

A *Schizosaccharomyces pombe* gene that promotes sexual differentiation encodes a helix–loop–helix protein with homology to MyoD

B.K.Benton¹, M.S.Reid and H.Okayama²

Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

¹Present address: The Rockefeller University, New York, NY 10021, USA

²Corresponding author

Communicated by P.Nurse

Nitrogen starvation of *Schizosaccharomyces pombe* induces a differentiated state in which haploid cells mate and sporulate. *esc1*⁺, a newly isolated *S.pombe* cDNA that promotes this sexual differentiation, encodes a putative transcription factor with a helix–loop–helix (HLH) motif similar to those of the human MyoD and Myf-5 myogenic differentiation inducers. Disruption of *esc1*⁺ in wild-type cells leads to a decrease in the efficiency of sexual conjugation, an early step in sexual differentiation. The disruption was also able partially to substitute for cAMP, an inhibitor of differentiation, to suppress the lethal, constitutive differentiation induced by the *pat1* mutation. Conversely, overexpression of this cDNA conferred partial resistance to cAMP-mediated inhibition of differentiation. Transcription from this novel gene was induced early in response to nitrogen starvation and is largely independent of the *ste11*⁺ gene product, which is required for the differentiation-specific expression of other genes. Thus, this MyoD/Myf-5-like protein appears to promote sexual differentiation by modulating responses to decreases in cAMP, a part of the nitrogen starvation signal that induces differentiation. Key words: differentiation/helix–loop–helix/MyoD/*pat1*/*Schizosaccharomyces pombe*

Introduction

Differentiation is the generation of cellular diversity by asymmetric cell divisions that produce daughters with different cell fates, and this process has been conserved from prokaryotes to humans (Horvitz and Herskowitz, 1992). In the fission yeast *Schizosaccharomyces pombe*, differentiation involves the sexual process by which vegetatively dividing haploid cells are induced by nitrogen starvation to exit the cell cycle, mate and sporulate (Egel and Egel-Mitani, 1974, see Egel, 1989). *S.pombe* cells differentiate to either of two distinct mating types, *h*⁺ or *h*⁻ (alternatively called Plus [P] or Minus [M]). In an *h*⁹⁰ homothallic strain, the population contains cells of both mating types and, therefore, can mate with itself. In contrast, *h*⁺ or *h*⁻ heterothallic strains contain cells of only one mating type and can only mate with strains of the opposite mating type. Sexual differentiation can be divided into two stages. The early stage involves exit from the cell cycle at G₁, induction of mating competency, and sexual conjugation of cells of opposite mating type to

form a diploid zygote. Up to this time the cells can re-enter the cell cycle as diploids or haploids upon addition of a nitrogen source, though the diploids are unstable and easily commit to the late stage of differentiation. This late stage of differentiation involves an irreversible commitment of the zygote to pre-meiotic DNA synthesis, meiosis, and sporulation.

This sexual differentiation is controlled by a complex regulatory cascade (Egel *et al.*, 1990), but the final commitment to meiosis and sporulation is primarily governed by the antagonistic actions of two gene products, Pat1 and Mei2. Pat1 (also known as Ran1) is a serine/threonine kinase (McLeod and Beach, 1988) that negatively regulates differentiation (Beach *et al.*, 1985; Iino and Yamamoto, 1985). This negative regulation involves transcriptional repression of genes at the mating type locus (Nielsen and Egel, 1990) and probably a post-transcriptional repression of Mei2 (McLeod and Beach, 1988), which is necessary and probably sufficient for induction of meiosis (Watanabe *et al.*, 1988). Mutations in *pat1*⁺ cause the cell to differentiate constitutively, i.e. *pat1* mutants undergo sporulation regardless of ploidy, nitrogen conditions or pheromone cross-induction by a mating partner (Beach *et al.*, 1985; Iino and Yamamoto, 1985). These cells form aneuploid spores because they are not required to undergo meiosis from a diploid state and thus the mutation is lethal. In contrast, Mei2 is a positive effector of differentiation as mutations in *mei2*⁺ are sterile, i.e. they cannot form spores (Bresch *et al.*, 1968). Mutations in *mei2*⁺ also rescue the lethal phenotype of *pat1*, which demonstrates that Mei2 is required for the constitutive differentiation induced by the *pat1* mutation and works downstream of Pat1 in the sexual differentiation cascade (Beach *et al.*, 1985). Currently, the biochemical function of Mei2 remains elusive.

When *S.pombe* cells are induced to differentiate by nitrogen starvation, intracellular concentrations of cAMP decrease (Fukui *et al.*, 1986; Maeda *et al.*, 1990) and, conversely, differentiation can be inhibited by increasing intracellular cAMP (Beach *et al.*, 1985; Maeda *et al.*, 1990). This initial nitrogen starvation signal and its concomitant decrease in intracellular cAMP presumably starts the regulatory cascade that gradually inactivates Pat1 kinase and that activates Mei2 during mating (McLeod and Beach, 1988; Beach *et al.*, 1985; Nielsen and Egel, 1990). The gradual inactivation of Pat1 ensures that sexual conjugation to form a diploid zygote proceeds before meiosis and sporulation and indeed, partial inactivation of Pat1 allows conjugation, but not sporulation (Nielsen and Egel, 1990). An important mediator between the initial nitrogen signal and activation of this regulatory cascade appears to be the Ste11 transcription factor, which is required for sexual differentiation (Sipiczki, 1988; Kitamura *et al.*, 1990; Sugimoto *et al.*, 1991). Ste11⁺ transcription is induced by nitrogen starvation and repressed by exogenous cAMP addition and Ste11

is required to induce the transcription of many other genes during differentiation, including that of *mei2*⁺ (Watanabe *et al.*, 1988; Sugimoto *et al.*, 1991).

Although many gene products involved in the regulatory cascade of sexual differentiation in *S.pombe* have been isolated and characterized, it is still unclear exactly how they exert their effects on each other. In order to isolate new regulators of sexual differentiation, we have used an expression cloning system to isolate novel extragenic *S.pombe* cDNAs that rescue the lethality of a *ts pat1* mutation. Here we characterize one such cDNA, *esc1*⁺ (early in sexual conjugation), which influences the early stages of sexual differentiation and codes for a putative transcription factor with a helix-loop-helix (HLH) motif similar to that of human MyoD and Myf-5, two genes that can induce mammalian myogenesis *in vitro* (Braun *et al.*, 1989).

Results

Isolation of a novel extragenic suppressor of *pat1*

In order to isolate novel factors involved in the sexual differentiation of *S.pombe*, *h⁻ pat1-114^{ts}* cells, which exit the cell cycle at G₁ and undergo constitutive sexual differentiation at the non-permissive temperature (Beach *et al.*, 1985; Iino and Yamamoto, 1985), were transformed with an *S.pombe* cDNA library (Tanaka *et al.*, 1992). One of the rescued cDNAs that was able to suppress *pat1-114* at the non-permissive temperature was *esc1*⁺.

One clue that *esc1*⁺ was an extragenic suppressor of the *pat1* mutation was that it could not fully complement that mutation. When the *esc1*⁺ cDNA, transcribed from the SV40 promoter (pcD2 and pcU; Nagata *et al.*, 1991), was transformed into the *pat1* mutant, it could complement at 35°C, but not at 37°C (pcD2esc1, pcUesc1; Figure 1). In contrast, the *pat1*⁺ cDNA transcribed from the same promoter (pcUpat1) could complement up to 37°C. As cDNAs under the SV40 promoter have been shown to be strongly transcribed in *S.pombe* cells (Jones *et al.*, 1988;

B.K.Benton, unpublished data), these differences were not due to inefficient transcription. The ability of *esc1*⁺ to rescue *pat1* was specific as pcD2esc1 was unable to rescue *ts* alleles of *cdc10*, *cdc2* or *cdc25* (data not shown). Additionally, while expression of the *pat1*⁺ cDNA efficiently prevented sporulation of *h⁻ pat1-114* cells under nitrogen-rich conditions, the *esc1*⁺ cDNA was unable completely to prevent sporulation of these same cells under nitrogen-rich conditions as colonies of cells expressing the plasmid had 20–30% spore asci on minimal media with nitrogen. Thus, the *esc1*⁺ cDNA was not behaving like the *pat1*⁺ cDNA and was a candidate for an extragenic suppressor of *pat1*.

