Association of HSP70 with endonucleases allows the expression of otherwise silent mutations

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Abstract A subpopulation of the 70 kDa heat shock protein (HSP70) found within the mitochondria of *Saccharomyces cerevisiae* functions as a stable binding partner of the endonuclease *Sce*I. We have previously found that the *Sce*I endonuclease monomer recognizes and cleaves a unique, 26 bp sequence in vitro. Dimerization with HSP70 changes the specificity of *Sce*I, allowing it to cleave at multiple sequences. This study shows that *Suv*I, an ortholog of *Sce*I isolated from a different yeast strain, contains two amino acid substitutions, yet it shows the same uni-site specificity in its monomeric form. Binding of HSP70 to the *Suv*I monomer confers multi-site specificity that is different from that exhibited by the HSP70/*Sce*I heterodimer. Mutation of single residues of *Sce*I to the corresponding residue in *Suv*I provides enzymes with specificities intermediate between *Sce*I and *Suv*I when complexed with HSP70. These results suggest that HSP70 interaction with certain endonucleases allows the expression of otherwise silent mutations in them, causing a change in enzyme cleavage specificity.

Key words: 70 kDa heat shock protein; Molecular chaperone; Endonuclease; Mitochondrion; Molecular evolution; *Saccharomyces cerevisiae*

1. Introduction

The major function of the 70 kDa heat shock protein (HSP70) is to transiently bind partially or totally unfolded proteins and thereby maintain them in a refolding-competent state (reviewed in [1]). More than 10 homologs of HSP70 have been cloned from or detected in the genomic sequence of the yeast *Saccharomyces cerevisiae* [2–6]. The HSP70 family is part of the group of molecules that function as chaperones. HSP70 family members function in a diverse set of processes, including regulation of the heat shock response, quality control of proteins, protein translocation across organellar membranes and protein folding [1,7]. We have previously shown that a subpopulation of HSP70 molecules found in the mitochondria of *S. cerevisiae* function as a stable binding partner of the endonuclease *Sce*I [8]. The endonuclease monomer recognizes and cleaves a unique 26 bp sequence in vitro, producing cohesive ends with a four-base 3′ overhang. The HSP70/endonuclease heterodimer, which represents the quaternary structure of this enzyme in vivo, can cleave multiple sequences that deviate partially from the unique, 26 bp sequence [9]. The cleavage of mitochondrial DNA at multiple sites by the HSP70/*Sce*I heterodimer has been hypothesized to be a method of initiating genetic recombination of mitochondrial DNA at the cleaved sites [10].

Due to its broad sequence specificity, the HSP70/*Sce*I dimer cleaves, both in vitro and in vivo, at more than 30 randomly distributed sites within the yeast mitochondrial DNA ([9] and data not shown). Mitochondrial fusion, which occurs when haploid cells mate to form zygotic cells, provides the biological venue for this endonuclease to function. During mitochondrial fusion the HSP70/*Sce*I heterodimer-mediated cleavage induces genetic recombination between the heterogeneous mitochondrial DNAs inherited from each parent [10].

Molecular, genetic and biochemical analyses have revealed that in the mitochondria, HSP70 transiently binds unfolded, nuclear-encoded mitochondrial proteins as they translocate through the mitochondrial membrane. Thus, mitochondrial HSP70 is essential for the import and subsequent folding of these proteins [11,12]. The binding of *Sce*I to HSP70 is unique as it results in the formation of a stable heterodimer and is not the transient interaction typical between a chaperone and an unfolded protein.

Heterodimerization occurs when the 50 kDa *Sce*I monomer is incubated with HSP70 in the presence of ADP [9]. Conversion of *Sce*I from a uni-sequence-specific endonuclease to a multi-sequence-specific one can be studied by comparing the enzymatic activities of the 50 kDa monomer and the reconstructed dimer.

