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Using the *cre-lox* recombination system to assess functional impairment caused by amino acid substitutions in yeast proteins

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ABSTRACT

A method was developed to assess the functional significance of a sequence motif in yeast Upf3p, a protein required for nonsense-mediated mRNA decay (NMD). The motif lies at the edge of the Upf3p-Upf2p interaction domain, but at the same time resembles the canonical leucine-rich nuclear export sequence (NES) found in proteins that bind Crm1p exportin. To test the function of the putative NES, site-directed mutations that cause substitutions of conserved NES-A residues were first selected to identify hypermorphic alleles. Next, a portable Crm1p-binding NES from HIV-1 Rev protein that functions in yeast was fused *en masse* to the C-terminus of variant Upf3 proteins using *loxP* sites recognized by bacterial *cre*-recombinase. Finally, variant Upf3-Rev proteins that were functional in NMD were selected and examined for the types of amino acid substitutions present in NES-A. The mutational analysis revealed that amino acid substitutions in the Upf3 NES impair both nuclear export and the Upf2p-Upf3p interaction, both of which are required for Upf3p to function in NMD. The method described in this report could be modified for the genetic analysis of a variety of portable protein domains.

INTRODUCTION

UPF3 is one of three genes in the yeast *Saccharomyces cerevisiae* that is required for the nonsense-mediated mRNA decay pathway (NMD) (1-5) Aberrant mRNAs where translation is prematurely terminated become substrates in the NMD pathway and are rapidly degraded. Mutations in *UPF3* stabilize nonsense mRNAs resulting in rates of decay similar to wild-type transcripts (5). We identified a sequence motif in Upf3p called NES-A that resembles the canonical leucine-rich nuclear export sequence (NES) found in many other proteins that binds to Crm1p, the exportin that carries cargo through nuclear pores into the cytoplasm (6). However, NES-A is located in a complex region at one edge of a domain that mediates a protein-protein interaction between Upf3p and Upf2p. This region also shows weak sequence similarity to canonical RNA recognition domains (RRM) (6, 7).

The segment containing NES-A was shown to direct a nuclear-localized reporter to the cytoplasm (6). Alanine substitutions of evolutionarily conserved leucine and isoleucine residues in NES-A abolished export of the same reporter. Three simultaneous alanine substitutions in NES-A (*upf3-triple*) inactivated NMD and caused the accumulation of Upf3p in the nucleolus, a region within the nucleus. These findings support a role for NES-A in

nuclear export. However, there were three unexplained features of NES-A that distinguished it from the canonical NES known to drive export of other proteins (6, 7). Although the NES-A sequence matches the consensus leucine-rich NES sequence, it is present in reverse orientation relative to the N- and C- terminus compared to all other NES domains that direct export. Also, the canonical NES binds to the Crm1p exportin (8-10), but Upf3p is not dependent on Crm1p for export and presumably does not bind to Crm1p. Finally, when export was fully restored by adding a heterologous NES at the C-terminus of Upf3-Triple, NMD was only partially restored, suggesting that NES-A may have one or more functions in addition to promoting export (6).

In order to resolve these conflicting observations and to separate the possible functions of NES-A, we developed a unique way to analyze mutations in NES-A. The method, which we refer to as the dual selection for loss/regain of function, requires two steps. The first is a selection for mutations in NES-A that impair the function of *UPF3*. The second step is based on the observation that the canonical leucine-rich NES is a self-contained and portable binding site for Crm1p (10). Mutations in NES-A that specifically impair NMD by blocking Upf3p export were identified by fusing a *bona fide* Crm1p-binding NES to the mutant Upf3p proteins and then selecting for those that regain function in NMD.