Sequencing of the *esc1*⁺ full-length cDNA revealed that indeed it was an extragenic suppressor of *pat1*. The *esc1*⁺ cDNA contained a 1139 bp 5' untranslated region, a 1239 bp open reading frame (ORF) encoding 413 amino acids from the putative initiating methionine codon, and a 370 bp 3' untranslated region (Figure 2). The long 5' untranslated region contains no sustained reading frames and is not required for *pat1* complementation (pcUesc1, Figure 1). However, it may serve in some yet undiscovered translational regulation. The first 200 amino acids of the ORF are serine/threonine rich (40%), which are followed by a 77 amino acid glutamine-rich stretch (19%). These motifs are both indicative of the transcriptional activator region of some transcription factors (Courey *et al.*, 1989; Theill *et al.*, 1989). An amino acid homology search revealed that the carboxyl terminus contains a region with similarity to the basic-HLH region of the human MyoD (31% identity; 70% similarity) and Myf-5 (26% identity; 69% similarity) myogenic inducers (Figure 3). This region in MyoD mediates dimerization with other HLH proteins and binds to specific DNA elements (Davis *et al.*, 1990). However, there is no homology between *Esc1* and these myogenic inducers outside the HLH domain, indicating that they are not exact homologs. Also, immediately following the HLH region of *Esc1* is a putative leucine zipper of three heptad leucine repeats. These leucine zippers are found either by

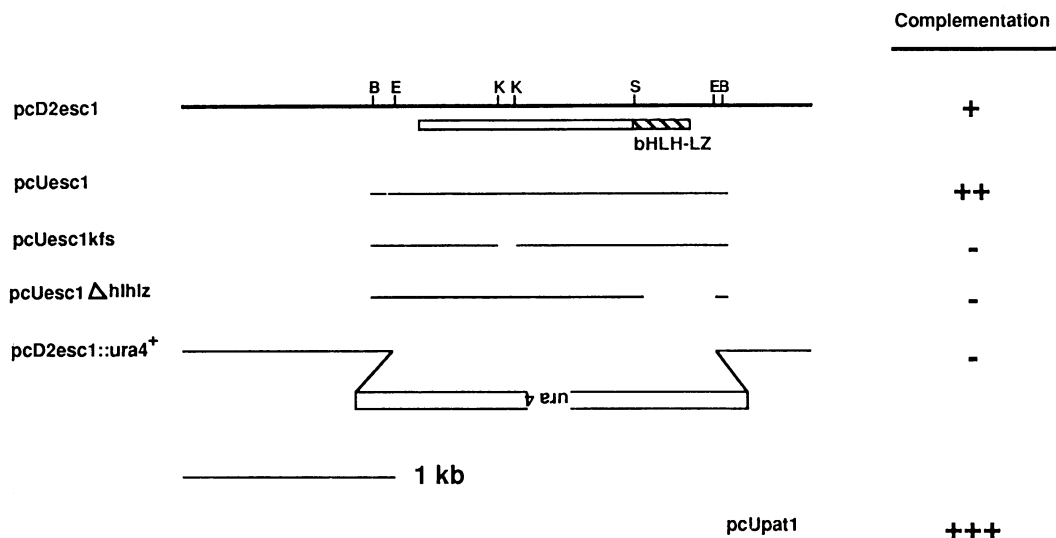


Fig. 1. Restriction map and complementation of *pat1* by various *esc1* constructs. Only the *esc1* inserts are shown. The ORF of 413 amino acids is depicted below the restriction map with the basic-HLH/leucine zipper region stippled (bHLH-LZ). The upside down *ura4* in pcD2esc1::ura4⁺ denotes the *ura4*⁺ gene in the opposite transcriptional orientation of that of the *esc1* cDNA. Complementation scores are as follows: -, no complementation at or above 34°C; +, weak to moderate complementation at 35°C, none at 37°C; ++, moderate complementation at 35°C, none at 37°C; +++, strong complementation at 35°C, moderate complementation at 37°C. B, *Bam*HI; E, *Eco*RV; S, *Sna*BI; K, *Kpn*I.

themselves such as in Fos and Jun or in combination with an HLH motif such as in the Myc family and also appear to mediate dimerization specificity (see Busch and Sassone-Corsi, 1990). The importance of the HLH-leucine zipper domain for Escl function was also demonstrated as pcUesc1Δhlhlz, which is missing this region, could not complement *pat1* (Figure 1).

Disruption of *esc1*⁺ lowers conjugation efficiency and can partially substitute for cAMP to inhibit differentiation of *pat1* mutants

To characterize further the role of Escl in the sexual differentiation of *S.pombe*, we isolated strains that contained a disruption of the *esc1*⁺ gene. A non-functional 3.3 kb

disrupted *esc1* cDNA fragment (Figure 1) was used for one-step gene replacement to generate such strains (Grimm *et al.*, 1988; Figure 4). Disruption of the *esc1*⁺ gene was not lethal and the growth rates of the *esc1* disruptants were similar to strains that are *esc1*⁺ (data not shown).

However, because the *esc1*⁺ cDNA inhibited sexual differentiation in a *pat1* mutant, we examined the effect of the *esc1* disruption on sexual differentiation (Figure 5). Cells of each mating type were grown separately in nitrogen-rich liquid media (SSL+N) and then mixed together in nitrogen-free liquid media (SSL-N) to induce sexual differentiation (Egel and Egel-Mitani, 1974). When cells of opposite mating types containing the *esc1* disruption were grown to high densities in SSL+N (1.0×10⁷/ml), mixed and shifted to