We identified *Suv*I as an ortholog of the *Sce*I endonuclease in *Saccharomyces uvarum* [13]. Both *Sce*I and *Suv*I monomers contain 476 amino acid residues. The two orthologs differ from each other in their primary sequence by only two amino acids. In this study, we have analyzed the difference in sequence specificity between the *Suv*I monomer and the HSP70/*Suv*I heterodimer. The *Suv*I monomer shows the same uni-site specificity as the *Sce*I monomer even though it differs in sequence by two amino acids. Binding of HSP70 to the *Suv*I monomer confers multi-site specificity that is different from that of the HSP70/*Sce*I heterodimer [13]. These results indicate a novel function for HSP70 whereby its binding allows the expression of silent mutations resulting in the alteration of the enzyme's properties. Furthermore, analysis of chimeric endonucleases that contain only one of the two ami-

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Abbreviations: HSP70, 70 kDa heat shock protein; GST, glutathione-*S*-transferase

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no acid substitutions reveal that each of the substitutions contributes to a unique difference in sequence specificity.

2. Materials and methods

2.1. Synthesis and purification of recombinant endonuclease monomer protein

The gene encoding each endonuclease was cloned into the pRSET expression vector (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* BL21(DE3) pLysS cells transformed with each endonuclease expression construct were induced with 0.4 mM isopropylthiogalactopyranoside at 18°C for 12 h to enable the expression of the corresponding N-terminal hexahistidine-tagged recombinant endonuclease monomer [9]. Partial purification of the overexpressed protein was achieved using a ProBond Ni-column (1×5 cm, Invitrogen), according to the manufacturer's protocol. The partially purified protein was subjected to a second chromatographic separation on an SP-Sepharose FF ion exchange resin column (1×5 cm, Amersham Pharmacia, Uppsala, Sweden) in the presence of a 200–500 mM NaCl gradient in 50 mM sodium phosphate buffer (pH 8.0). The endonuclease monomer eluted at a NaCl concentration of 300 mM in the salt gradient. The purified protein was stored in a solution of 50 mM Tris–HCl (pH 8.0) and 500 mM NaCl at 0 or −20°C, in the same buffer supplemented with 50% glycerol.

2.2. Reconstitution of the heterodimer

The mitochondrial HSP70 protein was overexpressed in *S. cerevisiae* and purified as previously described [14]. The purified HSP70 protein was incubated overnight, at 4°C in a solution containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10 mM ADP. To enable complex formation, the ADP-bound HSP70 and the 50 kDa endonuclease monomer subunit were incubated at a 1:1 molar ratio for 90 min at 20°C in 50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 1 mM dithiothreitol and 30 mM $MgCl_2$.

2.3. Binding assay

The HSP70 gene was cloned into the pGEX4T-1 expression vector (Amersham Pharmacia). This construct was overexpressed in *E. coli* JM109 cells and the resulting glutathione-*S*-transferase (GST)-fusion protein was purified according to the manufacturer's protocol. ADP was loaded onto the fusion protein by incubating it in the presence of 10 mM ADP at 4°C, overnight. The fusion protein was adsorbed onto glutathione-Sepharose 4B (Amersham Pharmacia). The glutathione-adsorbed fusion protein was allowed to bind the 50 kDa endonuclease protein at 20°C for 90 min in 50 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl, 30 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100 and 5 mM ADP. After this incubation the Sepharose resin was washed five times with 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM NaCl and 0.05% Triton X-100. The washed resin was boiled in Laemmli buffer [15] which was then subjected to Western blot analysis. Positive signal was detected using the ECL kit (Amersham Pharmacia).

2.4. Endonuclease assay

*Sce*I cleavage activity was assayed under the following conditions. The 1.8 kb *Eco*RI fragment of the *oli2* gene was cloned into a pUC vector (pMITO, [9]). pMITO DNA linearized by *Sca*I was incubated with 1–2 μl of the protein solution to be tested, in 20 μl of 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl, 1 mM dithiothreitol, and 10 mM $MgCl_2$. Reactions were performed at 37°C for 60 min unless otherwise stated. Reactions were stopped by the addition of ethylenediaminetetraacetic acid and proteinase-K [16]. Each reaction was phenol extracted and then analyzed by electrophoresis through a 0.8% agarose gel in TAE buffer [17]. The DNA in the gels was visualized by staining with 0.5 μg/ml of SYBR green I nucleic acid stain (Molecular Probes, Inc., Eugene, OR, USA) with gentle agitation at room temperature for 30 min. DNA cleavage was quantitated using an FM-BIO imaging scanner (Takara Biomedicals, Kyoto, Japan).