To accomplish this, we utilized the *cre-lox* recombination system commonly used for genomic manipulation. *Cre*-mediated recombination between *loxP* sites has been used successfully to eliminate or activate gene expression in a tissue-specific and time-specific manner, reduce the number of copies of integrated transgene arrays, and to direct the site-specific insertion of transgenes (11, 12). In our system, we used *cre-lox* recombination to cause a fusion of the leucine-rich NES from the mammalian HIV-1 Rev protein (13-16), which directs export in yeast, to the C-terminus of Upf3 mutant proteins. Two *loxP* sites were positioned such that induction of *cre*-recombinase eliminated the intervening sequence resulting in an in-frame translational fusion between sequences coding for the Rev NES and Upf3p. Following recombination, the alleles were analyzed to see if defects in nuclear export and NMD were corrected by the C-terminal addition.

In this report we focus on the details of the two-step selection and describe the characterization of three mutations causing amino acid substitutions in NES-A that impair the function of Upf3p. We suggest that a similar approach could yield valuable information when applied to the analysis of other sequence motifs and domains in other proteins.

MATERIALS AND METHODS

Strains, genetic methods, and supplemental information

Strain RSy5 (*MATa ade2-1 leu2-1 tyr7-1 can1-100 upf3-Δ1 trp1 ura3 his3 GAL2*), which carries a null allele of *UPF3* (7), was used as a transformation recipient in the dual selection for loss/regain of function. Further information on plasmids, strains, and mutant alleles of *UPF3* beyond what is described in this report can be found in Shirley *et al.* (7). Yeast was transformed by electroporation (17), which is described in the Protocol section. Supplemental information including the complete sequences for the plasmids pRR2 and pRLS207 described below can be found at http://www.molbio.wisc.edu/culbertson/creloxplasmids/.

cre/lox–mediated recombination of plasmid pRR2

The plasmid pRR2 (Figs. 1 and 3) was designed to analyze the functional effects of mutations in NES-A. pRR2 contains two *loxP* recognition sites oriented in the same direction. In cells expressing the site-specific *cre*-recombinase, double-strand breaks occur at both *loxP* sites. The free DNA ends recombine, resulting in a re-circularized plasmid in which the DNA between the two *loxP* sites is deleted. Recombination also results in the deletion of one of the *loxP* sites. *loxP* sites must be in the same orientation in order to delete the intervening segment. If they are in opposite orientation, recombination causes an inversion of the intervening segment. In pRR2, one *loxP* site is located within the *UPF3* open reading frame directly 5' of the stop codon (designated *UPF3-loxP*). The second *loxP* site is located 5' of sequences coding for a functional leucine-rich NES from the viral HIV-1 Rev protein and the 3'-UTR of *UPF3* (*loxP-Rev-UTR*). In *S. cerevisiae,* the HIV-1 Rev-NES domain binds to yeast Crm1p and promotes the nuclear export of any protein to which the NES is fused (8, 9). Expression of *cre*-recombinase results in the excision of the DNA sequence between the *loxP* sites and the fusion of the Rev-NES and *UPF3* 3'-UTR to *UPF3-loxP*. The recombined plasmid expresses a version of Upf3p that has a functional NES at the C-terminus. The pre-recombined *UPF3-loxP* and post-recombined *UPF3-loxP-Rev* alleles both express functional versions of an otherwise wild-type Upf3p as evidenced by the ability of *UPF3-loxP* and *UPF3-loxP-Rev* to complement a *upf3-Δ1* null allele (data not shown). Plasmid-mediated complementation was assayed by suppression of nonsense mutations and by accumulation of *CYH2* pre-mRNA as described below. The *S. cerevisiae ADE2* gene is located in the segment between the two *loxP* sites. In the *ade*$^-$ strain RSy5, the *ADE2* gene on the plasmid allows selection of the pre-recombined plasmid and the ability to monitor recombination following *cre* expression (Fig. 3). Colonies expressing the pre-recombined *UPF3-loxP* plasmid are white. After recombination and excision of the *ADE2* gene, colonies turn red because *ade2* mutants accumulate a red pigment.

