620	585	585	610	650	590	620	590	580	640	590	590	599	24	4.0	13.1

[a]Strains representing the 14 races of U. hordei (McCluskey & Mills 1990).

[b]Chromosome band sizes, in kilobase pairs (kb) identified by the probe.

[c]ND = Not determined.