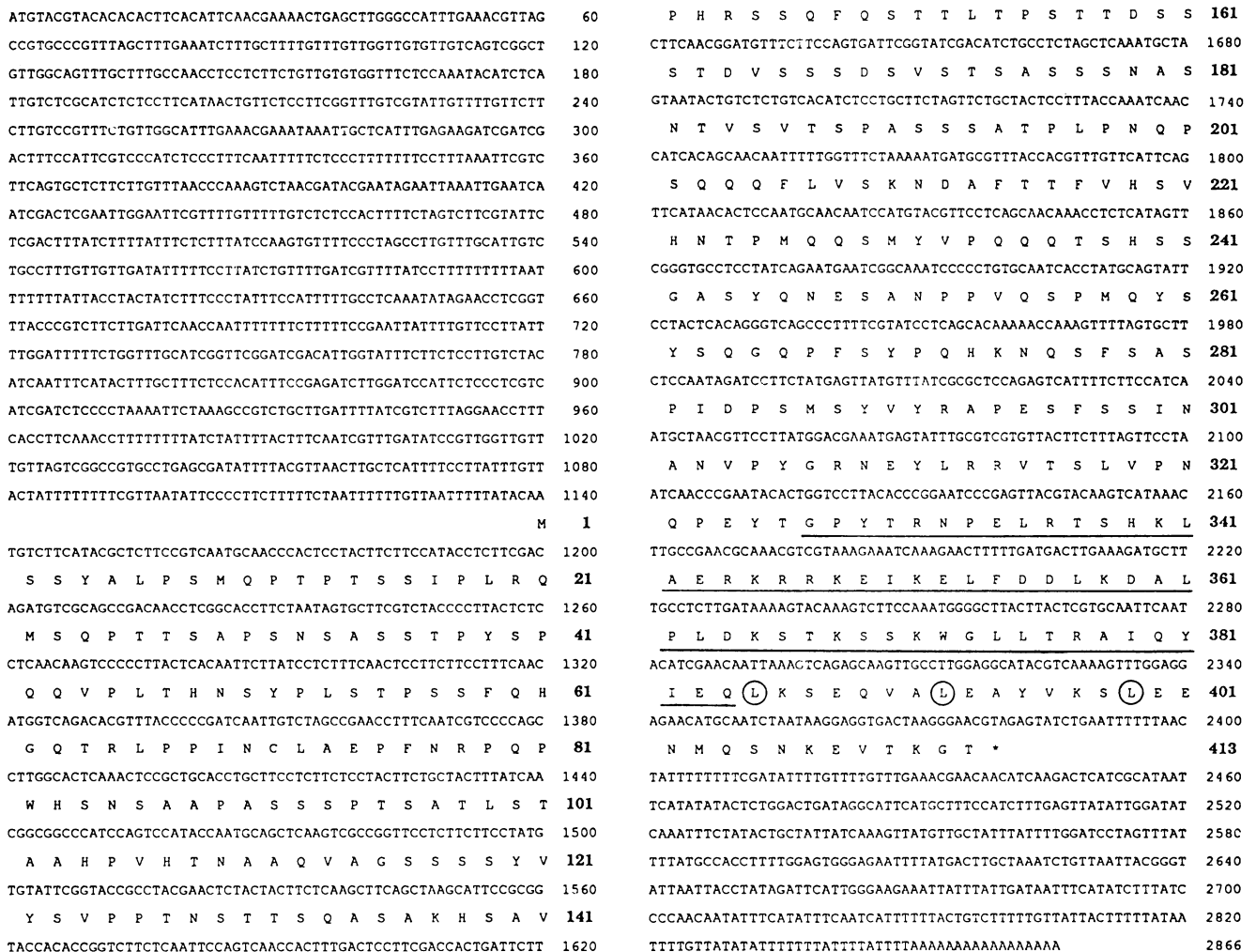


Fig. 2. Nucleotide and deduced amino acid sequence of the *esc1*⁺ cDNA. Normal contrast numbers are nucleotide positions and bold contrast numbers are amino acid positions. The region with homology to the basic-HLH region of MyoD and Myf-5 is underlined. The leucines in the leucine heptad repeat are circled.

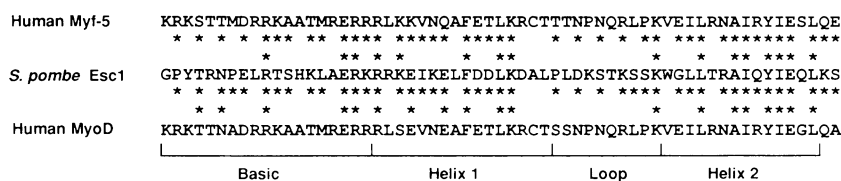


Fig. 3. Amino acid homology among the deduced amino acid sequences of *S.pombe* Esc1, human MyoD and human Myf-5. Two asterisks indicate exact identities between the human proteins and Esc1 and one asterisk indicates similarities. The basic, helix 1, loop and helix 2 regions of the HLH proteins are shown.

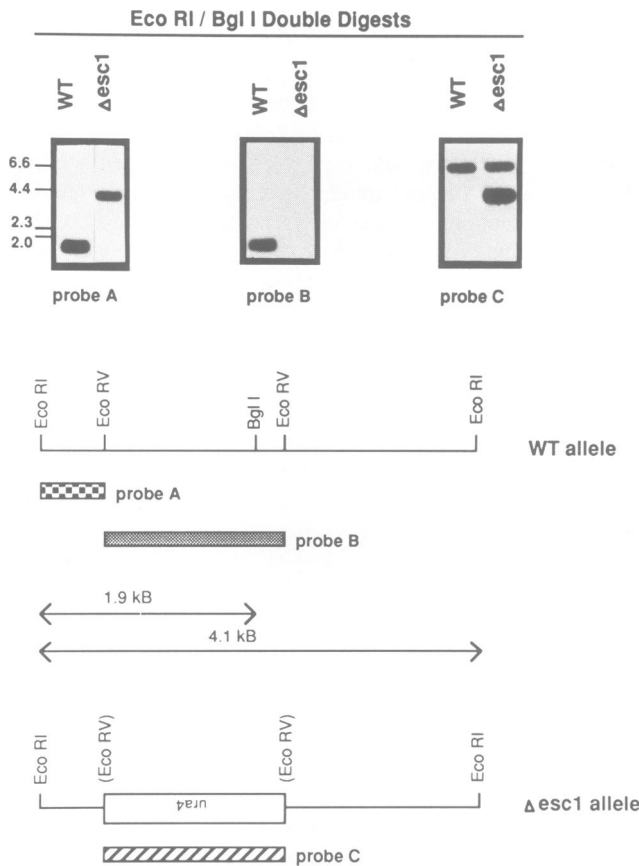


Fig. 4. Disruption of the endogenous *esc1*⁺ gene in *S.pombe*. The disrupted *esc1* allele described in Figure 1 was excised from the pcD2 vector and was used for one-step gene disruption of diploids. One candidate diploid was sporulated and Southern analysis of a viable haploid *ura4*⁺ cell strain (SB1) was performed. Probe A is specific for both the endogenous and disrupted alleles, probe B is specific for the endogenous allele since it covers the region deleted in *esc1::ura4*⁺ and probe C is the 1.8 kb *ura4*⁺ *Hind*III fragment which is specific for the disrupted allele. The higher mol. wt band in both lanes that hybridized with probe C represents the *Eco*RI fragment of the endogenous *ura4*⁺ gene (Grimm *et al.*, 1988).

SSL-N, they exhibited a modest decrease in conjugation efficiency when compared to a control mating of *h*⁺ and *h*⁻ *esc1*⁺ cells grown to the same density in SSL+N before mixing (Figure 5). As a negative control *h*⁻ *esc1*⁺ cells were shifted to SSL-N, which did not conjugate because all cells are of the same mating type (data not shown). Despite its inhibition of the early stage of differentiation manifested through the conjugation event, the *esc1* disruption appeared not to have an effect on sporulation—the final stage of sexual differentiation. When the percentage of asci to total conjugates (diploids and asci) was determined at 24 and 48 h for the experiment in Figure 5, the mating of *esc1* disruptant strains and *esc1*⁺ strains showed similar degrees of sporulation (84 and 86% sporulation respectively at 24 h, 97 and 99% sporulation respectively at 48 h). Also, sporulation of diploids (representing a post-conjugation event) containing either a heterozygous or homozygous disruption of *esc1* was not different from that of an *esc1*⁺/*esc1*⁺ diploid (data not shown).

Because disruption of *esc1*⁺ inhibits the early stage of sexual differentiation, we investigated whether the disruption could rescue the differentiation-induced lethality of the *pat1*

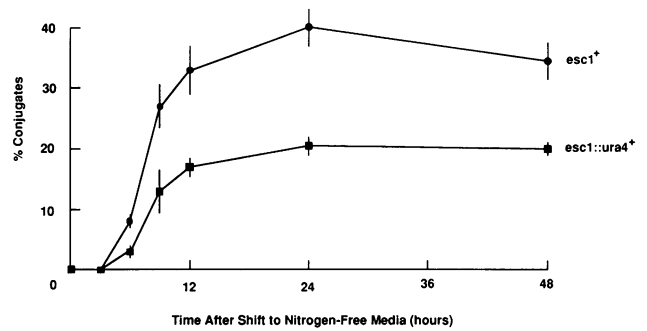


Fig. 5. Conjugation efficiency of the *esc1* disruptants. SB21, SB74, SO3 and SO4 (Table I) were grown separately in SSL+N to 1.0×10^7 /ml. SB21 and SB74 (*esc1::ura4*⁺) were mated and SO3 and SO4 (*esc1*⁺) were mated in SSL-N and the percentage of conjugates calculated at the indicated times as described in Materials and methods. Each determination was done in triplicate and error bars are \pm one standard error of the mean.