The pRR2 plasmid, which was constructed starting with the vector pRS316 (7, 18), is a single copy plasmid containing an *S. cerevisiae* origin of replication (*ARS4*), the centromere of *S. cerevisiae* chromosome IV (*CEN4*), and the *S. cerevisiae URA3* gene which confers selective growth of Ura$^+$ transformants in medium lacking uracil. pRR2 carries a bacterial ampicillin resistance gene and the pBR322 origin of replication allowing selection of *E. coli* transformants.

Expression of *cre* recombinase

Plasmid pRLS207 expresses *cre*, the site-specific recombinase from bacteriophage P1 (19) (Fig. 1). In pRLS207, the *cre* open reading frame is flanked by the *S. cerevisiae GAL1* promoter and *CYC1* transcription termination signal sequence. When cells harboring pRLS207 are grown in glucose, $GAL1^{P}$-*cre*-$CYC1^{T}$ is not expressed, but in the presence of galactose transcription is induced. pRLS207, which was constructed starting with the vector pRS423 (18) is a multicopy plasmid carrying the origin of replication from the *S. cerevisiae* 2µm endogenous plasmid and the yeast *HIS3* gene, which confers selective growth of His^{+} transformants in medium lacking histidine. pRLS207 carries the ampicillin resistance gene and the pBR322 origin of replication allowing selection of *E. coli* transformants.

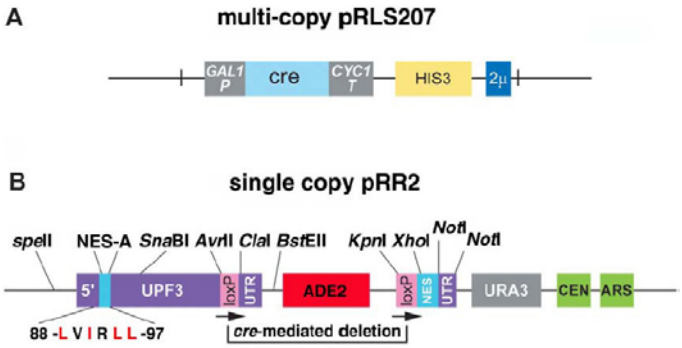

Fig. 1: Plasmids used in the dual screen for loss/regain of function. Both plasmids are circular with critical elements shown in linear format. Both plasmids are shuttle vectors that replicate in both yeast and *E. coli* **(A)** The diagram shows the organization of elements important for expression of *cre* recombinase in pRLS207. **(B)** The diagram shows the organization of elements in pRR2 used in mutagenisis and *cre*-mediated recombination. Wild-type amino acids targeted by mutagenesis are shown in red.

Phenotypic assays

The phenotypes of mutations in NES-A were assessed by three different assays: growth tests (suppression of nonsense mutations), accumulation of a pre-mRNA whose abundance and stability depends on *UPF3* function, and cytological localization of the protein products of mutant *upf3* alleles. All three assays summarized here are described in greater detail in previous reports (6, 7).

Nonsense suppression was monitored in strains carrying the *leu2-1* (UAA) and *tyr7-1* (UAG) nonsense mutations, which prevent growth on medium lacking leucine and tyrosine. Mutations in *UPF3* that cause loss of function suppress *leu2-1* and *tyr7-1* and allow growth on medium lacking leucine and tyrosine. The *can1-100* (UAA) nonsense mutation confers resistance to canavanine (Can^{r}) in a wild-type *UPF3* strain, whereas loss-of-function mutations in *UPF3* suppress *can1-100* and confer sensitivity to canavanine (Can^{S}). In each of these three cases, suppression results from increased stability of the nonsense mRNA and from increased read-through of translation termination at the premature stop codon.

CYH2 coding for ribosomal protein L29 contains an intron that is inefficiently spliced (20). An in-frame stop codon at position 19 in the intron triggers rapid decay of unspliced pre-mRNA (21). The pre-mRNA accumulates to a 5-6 fold higher level in *upf3*⁻ strains due to an increase in the pre-mRNA half-life but without any change in the mature mRNA half-life. The *CYH2* pre-mRNA/mRNA ratio therefore provides a quantitative assessment of function of the NMD pathway. Methods for quantitative measurement of RNA accumulation using Northern blotting are described by Leeds *et al.* (1) and Atkin *et al.* (22). To determine relative accumulation, the *CYH2* pre-mRNA/mRNA ratio in *upf3* mutants was normalized to the ratio in a wild-type *UPF3* strain to calculate the fold change. Fold changes were averaged across three or more trials and evaluated statistically as described previously (7).