2.5. Digestion of mitochondrial DNA

Mitochondrial DNA was purified from the *S. cerevisiae* strain, YKN2673, as previously described [10]. 140 ng of this mitochondrial DNA was subjected to digestion by the 50 kDa protein in the presence or absence of ADP-bound HSP70. After digestion the DNAs were treated with proteinase-K, phenol extracted and ethanol precipitated prior to being electrophoresed through a 0.6% agarose gel in TAE buffer. Mitochondrial DNA from the yeast strain YKN2673 was completely digested with *Bam*HI and *Pst*I. *Bam*HI has four restriction sites, while *Pst*I has a unique site within the YKN2673 mitochondrial DNA sequence (data not shown). The mitochondrial DNA restriction digests were subjected to phenol extraction followed by ethanol precipitation.

2.6. Determination of cleavage sites

The precise nucleotide sequences recognized by the endonucleases as their cognate cleavage sites were determined as follows. Substrate DNAs containing fragments of mitochondrial DNA were incubated with the endonuclease heterodimers. The four-base, 3′ protrusions generated at the ends of the DNA fragments cleaved by the endonuclease dimers were blunted by an incubation with T4 DNA polymerase in the presence of dNTPs. The blunted ends were subsequently ligated using T4 DNA ligase. These ligated DNAs were used to transform *E. coli*. Repaired plasmid DNAs obtained from the transformed bacteria were sequenced in both directions. Analysis of the sequence data allowed the cleavage sites to be determined at the nucleotide level in reference to the original sequences of the intact DNAs.

3. Results and discussion

3.1. Sequence specificity of the endonuclease orthologs

Our previous studies had determined that heterodimers of HSP70 and *Suv*I existed in the mitochondria of the yeast *S. uvarum* (NCYC74). *Suv*I is an ortholog of the *S. cerevisiae*, *Sce*I protein [13]. Mitochondrial extracts prepared from *S. uvarum* (NCYC74) exhibited an endonuclease activity that was multi-sequence-specific as well as different from that of the HSP70/*Sce*I heterodimer [13]. Both *Sce*I and *Suv*I monomers contain 476 amino acid residues. Only two amino acid changes were found when the primary sequence of *Sce*I was compared to that of *Suv*I, Gly to Lys at position 217 and Asn to Asp at position 346. *Sce*I and *Suv*I monomers share the consensus amino acid sequence LAGLIDADG [18] with other homing endonucleases known to be involved in genetic recombination in uni-cellular eukaryotes and archaeons [18]. The HSP70 binding ability of these homing endonucleases has not yet been determined, thus suggesting the possibility that multi-site-specific cleavage is specific to the *Sce*I-related endonucleases in yeast mitochondria. The two amino acid substitutions are in the vicinity of the conserved LAGLIDADG sequence (Fig. 1). This conserved sequence has been located within α-helices that are followed by β–(β)–α–β–β–α motifs in the homing endonucleases [19–21]. The α/β motifs form the endonuclease active site, suggesting that the difference in amino acid sequence between *Sce*I and *Suv*I could be responsible for the difference observed in their cleavage site sequence specificity [13].

The DNA cleavage activity of recombinant *Sce*I was compared to that of recombinant *Suv*I, in order to determine whether the two amino acid substitutions altered the sequence specificity of the endonuclease monomer or that of the HSP70/endonuclease heterodimer. *Sce*I single mutants, *Sce*I (G217K) and *Sce*I (N346D), which had either a Lys at position 217 or an Asp at position 346, respectively, were constructed in order to test the contribution of each amino acid substitution to cleavage site specificity (Fig. 2a).

Mitochondrial DNA (about 80 kb in total length), digested with both *Bam*HI and *Pst*I, was used for the DNA cleavage assays. The *Sce*I monomer gene contains a unique *Pst*I site [13]. This *Pst*I site is approximately 1.2 kb downstream of the cleavage site detected by the *Sce*I monomer in the *oli2* gene

Fig. 1. Sequence alignment of LAGLIDADG motifs in *Sce*I and *Suv*I with those found in homing endonucleases. The consensus sequences are shown at the top of the figure. The number next to each amino acid represents its position in the primary sequence of the protein. Amino acid residues that match the consensus sequence are depicted by white letters. Secondary structure elements of the homing endonucleases, I-*Dmo*I [19], PI-*Sce*I [20] and PI-*Pfu*I [21], are depicted as cylinders (α-helices) and arrows or bars (β-strands).