mutant. An *esc1::ura4*⁺ *pat1-114*^{ts} double mutant was tested for temperature sensitive lethality at 35°C. The double mutant behaved like the *pat1* single mutant as both were unable to grow at 35°C (Figure 6, 0+0). The control strains, the *esc1* disruptant and a *pat1*⁺ *esc1*⁺ (wild-type) strain, both grew at 35°C. However, it is still conceivable that Esc1 could be part of the nitrogen starvation signal that works early in differentiation, but by itself is unable to rescue fully the *pat1* lethality. Data suggest that decreases in cAMP levels at least in part mediate the nitrogen starvation signal to differentiate and high levels of exogenous cAMP or mutants that increase intracellular levels of cAMP block the constitutive differentiation induced by *pat1* and rescue its lethality (Beach *et al.*, 1985; Maeda *et al.*, 1990). If Esc1 is part of the nitrogen starvation signal that modulates decreases in cAMP with onset of differentiation, then disruption of *esc1*⁺ may inhibit signal transduction and, thus, *esc1* disruptants would require less extracellular cAMP to prevent onset of differentiation. This was indeed the case. When cells were streaked on plates with 1 mM caffeine (a phosphodiesterase inhibitor that increases the intracellular cAMP concentrations; Beach *et al.*, 1985) and varying concentrations of cAMP, the double mutant required at least 25-fold less cAMP than the *pat1* single mutant (Figure 6). Cells wild-type at both the *esc1*⁺ and *pat1*⁺ loci and the *esc1::ura4*⁺ strain grew at all concentrations of cAMP supplemented with caffeine at 35°C. If caffeine is not added to the media, the *pat1* single mutant requires 3 mM cAMP for efficient growth, while the *pat1 esc1* double mutant requires only 0.3 mM cAMP (data not shown). Thus, the *pat1-114* mutation is rescued by lower extracellular concentrations of cAMP in an *esc1::ura4*⁺ background as compared to an *esc1*⁺ background. This is consistent with the hypothesis that the disruptant does not require as much cAMP to suppress differentiation because the signal involving decreases in cAMP with onset of differentiation is impaired.

Overexpression of Esc1 increases resistance to cAMP-mediated inhibition of conjugation

To determine if overexpression of Esc1 could promote differentiation in wild-type cells, *h*⁺ *esc1*⁺, or *h*⁻ *esc1*⁺ strains were transformed with a plasmid containing the 1.8 kbp *Bam*HI fragment of the *esc1*⁺ cDNA subcloned under the SV40 promoter (pcUesc1). These transformants were

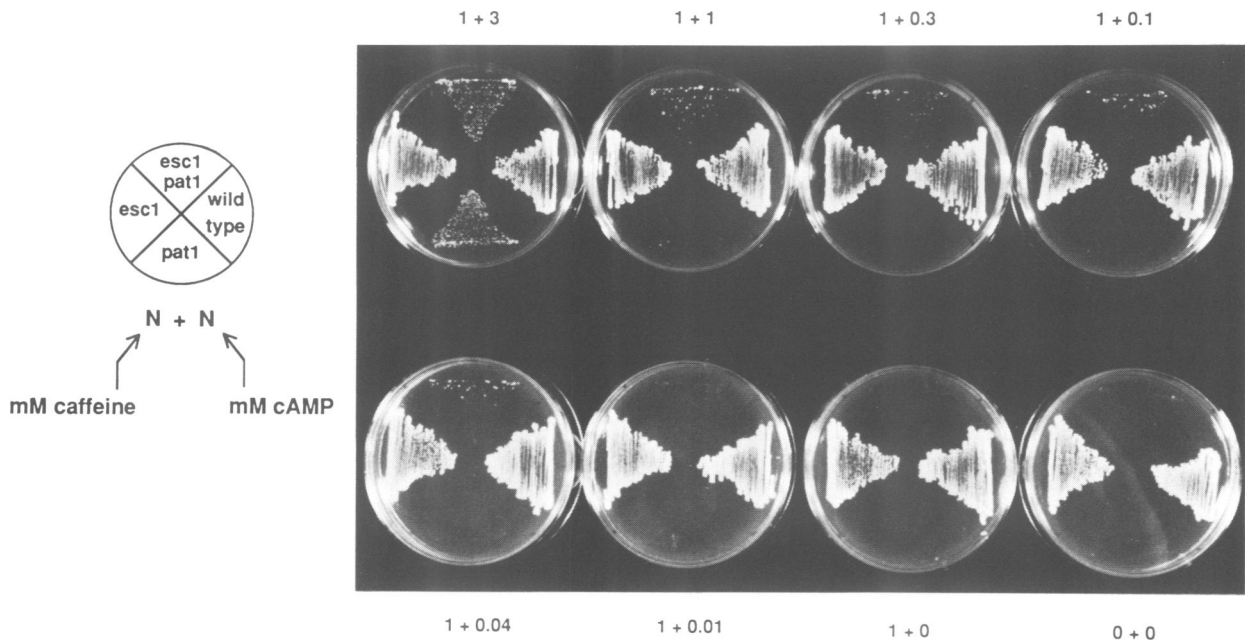


Fig. 6. The ability of the *esc1* disruption to rescue the *pat1* phenotype. $h^- leu1$ (wild-type), $h^- pat1-114 leu1$ (*pat1*), SB21 (*esc1*) and SB30 (*esc1 pat1*) were streaked out on MMA (nitrogen-rich) plates supplemented with 50 $\mu\text{g/ml}$ leucine and caffeine and cAMP at the concentrations indicated. The plates were incubated at 35°C for 3 days.

then used to investigate further the role of Esc1 in conferring sensitivity to cAMP-mediated inhibition of differentiation. Cell strains of opposite mating types either transformed with pcUesc1 or the pcU vector alone were grown separately in SSL+N overnight at 27°C and then mixed and shifted to SSL-N at 27°C. If overexpression of *esc1+* behaves conversely to disruption of *esc1+*, then we would expect that the overexpresser would differentiate more readily at higher concentrations of cAMP since the constitutive expression of the *esc1+* cDNA under the SV40 promoter could override the inhibition by exogenous cAMP addition. In three matings involving six independent pcUesc1 transformants, the cells were more resistant to cAMP-mediated inhibition of differentiation (Figure 7). While the concentration of cAMP in nitrogen-free media had to be <10 μM before control cells transformed with the pcU vector alone were able to conjugate significantly, the cells transformed with pcUesc1 could conjugate at cAMP concentrations between 10 and 40 μM . Even when the levels of cAMP dropped to 6 μM the pcUesc1 overexpressers still conjugated significantly more sufficiently than the control transformants ($P < 0.05$). These results are in complete agreement with the observation that the *esc1* disruptant is more sensitive to cAMP-mediated inhibition of differentiation in a *pat1* background. These data suggest that Esc1 is modulating some part of the nitrogen starvation signal, presumably manifested through decreases in intracellular cAMP, that leads to sexual differentiation. However, while Esc1 overexpression can override cAMP-mediated inhibition of differentiation, it has little effect on inhibition mediated by addition of an exogenous nitrogen source, $(\text{NH}_4)_2\text{SO}_4$ (data not shown). This suggests that the differentiation signal mediated by Esc1 is only part of the nitrogen starvation signal and the modulation of cAMP-responsiveness is insufficient to override the growth promoting effect of exogenous nitrogen (see Discussion).