Epitope-tagged Upf3p was visualized by immunofluorescence. Early log phase cells were processed as described previously (6, 23). The ability of mutant Upf3 proteins to interact with Upf2p was assayed using the yeast two-hybrid system as described by James *et al.* (24). The details of the two hybrid experiments are described by Shirley *et al.* (7). The results are not presented in detail here but are summarized in Table 2.

Mutagenesis

Multi-round PCR was used to generate mutations in the sequences coding for residues L88, I90, L92 and L93 in NES-A (Fig. 2). The mutagenic oligonucleotide RR03 was degenerate for nucleotides in each of four codons such that the mutations in each codon could potentially result in 15 different amino acid substitutions (not including Met, Phe, Tyr, Cys, and Trp). Variants where U was the first nucleotide of a codon were avoided so that UAA, UAG, and UGA termination codons would not be generated. Alternative schemes designed to produce all 20 amino acid substitutions were not attempted because they necessitate the inclusion of stop codons.

In the first round of PCR, oligonucleotides primers RRO3 and LSO183 (Fig. 2) and template plasmid pLS17 (*CEN URA3 UPF3*) (7) were used to amplify DNA fragments containing random mutations at each of the four codons. The first-round PCR products were used as a megaprimer in the second round along with oligonucleotide T7 (Fig. 2) and template pLS17. The PCR fragments amplified in the second round contained recognition sites for *Sna*BI and *Sac*II. After digestion with these enzymes, the resulting DNA fragments were used to replace the *Sac*II–*Sna*BI fragment containing wild-type NES-A in plasmid pRR2.

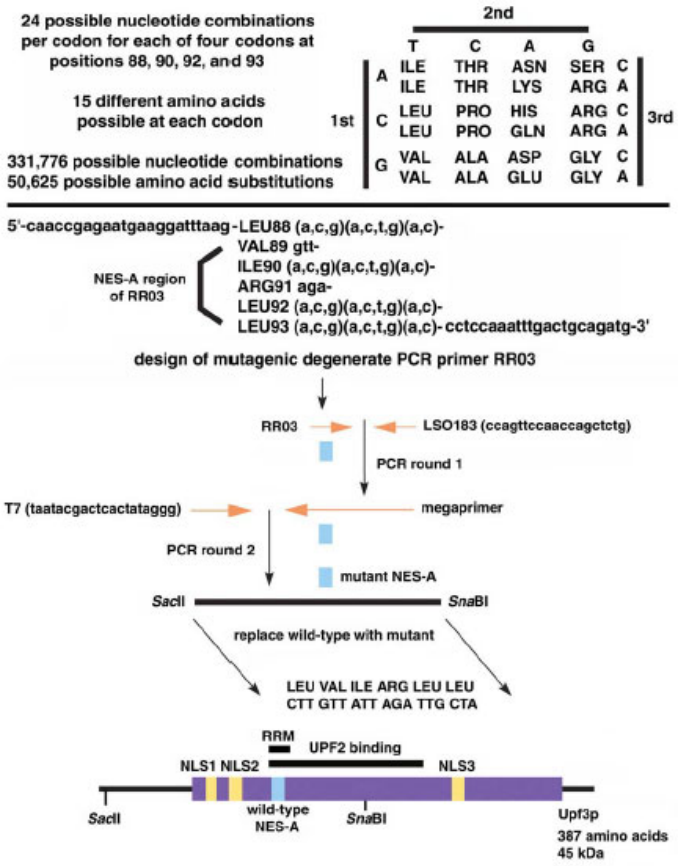

Fig. 2: Strategy for mutagenesis of NES-A in pRR2 using multi-round PCR. The structure of *UPF3* is shown at the top including known or suspected domains: NLS, nuclear localization signal sequence; Upf2p binding, the region required for binding to Upf2p; RRM, a region showing similarity to an RNA recognition motif identified in studies of the human ortholog to yeast Upf3p (27); NES-A, the motif resembling the leucine-rich nuclear export domain corresponding to the binding domain for the exportin Crm1p.