(oligomycin sensitive ATPase subunit 6 gene) [10]. Consistent with our previous observation, the *Sce*I monomer generated fragments of 1.2 and 8.4 kb from the 9.6 kb mitochondrial DNA restriction fragment (Fig. 2b). Generation of the 1.2 kb fragment by the other recombinant endonuclease monomers, *Suv*I, *Sce*I (G217K) and *Sce*I (N346D), was evidence that they shared a common uni-site sequence specificity with *Sce*I (Fig. 2b). The 1.8 kb *Eco*RI fragment of the *oli2* gene cloned into pUC19 vector (pMITO [9]) was used in a cleavage assay to confirm cleavage site location. Specific activities of the wild-type and chimeric endonuclease monomers were comparable with one another (described later).

3.2. Reconstitution of the HSP70/endonuclease heterodimer

We have previously shown that efficient in vitro reconstitution of the HSP70/endonuclease heterodimer can be achieved without denaturation of the monomer when HSP70 and the *Sce*I monomer are combined in the presence of ADP [9]. A GST pull-down assay was performed in order to examine the binding ability of the recombinant endonucleases to HSP70

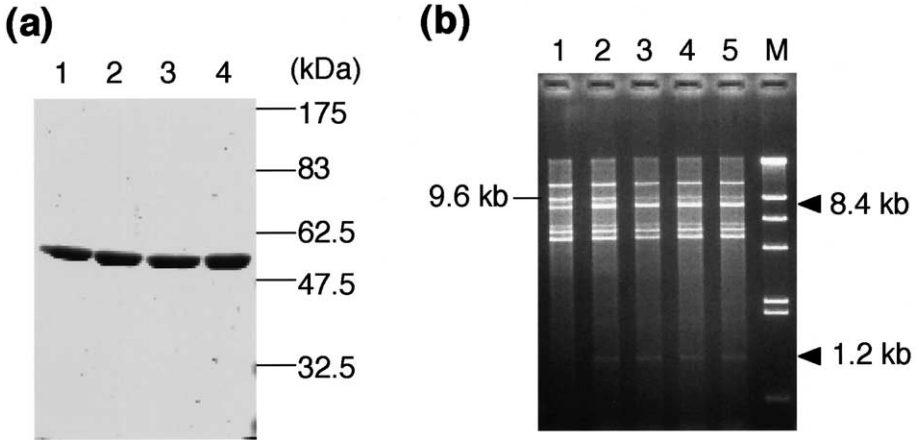

Fig. 2. Uni-site specificity of the endonuclease monomers. a: Preparation of the endonucleases. Endonuclease cDNAs were cloned into pRSET vectors and overexpressed in *E. coli* [9]. The recombinant endonucleases have N-terminal hexahistidine tags to enable their purification by Ni-column chromatography [9]. Purity and integrity of the endonucleases was examined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). 800 ng of each protein was separated on a 10% SDS–PAGE gel. Proteins were stained with Coomassie brilliant blue. Lane 1 contains *Sce*I; lane 2, *Suv*I; lane 3, *Sce*I (G217K) and lane 4, *Sce*I (N346D). b: Uni-site specific endonuclease activity of the endonuclease monomers. 500 ng of mitochondrial DNA was completely digested with *Bam*HI and *Pst*I and then treated with 60 ng of each endonuclease monomer at 37°C for 30 min. Lane 1 contains the *Bam*HI/I digests; lane 2, *Sce*I-treated mitochondrial DNA; lane 3, *Suv*I; lane 4, *Sce*I (G217K) and lane 5, *Sce*I (N346D). M represents λ DNA digested with *Hin*dIII. The reaction mixture for each endonuclease contained 50 mM Tris (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol [9]. The DNA fragments generated by the cleavage of the *oli2* sequence are indicated by black arrowheads.