***Esc1+* transcription is induced quickly after nitrogen starvation**

To examine further the role of Esc1 in differentiation, the level of *esc1+* steady-state transcripts was determined in cells which were induced to differentiate by nitrogen starvation. The two *esc1+* transcripts of ~3.0 kb and ~1.8 kb were strongly induced by nitrogen starvation after either h^- or h^+ *esc1+* cells alone were shifted to SSL-N, indicating that *esc1+* induction is pheromone-independent and is not mating type-specific (Figure 8A). Similarly, when h^+ and h^- *esc1+* cells were grown separately in SSL+N and then mixed in SSL-N, the *esc1+* transcripts were induced rapidly and strongly (Figure 8B). Thus, *esc1+* responds like other differentiation-specific genes such as *mei2+* and *stell1+* upon nitrogen starvation (Watanabe *et al.*, 1988; Sugimoto *et al.*, 1991). It has been previously shown that the increased transcription of many genes during differentiation requires the Ste11 gene product. However, Figure 8B shows that the *esc1+* gene is turned on somewhat before *stell1+* transcription is induced. In addition, *esc1+* is still induced in the absence of Ste11 function (Figure 8B, $h^{90} stell1 leu1$ cells) indicating that Ste11 is not required for *esc1+* transcription, although the level of induction is slightly lower. These data suggest that the *esc1+* transcription is turned on as a direct consequence of nitrogen starvation and is largely independent on the Ste11 transcription factor for its induction.

Discussion

From the above data it is apparent that *esc1+* is involved in the sexual differentiation process of *S.pombe* and is the first yeast gene isolated that encodes an HLH protein involved in differentiation. Although disruption of *esc1+* did inhibit conjugation, it is not the classical sterile mutant as spores can eventually form. However, *esc1+* gene function

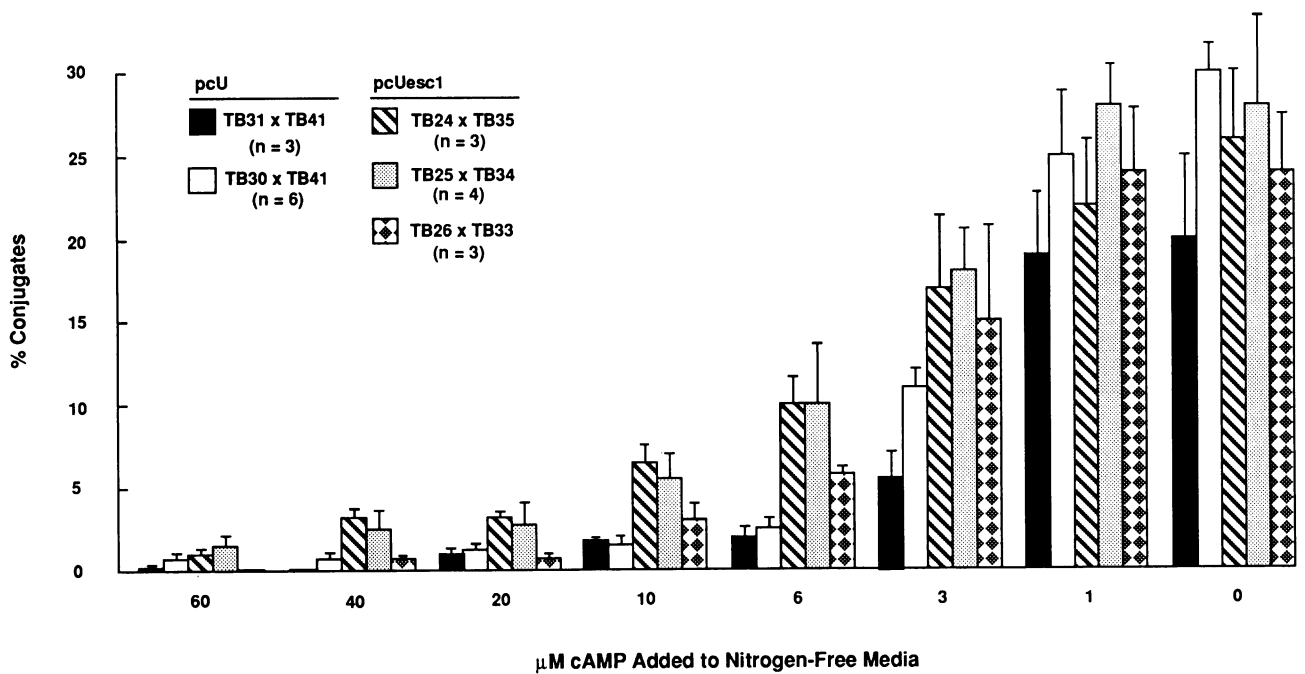


Fig. 7. Resistance to cAMP-mediated inhibition of differentiation of cell strains overexpressing Esc1. Cells were grown separately in SSL+N, mixed, shifted to SSL-N with the indicated concentrations of cAMP and incubated for 28 h at 27°C. Percent conjugates were calculated as in Figure 5. Error bars are one standard error of the mean. The number of determinations (*n*) for each mating is in parenthesis.

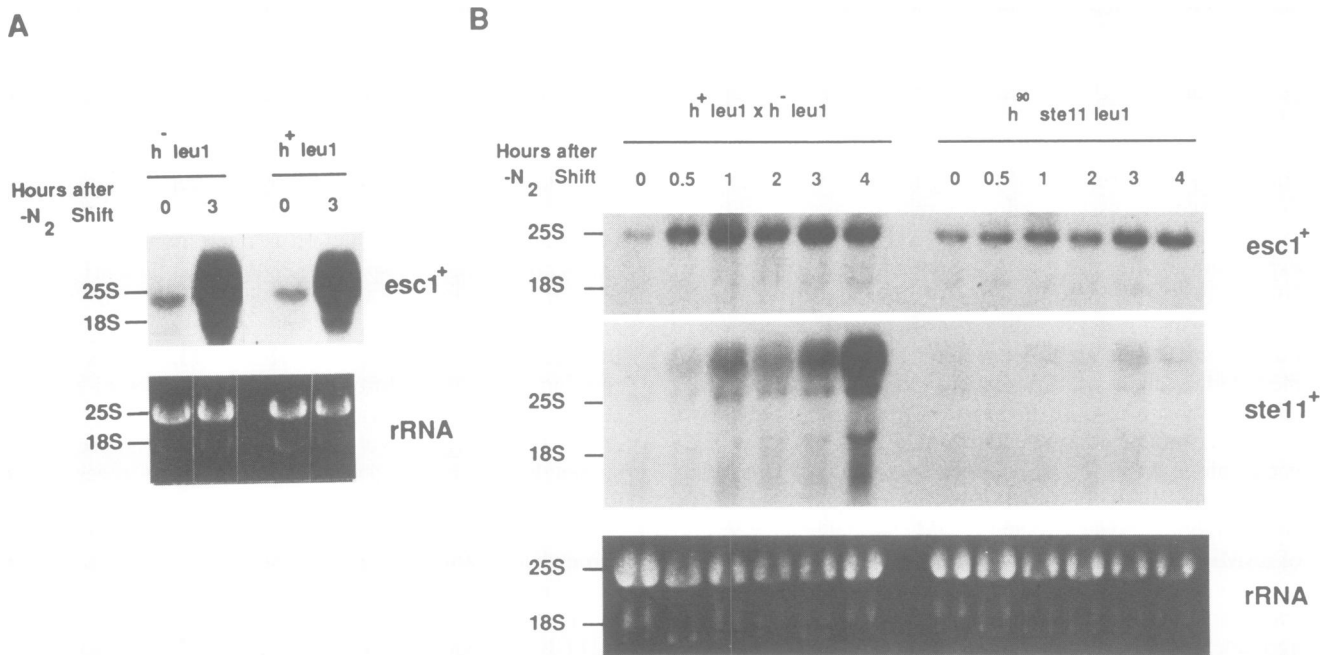


Fig. 8. (A) Expression of *esc1+* RNA in heterothallic *h+* and *h-* cells starved for nitrogen. The 2.0 kb *Bam*HI fragment of *esc1+* hybridizes to two mRNAs—a major 3.0 kb RNA and a minor 1.8 kb mRNA. Below the autoradiograph is the ethidium bromide-stained gel before transfer. The 28S rRNA is 3.3 kb and the 18S rRNA is 1.7 kb as determined by mol. wt standards. (B) Time course of *esc1+* and *ste11+* induction in mating of SO3 and SO4 cells and of *h⁹⁰ ste11 leu1* (C756-1A) after nitrogen starvation. The 1.4 kb *Pvu*II fragment of *ste11+*, covering the ORF, hybridizes to three RNAs as reported previously (Sugimoto *et al.*, 1991). The same membrane was probed with the *esc1+* probe, stripped and re-probed with the *ste11+* probe.

could be redundant in the *S.pombe* genome and, thus, disruption of only one *esc1+*-like gene might give a partial phenotype. If Esc1 is analogous to MyoD, then one might expect that another dimerization partner involved in conjugation may be sufficient by itself for inefficient conjugation (Lassar *et al.*, 1991). In contrast to its effect

on early stages of sexual differentiation, Esc1 did not appear to have an effect on sporulation, a later stage of differentiation.