The ligation reaction between *Sac*II-*Sna*BI PCR fragments and *Sac*II-*Sna*BI-digested pRR2 was transformed into XL10-Gold ultra-competent *E. coli* cells (Stratagene, La Jolla, CA) and plated on standard Luria-Bertani (LB) medium plus 100 µg/ml ampicillin. Plasmid DNA isolated from pools of *E. coli* transformants were used to transform yeast strain RSy5 as described below. The number of *E. coli* transformants to be pooled in order to obtain with 95% confidence every possible amino acid combination caused by the mutagenic oligonucleotide was estimated using the formula:

$$N = \frac{\ln(1-P)}{\ln(1-n/T)}$$

where P = the probability that each combination of amino acid substitutions will be represented once, N = the number of bacterial colonies pooled, n = the number of the rarest combination, and T = the total number of combinations. Since some of the amino acids are represented by only one of the 24 possible codon combinations, 70 colonies must be pooled in order to have a 95% probability of obtaining at least one of each of the 24 combinations at a given codon. This assumes that each combination is represented equally after PCR amplification, which might not occur if any fragments are preferentially amplified. When four codons are mutated, a pool of ~10^6 *E. coli* transformants would be required to ensure detection of the rarest combinations. In the experiments described below, we pooled far fewer than this number and did not, therefore, exhaust the potential to uncover mutants of interest. In designing a screen of this type, there are trade offs between the advantages of mutating multiple codons and the disadvantages of needing to screen large numbers of transformants to recover all possible mutant combinations.

RESULTS AND DISCUSSION

Rationale

The combined mutagenesis and dual selection requires five steps (Figs. 2 and 3): (1) mutagenic PCR to create mutations in pre-chosen residues in NES-A, (2) selection for yeast co-transformants that carry both a mutagenized pRR2 plasmid and the pRSL207 plasmid that codes for *cre*-recombinase, (3) plate tests for loss of function before recombination, (4) induction of *cre*-mediated plasmid recombination that places the HIV-1 Rev-NES at the C-terminus of mutant Upf3 proteins, and (5) plate tests to assess whether the C-terminal Rev NES improves function.