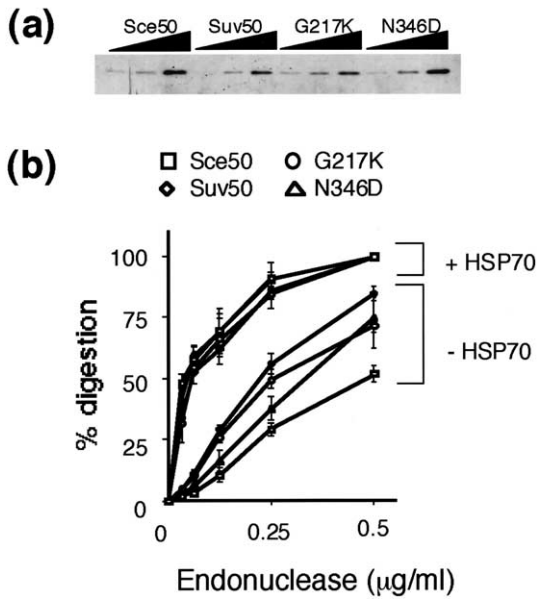

Fig. 3. Reconstitution of the HSP70/endonuclease heterodimers. a: Western blot analysis of the endonucleases coprecipitated with the GST–HSP70 fusion protein [9]. 1 μg of GST–HSP70 was incubated with 25, 50 or 100 ng of the endonuclease at 25°C for 90 min [9]. The blots were immmunostained with an anti-hexahistidine monoclonal antibody (Clontech) to detect the endonuclease. b: Enhancement of the endonuclease activity by binding of HSP70. The endonuclease complexes, with or without HSP70, were incubated with pMITO (5 μg/ml) at 37°C for 30 min.

[9]. Fig. 3a shows that all the endonuclease monomers, *Sce*I, *Suv*I, *Sce*I (G217K) and *Sce*I (N346D), associate efficiently with the GST–HSP70 fusion protein in the presence of ADP. The amount of each endonuclease pulled down by GST–HSP70 increased in a dose-dependent manner, but was comparable among all the endonucleases under the same conditions. This suggests that neither the amino acid substitution at position 217 nor that at 346 affects the ability of the endonuclease monomer to bind HSP70.

We reconstituted the HSP70/endonuclease heterodimer using purified HSP70 [9,14] and endonuclease monomer protein under the same conditions that were used for the GST fusion assay. The specific activity of the *Sce*I endonuclease monomer was not significantly different from that of the *Suv*I monomer (Fig. 3b). Previous studies indicated that specific activity increased when HSP70 bound the *Sce*I monomer [9]. Similarly, the specific activity of the reconstituted heterodimers, HSP70 with *Sce*I, *Suv*I, *Sce*I (G217K) or *Sce*I (N346D), was approximately three- to four-fold higher than that of the corresponding recombinant monomers, when pMITO was used as the substrate in these assays. The specific activity of each heterodimer was comparable to that of the others. This result indicates that the specific amino acid substitution at either position 217 or 346, or both, does not affect the binding of the monomer to HSP70 or the enhancement of specific activity when the *oli2* gene is used as a substrate.

3.3. Contribution of cryptic mutations to divergent sequence specificity in the endonuclease heterodimer

Next we determined whether the difference in cleavage site specificity of the reconstituted dimers was a consequence of the amino acid substitutions. Both reconstituted heterodimers