The data presented suggest that Esc1 is modulating the ability of the cell to differentiate in response to the nitrogen starvation signal and in particular, in response to decreases

in cAMP. *Esc1*⁺ is one of the first, if not the first, gene induced by nitrogen starvation. Furthermore, disruption of *esc1*⁺ affected the conjugation efficiency of the cells in response to nitrogen starvation and the disruption and overexpression of *esc1*⁺ affected the cell's sensitivity to cAMP-mediated inhibition of differentiation. Since cAMP is probably one mediator of the nitrogen starvation signal (Beach *et al.*, 1985; Fukui *et al.*, 1986; Maeda *et al.*, 1990), these data suggest that *Esc1* is modulating signal transduction from the nitrogen starvation stimulus to the onset of differentiation. Possible mechanisms for the effect of *Esc1* in response to nitrogen starvation are that *Esc1* induces a decrease in intracellular cAMP or that it inactivates cAMP-dependent protein kinase. However, neither of these seem likely. The *esc1* disruptant (which partially substitutes for cAMP) still became 'wee' in stationary phase (data not shown), which is a general response to nutrient deprivation (Fantès and Nurse, 1977). In contrast, inactivating mutations in either the *S.pombe* cAMP-dependent protein kinase regulatory subunit or cAMP phosphodiesterase, both of which result in a constitutively activated cAMP signal, cause the cells to become elongated when starved (DeVoti *et al.*, 1991). This suggests that *Esc1* is working either downstream or independent of cAMP-dependent protein kinase. However, while *Esc1*⁺ overexpressers could overcome cAMP-mediated inhibition of differentiation, they could not overcome the same inhibition induced by an exogenous nitrogen source. This indicates the inhibition due to addition of nitrogen is not due only to the concomitant increase in intracellular cAMP levels that is presumed to occur (Fukui *et al.*, 1986; Maeda *et al.*, 1990). The data here are consistent with a model in which the nitrogen starvation signal pathway contains two rate-limiting branches. One branch modulates decreases in cAMP with onset of differentiation and includes *Esc1*. The other branch is dependent on nitrogen starvation, but independent of cAMP levels. Thus, when the *esc1*⁺ overexpressers are only partially starved for nitrogen their ability to differentiate is still dependent on the cAMP-independent pathway and *esc1*⁺ overexpression cannot overcome differentiation inhibition by nitrogen. However, when the cells are completely starved for nitrogen in the presence of exogenous cAMP, then the cAMP-independent pathway is fully activated and differentiation becomes dependent on the balance between levels of cAMP and *Esc1*. In the *esc1*⁺ overexpressers, more *Esc1* is present in the cell and thus can better overcome cAMP-mediated inhibition of differentiation.

Previously, it was shown that nitrogen starvation, probably through a decrease in cAMP, induced the *Ste11* gene product and that the increase of other differentiation-specific genes, including *mei2*⁺, was mediated through *Ste11* (Watanabe *et al.*, 1988; Sugimoto *et al.*, 1991). Moreover, since the *ste11* mutation, like *mei2*, can also suppress the *pat1* mutation completely (Watanabe *et al.*, 1988; Sipiczki, 1988; Kitamura *et al.*, 1990) and since overexpression of *ste11*⁺ under a constitutive promoter can induce differentiation independent of nutritional status in the presence of mating factors (Sugimoto *et al.*, 1991), it suggests that *Ste11* is necessary and sufficient to mediate the nitrogen starvation signal into a differentiation response. However, *Esc1* is also required for efficient conjugation. Also, *esc1*⁺ transcription is largely independent of *Ste11* and occurs at the same time

if not earlier than *ste11*⁺ transcription. Possibly, *Esc1* may work together with *Ste11* to induce differentiation. When cells are starved for nitrogen, *ste11*⁺ and *esc1*⁺ transcription is rapidly induced. *Ste11* then induces transcription of many differentiation-specific genes which promote conjugation and meiosis and repress *Pat1* function. As *Esc1* is a putative transcription factor, it may modulate *Ste11*-controlled differentiation by either complexing with *Ste11* to make a more efficient transcription factor complex or inducing transcription of genes that may increase the *Ste11* transcriptional efficiency, or inhibit *Pat1* repression of differentiation. Indeed, preliminary evidence suggests a dysregulation of *ste11*⁺ and *mei2*⁺ transcription during nitrogen starvation in the *esc1* disruptants (B.K.Benton, unpublished data). Although the *Ste11* transcription factor is related to the HMG group of transcription factors (Sugimoto *et al.*, 1991) that probably do not dimerize to HLH proteins, it is not hard to envision that a *Ste11* – *Esc1* responsive promoter could have separate elements that bind each of these different transcription factors.

It is still unclear why overexpression of the *esc1*⁺ cDNA, whose gene disruption inhibits an early stage of differentiation, should be able partially to prevent the constitutive differentiation of the *pat1* mutant. One possible explanation is that *Esc1* has a bifunctionality that is also regulated by nitrogen starvation. In the presence of nitrogen, overexpression of *Esc1* may be growth promoting. In support of this, the ability of *esc1*⁺ to rescue *pat1* is 2- to 3-fold lower on media with low levels of a nitrogen source compared to the ability of the *pat1*⁺ cDNA to rescue *pat1* on the same media (data not shown). In a wild-type background, this growth promoting activity may be masked by the powerful growth promoting effects of *Pat1*. However, in a mutant with an inactive *Pat1* kinase, this growth promoting activity from constitutive *Esc1* overexpression is no longer masked and is sufficient to partially rescue *pat1*. This growth-promoting effect is specific for the *pat1*⁺ pathway as overexpression of *esc1*⁺ could not rescue *cdc25*, *cdc10* and various mutant alleles of *cdc2*. When the cells are starved for nitrogen, the nitrogen starvation signal may then change the function of *Esc1* so that it becomes a differentiation inducer as discussed above.

Finally, the requirement of the *Esc1* HLH motif for function and its structural similarity with the HLH motif of *MyoD* suggests that a common feature of the differentiation program has been conserved from yeast to man. The basic-HLH region of *MyoD* is both necessary and sufficient to induce differentiation *in vitro* (Tapscott *et al.*, 1988). The helices of the HLH region, which mediate dimerization, and the basic region, which is a DNA binding domain, are both required for transcriptional activation by *MyoD* (Davis *et al.*, 1990). The strongest homology within the HLH region among *Esc1*, *Myf-5* and *MyoD* is within the two helices while some divergence occurs in the loop. However, *MyoD* has been shown to tolerate numerous alterations within the loop region without affecting its activity (Davis *et al.*, 1990). Alternatively, *Esc1* could be acting molecularly like *Id*, a negative regulator of HLH proteins (Benezra *et al.*, 1990), by repressing the function of another HLH protein. The hallmark of *Id*-like proteins is that they lack a basic region and inhibit the function of other HLH proteins by dimerizing with them and preventing their binding to DNA targets. The *Esc1* basic region has fewer basic residues than that of *MyoD*

(Figure 3) and thus, it is unclear whether it can bind DNA (Davis *et al.*, 1990). Further investigation into the DNA binding activity of Esc1 will help elucidate its biochemical function.

However, regardless of whether or not Esc1 works as an enhancer or repressor of transcription, it does appear to elicit phenotypes similar to those induced by other HLH proteins that are differentiation inducers. Both MyoD and Esc1 appear to work early in differentiation induction, but neither can be considered an independent inducer as cells must be starved for growth factors for MyoD to induce differentiation (Braun *et al.*, 1989), while overproduction of Esc1 has only modest effects on the cell's sensitivity to cAMP-mediated inhibition. MyoD can induce transcription of many muscle-specific genes that have the CANNTG consensus sequence in their promoters (see Olson, 1990). While we have not yet identified a gene directly regulated by Esc1, preliminary evidence suggests a dysregulation in the *esc1* disruptants of some differentiation-specific genes with CANNTG sequences upstream of their transcription initiation sites (B.K.Benton, unpublished data). Finally, homozygous mutations in the only homolog of *myoD* in the nematode *C.elegans* may only partially block muscle formation (Chen *et al.*, 1992) similar to the effect of *esc1*⁺ disruption on sexual conjugation. Further analysis of the biochemistry of Esc1 and of the ability of mammalian HLH proteins to function in *S.pombe* may further substantiate the similarity of these differentiation inducers.

The discovery of an HLH protein in yeast that is involved in differentiation suggests that one aspect of the differentiation mechanism is conserved from yeast to man. If the biochemical and phenotypical properties of the mammalian HLH differentiation inducers and this yeast differentiation enhancer are indeed similar, then the fission yeast may become a genetically amenable host to dissect a differentiation switch that may be as well conserved as has been demonstrated for cell cycle regulation involving Cdc2.

Materials and methods

Yeast strains and media

All strains used in this study are listed in Table I. Minimal medium (MMA), malt extract (MEA) and yeast extract (YEA) agar were prepared as described by Gutz *et al.* (1976). MMA is nitrogen-rich. Crosses were performed as described by Gutz *et al.* (1976). SSL+N and SSL-N were the same as described previously (Egel and Egel-Mitani, 1974). For the cAMP suppression of *pat1* mutants, MMA was supplemented with 50 µg/ml leucine and the indicated concentrations of cAMP (Kohjin Co. Ltd) and caffeine (Nacalai Tesque).

Sequencing of the *esc1*⁺ cDNA

Both strands of the 2.9 kb *esc1*⁺ cDNA were sequenced by subcloning into M13 phage vectors (Yanisch-Perron *et al.*, 1985) and by dideoxynucleotide sequencing (Sanger *et al.*, 1977) using Sequenase version 2.0 (US Biochemical) according to the manufacturer's instructions.

Plasmid constructions and transformations

The 1.7 kb *Bam*HI fragment of the *esc1*⁺ cDNA containing the entire ORF was subcloned into the *Bam*HI site of pcU (Nagata *et al.*, 1991) downstream of the SV40 promoter to produce pcUesc1. To construct pcUesc1kfs, pcUesc1 was then digested with *Kpn*I and the cohesive ends were filled with Klenow and dNTPs (Sambrook *et al.*, 1989). This filled fragment was then self-ligated to introduce a 50 bp deletion and frameshift that putatively terminates translation at amino acid position 141. This is upstream of both the glutamine-rich and HLH regions. pcUesc1Δhlhlz was constructed by first isolating the 1.1 kb *Eco*RV-SnaBI fragment from pcD2esc1 and using it to replace the 1.4 kb *Eco*RV fragment from pcD2esc1. Then the 1.4 kb *Bam*HI fragment from this pcD2esc1Δhlhlz was subcloned into pcU to give pcUesc1Δhlhlz. Finally, pcD2esc1::ura4⁺ was constructed by replacing the

Table I. Strains used in this study

Strain	Genotype
SO1	<i>h</i> ⁻ <i>ura4-294</i>
SO2	<i>h</i> ⁺ <i>ura4-294</i>
SO3	<i>h</i> ⁻ <i>leu1-32</i>
SO4	<i>h</i> ⁺ <i>leu1-32</i>
SO5	<i>h</i> ⁻ <i>pat1-114 ura4</i>
SO6	<i>h</i> ⁻ <i>pat1-114 leu1</i>
C756-1A	<i>h</i> ⁹⁰ <i>ste11 leu1</i>
DK1	<i>h</i> ⁻ / <i>h</i> ⁺ <i>ade6-M210/ade6-M216 ura4/ura4 leu1/leu1</i>
SB1	<i>h</i> ⁺ <i>esc1::ura4⁺ ade6-M210 ura4 leu1</i>
SB21	<i>h</i> ⁺ <i>esc1::ura4⁺ ura4 leu1</i>
SB30	<i>h</i> ⁻ <i>esc1::ura4⁺ pat1-114 ura4 leu1</i>
SB74	<i>h</i> ⁻ <i>esc1::ura4⁺ ura4 leu1</i>
TB24	<i>h</i> ⁻ <i>ura4</i> (pcUesc1)
TB25	<i>h</i> ⁻ <i>ura4</i> (pcUesc1)
TB26	<i>h</i> ⁻ <i>ura4</i> (pcUesc1)
TB33	<i>h</i> ⁺ <i>ura4</i> (pcUesc1)
TB34	<i>h</i> ⁺ <i>ura4</i> (pcUesc1)
TB35	<i>h</i> ⁺ <i>ura4</i> (pcUesc1)
TB30	<i>h</i> ⁻ <i>ura4</i> (pcU)
TB31	<i>h</i> ⁻ <i>ura4</i> (pcU)
TB40	<i>h</i> ⁺ <i>ura4</i> (pcU)
TB41	<i>h</i> ⁺ <i>ura4</i> (pcU)

1.4 kb *Eco*RV fragment containing the entire ORF of the *esc1*⁺ cDNA with the 1.8 kb blunted *Hind*III fragment of *ura4*⁺ (Grimm *et al.*, 1988). The transcription of *ura4*⁺ is in the opposite direction to the SV40-directed transcription of the *esc1*⁺ cDNA. Transformations were performed as described previously (Okazaki *et al.*, 1990), except that they were scaled down 1:5 using 300 ng of DNA. For the pcD2 plasmids, 300 ng of pAU21 linearized with *Pst*I was co-transformed as a selectable marker into *ura4* cells.

Disruption of the *esc1*⁺ gene

Disruption was performed as described for *S.pombe* cells (Grimm *et al.*, 1988). The *esc1*-disrupted allele was excised from pcD2esc1::ura4⁺ by *Xho*I digestion and transformed into diploid DK1. One transformant from ~1300 transformants was isolated that contained one *esc1*-disrupted allele. This diploid was sporulated on MEA, and the spores were collected and plated on YEA (Gutz *et al.*, 1976). Approximately 100 random spores were then tested for the *ura4*⁺ phenotype (Grimm *et al.*, 1988). To confirm the presence of the disruption, DNA was prepared from DK1 and one of the haploid disruptants, SB1, as described previously (Moreno *et al.*, 1991) except that 1 M sorbitol buffer (1 M sorbitol, 0.1 M EDTA, 10 mM sodium sulfite and 100 mM lithium acetate) was used to suspend the cells for zymolyase treatment. Between 3 and 4 µg of DNA were digested with both *Bgl*II and *Eco*RI restriction endonucleases and Southern hybridizations performed (Sambrook *et al.*, 1989). Probes were prepared using the Amersham Multiprime DNA Labelling System. All disruptant strains were confirmed by Southern hybridization.

Conjugation assays

h⁺ and *h*⁻ cells of the appropriate genotypes were cultured separately overnight with shaking to the indicated concentrations in SSL+N media with the appropriate supplements at 27°C. Five million cells of each indicated mating partner were then mixed together (for *h*⁻ controls, 1 × 10⁷ cells were used) in SSL-N media and 1 ml aliquots were transferred to sterile glass culture tubes and incubated with shaking at 27°C. At the indicated times aliquots were sonicated and the number of conjugates formed counted under the microscope. The percentage conjugates are those conjugates formed divided by the total number of cells (conjugates counted as one cell) and converted to a percentage.