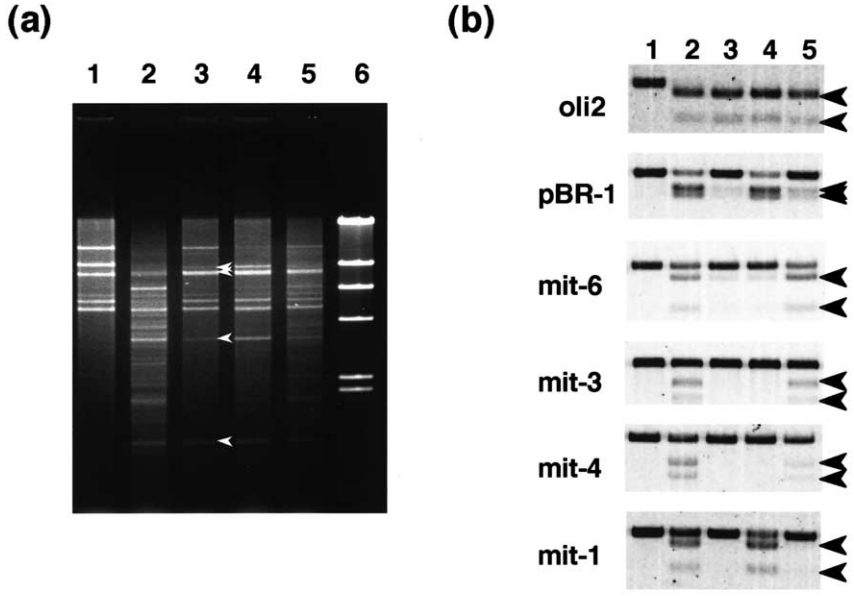

Fig. 4. Multi-site specificity of the endonuclease was generated by binding of HSP70. a: Reconstituted heterodimers exhibit multi-site cleavage specificity on mitochondrial DNA substrates. The HSP70/endonuclease heterodimers containing either *Sce*I or *Suv*I cleave *Bam*HI/*Pst*I-digested mitochondrial DNA at multiple sites. Lane 1, untreated; lane 2, mitochondrial DNA treated with *Sce*I; lane 3, *Suv*I; lane 4, *Sce*I (G217K); lane 5, *Sce*I (N346D) and lane 6, λDNA/*Hin*dIII digests. The fragments generated by *Suv*I digestion are indicated by white arrowheads in lane 3. b: Dependence of sequence specificity on residues 217 and 346. Short fragments (400–1000 bp) of mitochondrial DNA were cloned into pUC vector. After purification of the insert DNAs by restriction digestions and preparative gel electrophoresis (lane 1), the DNAs (2 ng) were subjected to cleavage by the HSP70/endonuclease heterodimers at 37°C for 1 h. Lane 2, *Sce*I; lane 3, *Suv*I; lane 4, *Sce*I (G217K) and lane 5, *Sce*I (N346D). 60 pg of endonuclease were used for each assay. Cleavage products are indicated by arrowheads. Difference in sequence specificity is also seen for the HSP70/*Sce*I heterodimer cleavage sites previously identified within the pBR322 vector (pBR-1).

consisting of either HSP70 and *Sce*I, or HSP70 and *Suv*I, exhibited multi-site specificity, generating multiple fragments from the restriction-digested mitochondrial DNA substrate (Fig. 4a). The HSP70/*Sce*I heterodimer generated more than 40 fragments. The digestion at each cleavage site was not necessarily complete under the experimental conditions used [9]. The actual number of cleavage sites is probably much lower than that suggested by the number of fragments obtained from the cleavage assay. However, our preliminary results suggest that the *Sce*I heterodimer can cleave at about 35 sites within the mitochondrial DNA. The HSP70/*Suv*I heterodimer also cleaves mitochondrial DNA at several sites. However, this heterodimer generates only four major fragments, which is far fewer than the number of fragments generated by the HSP70/*Sce*I heterodimer. This suggests that the HSP70/*Suv*I heterodimer is less tolerant of changes in sequence at the cleavage site and can cleave primarily the *oli2* sequence and one other site.

The assay used to locate the cleavage sites and determine the cleavage sequences used relatively small restriction fragments (<4 kb) of mitochondrial DNA cloned into a pUC vector as substrate. The fragmented mitochondrial DNAs were subjected to digestion with *Sce*I, *Sce*I (G217K), *Sce*I (N346D) or *Suv*I, and the resulting cleaved fragments were used to transform bacteria after being blunt-ended and ligated. Eight digested and repaired plasmid DNAs were randomly chosen for cleavage site sequence determination. The *oli2* gene and the major cleavage site for *Suv*I were also included in the analysis. Sequences flanking the cleavage sites were obtained by comparing the cleavage site sequences to published sequences. Based on these data, oligonucleotide primers were designed to amplify small pieces of mitochondrial DNA (400–1000 bp) using the polymerase chain reaction. Each amplicon contained a single cleavage site. Susceptibility of these mitochondrial DNA fragments to the four different endonuclease heterodimers was confirmed by repeating the cleavage assay. Representative examples of such cleavage assays are shown in Fig. 4b. Interestingly, all these randomly chosen fragments were cleaved by the *Sce*I heterodimer (Table 1). Although some fragments are cleaved by two or more endonucleases, no sequence is specific only to *Suv*I, *Sce*I (G217K), or *Sce*I (N346D). Two cleavable sequences previously identified in the pBR322 vector [9], the eight randomly chosen repaired sequences, the *oli2* gene, and the *Suv*I site, can be grouped into three categories depending on their ability to be cleaved by the different endonucleases (Table 1). Sequences in Group I were cleaved by all the endonucleases, while those in groups II (mit-3, -4, -5, -8, and -9) and III (mit-1 and mit-2) required an endonuclease containing Gly and Asn, respectively. *Suv*I, which lacks both Gly217 and Asn346, can cleave only the *oli2* and mit-7 mitochondrial DNA sequences. The mit-7 sequence is present in the second intron of the cytochrome oxidase gene. These data demonstrate that the specificity of the HSP70/*Suv*I heterodimer can be broadened by altering amino acids 217 and/or 346, thereby enabling the cleavage of additional sequences. Additionally, these results suggest that the substitution of Lys for Gly at position 217, or Asp for Asn at position 346 broadens the sequence specificity of a *Sce*I-related heterodimer (*Sce*I, *Sce*I (G217K) or *Sce*I (N346D)) as compared to that of the *Suv*I heterodimer.

The present study has revealed that the amino acid substitutions found between *Sce*I and *Suv*I are silent mutations in terms of sequence specificity of the monomeric endonuclease. However, binding of HSP70 to the endonuclease allows the expression of these otherwise silent mutations, resulting in a difference in cleavage site sequence specificity. This is a previously unidentified role for HSP70, which has primarily been characterized as a molecular chaperone. We have shown that the endonuclease binds to the N-terminal ATPase domain of HSP70 and not to its unfolded substrate binding domain [9]. However, the mechanism by which modulation of *Sce*I and *Suv*I endonuclease activity is achieved has not yet been determined.

Table 1
Divergence of substrate specificity of endonuclease heterodimers

		SceI	SuvI	217K	346D	
		217 Gly	Lys	Lys	Gly	
		346 Asn	Asp	Asn	Asp	Initial detection
Group A	▼					
oli2	**AATAGCCCAGAC ATATCCCTGAATGA**	100	100	100	100	SceI
pBR-1	g**AT**gctgt**AG**g**C ATA**gg**C**t**TG**gt**T**at	42	2	41	5	SceI
pBR-2	gg**T**tc**C**g**C**gc**AC AT**t**T**CCC**C**g**AA**aag	15	15	21	9	SuvI
mit-6	**AATA**tatt**A**t**A**g tg**A**aCCC**C**g**AA**a**G**g	35	3	4	45	G217K
mit-7	ttcct**CCCA**t**AC ATA**ag**C**a**TGAAT**c**A**	26	22	28	18	SuvI
Group B						
mit-3	g**A**atatat**A**t**A**a **ATA**a**CCC**c**GAA**a**G**g	16	0	0	12	N346D
mit-4	gcc**A**cggg**AG**c**C** gg**A**a**CCC**c**G**g**A**a**G**g	23	0	0	5	SceI
mit-5	**A**tctaa**C**a**A**t**AC AT**t**T**atC**TGA**t**T**a**A**	3	0	0	1	SceI
mit-8	**AAT**tatt**CA**t**A**t **A**gt**TCCC**c**GAA**a**G**g	27	0	0	8	SceI
mit-9	t**A**attgat**A**a**AC ATATC**tC**TG**tt**T**at	27	0	0	23	N346D
Group C						
mit-1	tccccc**A**c**T**t**AC ATA**aa**C**t**T**acg**T**at	20	0	52	0	SceI
mit-2	**A**tgtata**C**t**AC** g**TAT**a**C**t**T**ac**AT**at	3	0	17	0	G217K

An arrowhead above the top strand of sequence indicates a cleavage site. Numbers represent the percentage of digestion at each cleavage site used in the substrate. 'Initial detection' indicates which endonuclease was used first in order to detect cleavage. Bold type upper case letters are used to denote nucleotides that match the cleavage sequence within the *oli2* gene.

Phylogenetic analysis of yeast mitochondrial DNAs has suggested that the mitochondrial genome of *S. cerevisiae* evolved from that of *S. uvarum* [22]. Since both the *Sce*I and *Suv*I monomers are encoded by the corresponding mitochondrial genomes [13], it is probable that *Suv*I represents the ancestral form of *Sce*I. It is also possible that the *Sce*I heterodimer evolved from the *Suv*I heterodimer by incorporating the two amino acid substitutions at residues 217 and 346, thereby broadening its sequence specificity. This study presents a different view on whether the increased divergence in sequence specificity of recombinogenic endonucleases provides an advantage during evolution of the mitochondrial genome under selective pressure.

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