RNA preparation and Northern analysis

h⁺ and *h*⁻ *leu1* cells were first grown separately in SSL+N with 50 µg/ml leucine at 27°C to a concentration of 2 × 10⁶/ml and shifted to SSL-N with leucine at a concentration of 4 × 10⁶/ml of each cell strain (final total concentration was 8 × 10⁶/ml). For starvation of *h*⁹⁰ *ste11 leu1* cells (Kitamura *et al.*, 1990), *h*⁺ *leu1* and *h*⁻ *leu1* cells separately, the cells were concentrated to 8 × 10⁶/ml in SSL-N with leucine. Cells were then incubated with shaking at 27°C for the indicated times. After incubation,

the cells were harvested by centrifugation in a Beckman J-6B centrifuge at 3000 r.p.m. for 3 min, the supernatant was removed, and the cell pellet resuspended in 1 M sorbitol buffer at a concentration of $4-8 \times 10^8$ cells/ml and transferred to a 1.5 ml microfuge tube. Twenty units of zymolyase-100T (Seikagaku Kogyo) were added and the cell suspension incubated at 37°C for 5 min. The cells were then pelleted in a microfuge at 10 000 r.p.m. for 1 min and the supernatant removed. The cell pellet was resuspended in 1 ml of 4 M GTC solution (4 M guanidine thiocyanate, 18 mM sodium citrate, 0.36% sodium lauryl sarkosine and 0.18 M 2-mercaptoethanol), vortexed for 1 min to lyse the cells and kept on ice for 5–10 min. To the lysate was added 200 μ l of 2 M sodium acetate, pH 4, 1 ml of water-saturated phenol and 200 μ l of chloroform. The tubes were vortexed briefly and iced for 15 min. Afterwards, the tubes were centrifuged at 15 000 r.p.m. at 4°C for 10 min in a microfuge and the aqueous phase was re-extracted with 1 ml of phenol and 200 μ l of chloroform. An equal volume of ethanol was added to the aqueous phase and the RNA was precipitated at -20°C for at least 2 h. The RNA precipitate was centrifuged at 15 000 r.p.m. at 4°C for 10 min, the ethanol removed and the pellet air dried. The pellet was resuspended in 100–200 μ l of 4 M GTC and an equal volume of ethanol was added. The RNA was precipitated again at -20°C for at least 2 h, pelleted as above and resuspended in a convenient volume of water. Northern analysis was done with 10 μ g of total RNA as described previously (Nagata *et al.*, 1991). RNA was stored at -80°C as a 50% ethanol solution.

Acknowledgements

We are grateful to M. Yamamoto for the *stel1+* plasmid and C. Shimoda, K. Okazaki, and N. Okazaki for cell strains. We also thank H. Shinagawa and R. Moerschell for critical evaluation of the manuscript. This work was supported by a Japan Society for the Promotion of Science Postdoctoral Fellowship (B.K.B.), Taniguchi Shogakusei Research Fellowships (B.K.B. and M.S.R.), and grants from the Ministry of Education, Science and Culture, Japan (H.O.).

References

- Beach, D., Rodgers, L. and Gould, J. (1985) *Curr. Genet.*, **10**, 297–311.
- Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. and Weintraub, H. (1990) *Cell*, **61**, 49–59.
- Braun, T., Bober, E., Buschhausen-Denker, G., Kotz, S., Grzeschik, K.-H. and Arnold, H.H. (1989) *EMBO J.*, **8**, 3617–3625.
- Bresch, C., Muller, G. and Egel, R. (1968) *Mol. Gen. Genet.*, **102**, 301–306.
- Busch, S.J. and Sassone-Corsi, P. (1990) *Trends Genet.*, **6**, 36–40.
- Chen, L., Krause, M., Draper, B., Weintraub, H. and Fire, A. (1992) *Science*, **256**, 240–243.
- Courey, A.J., Holtzman, D.A., Jackson, S.P. and Tjian, R. (1989) *Cell*, **59**, 827–836.
- Davis, R.L., Cheng, P.-F., Lassar, A.B. and Weintraub, H. (1990) *Cell*, **60**, 733–746.
- DeVoti, J., Seydoux, G., Beach, D. and McLeod, M. (1991) *EMBO J.*, **10**, 3759–3768.
- Egel, R. (1989) Mating-type genes, meiosis and sporulation. In Nassim, A., Young, P. and Johnson, B.F. (eds), *Molecular Biology of the Fission Yeast*. Academic Press, San Diego, pp. 31–73.
- Egel, R. and Egel-Mitani, M. (1974) *Exp. Cell Res.*, **88**, 127–134.
- Egel, R., Nielsen, O. and Weilguny, D. (1990) *Trends Genet.*, **6**, 369–373.
- Fantes, P.A. and Nurse, P. (1977) *Exp. Cell Res.*, **107**, 377–386.
- Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T. and Yamamoto, M. (1986) *Cell*, **44**, 329–336.
- Grimm, C., Kohli, J., Murray, J. and Maundrell, K. (1988) *Mol. Gen. Genet.*, **215**, 81–86.
- Gutz, H., Heslot, H., Leupold, U. and Loprieno, N. (1976) *Schizosaccharomyces pombe*. In King, R.C. (ed.), *Handbook of Genetics*. Plenum Press, New York, Vol. 2, pp. 395–445.
- Horvitz, H.R. and Herskowitz, I. (1992) *Cell*, **68**, 237–255.
- Iino, Y. and Yamamoto, M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2447–2451.
- Jones, R.H., Moreno, S., Nurse, P. and Jones, N.C. (1988) *Cell*, **53**, 659–667.
- Kitamura, K., Nakagawa, T. and Shimoda, C. (1990) *Curr. Genet.*, **18**, 315–321.
- Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) *Cell*, **66**, 305–315.
- Maeda, T., Mochizuki, N. and Yamamoto, M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7814–7818.
- McLeod, M. and Beach, D. (1988) *Nature*, **332**, 509–514.
- Moreno, S., Klar, A. and Nurse, P. (1991) *Methods Enzymol.*, **194**, 795–823.
- Nagata, A., Igarashi, M., Jinno, S., Suto, K. and Okayama, H. (1991) *New Biol.*, **3**, 959–968.
- Nielsen, O. and Egel, R. (1990) *EMBO J.*, **9**, 1401–1406.
- Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K. and Okayama, H. (1990) *Nucleic Acids Res.*, **18**, 6485–6489.
- Olson, E.N. (1990) *Genes Dev.*, **4**, 1454–1461.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sipiczki, M. (1988) *Mol. Gen. Genet.*, **213**, 529–534.
- Sugimoto, A., Iino, Y., Maeda, T., Watanabe, Y. and Yamamoto, M. (1991) *Genes Dev.*, **5**, 1990–1999.
- Tanaka, K., Okazaki, K., Okazaki, N., Ueda, T., Sugiyama, A., Nojima, H. and Okayama, H. (1992) *EMBO J.*, **11**, 4923–4932.
- Tapscott, S.J., Davis, R.L., Thayer, M.J., Cheng, P.-F., Weintraub, H. and Lassar, A.B. (1988) *Science*, **242**, 405–411.
- Theill, L.E., Castrillo, J.-L., Wu, D. and Karin, M. (1989) *Nature*, **342**, 945–948.
- Watanabe, Y., Iino, Y., Furuhashi, K., Shimoda, C. and Yamamoto, M. (1988) *EMBO J.*, **7**, 761–767.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103–119.

Received on July 3, 1992; revised on September 21, 1992

Note added in proof

The nucleotide and deduced amino acid sequences reported here have been submitted to the EMBO/GenBank databases under accession number X69389.