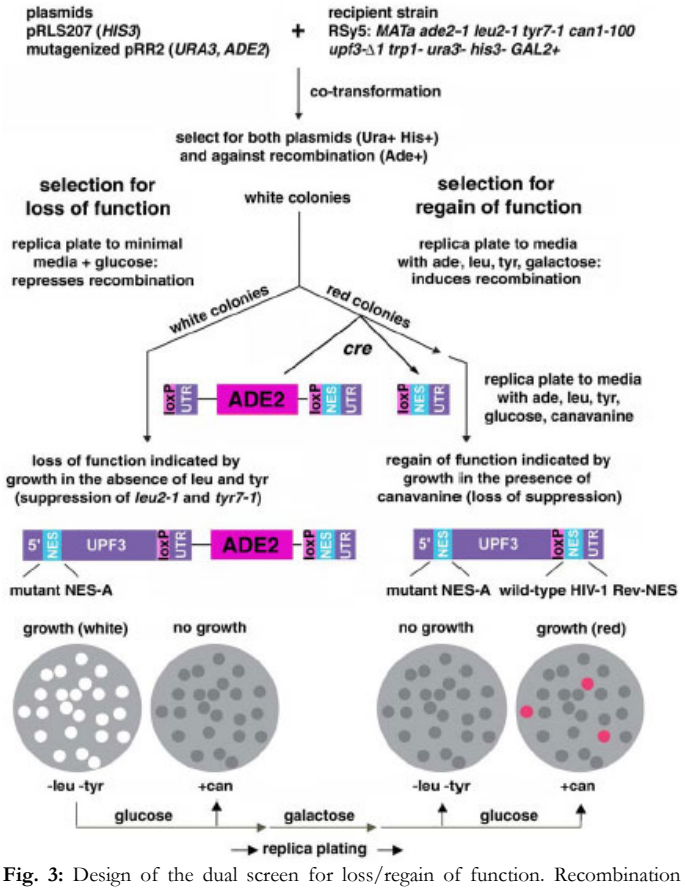

Fig. 3: Design of the dual screen for loss/regain of function. Recombination mediated by bacterial *cre* recombinase expressed in yeast places a function nuclear

export sequence (NES) derived from the mammalian HIV-1 Rev protein at the C-terminus of mutant Upf3p. Regain of function after recombination implies that a mutation in the Upf3p NES motif impairs nuclear export and that nuclear export is required for the function of Upf3p. The petri plates shown at the bottom represent idealized results. Loss of function is indicated by co-suppression of *leu2-1 tyr7-1* (white colonies before recombination that grow in the absence of leucine and tyrosine) and suppression of *can1-100* (grey imprints of colonies that fail to grow). Regain of function after recombination is indicated by loss of suppression allowing growth in the presence of canavanine (red colonies). White colony color results from the presence of the *ADE2* gene on plasmid pRR2 before recombination and red colony color results from deletion of the *ADE2* gene after recombination. The *cre*-recombinase expressed from plasmid pRLS207 is controlled by the *GAL1* promoter and is induced with galactose as carbon source.

Since mutant alleles were identified based on loss and regain of function of mutant Upf3 proteins in the absence or presence of C-terminal additions, respectively, it was important to determine whether the additions of loxP and Rev NES affect function by themselves. The addition of an HA epitope at the N-terminus was known to cause a slighty impairment of Upf3 function (6).

Appropriate alleles were tested for their ability to complement a *upf3-⊿1* null allele as measured by nonsense suppression and *CYH2* pre-mRNA accumulation (see below). The C-terminal addition of loxP and the HIV-1 Rev-NES had no significant effect on the function of wild-type Upf3p (data not shown), indicating that the NMD phenotype of the mutant Upf3-loxP and Upf3-loxP-Rev proteins would be due solely to their amino acid substitutions in NES-A.

Dual selection for loss/regain of function

Plasmid DNA was isolated from a pool of ~8,600 *E. coli* transformants carrying PCR-mutagenized pRR2 plasmids. The pRR2 DNA was mixed with pRLS207 plasmid DNA and used to co-transform strain RSy5 (*MATa ade2-1 leu2-1 tyr7-1 can1-100 upf3-⊿1 trp1 ura3 his3 GAL2*) to a Ura$^+$ His$^+$ phenotype (pRR2 carries *URA3* and pLRS207 carries *HIS3,* see Fig. 1). Omission of adenine in the growth medium and white colony color ensured that transformants contained pre-recombined plasmids. Transformants were plated directly onto medium lacking uracil, histidine, adenine, leucine and tyrosine in order to select for transformants harboring pRR2 plasmids that suppress the *tyr7-1* and *leu2-1* nonsense mutations. Out of ~84,000 yeast transformants plated, 7,200 had a Tyr$^+$ Leu$^+$ phenotype, indicating that at least one mutation causing impairment of *UPF3* function was carried on the pRR2 plasmids.

Two alternative approaches can be used to analyze the Tyr$^+$ Leu$^+$ transformants. The transformants can be replica-plated to medium containing canavanine. Complete loss of *UPF3* function leads to a Can^S phenotype due to suppression of the canavanine resistant nonsense allele *can1-100*. Mutations that partially impair function cause an intermediate phenotype of weak resistance (increased sensitivity) compared to wild-type. The strength of the phenotype can be adjusted by varying the concentration of canavanine. Growth on canavanine prior to recombination can be compared to growth of the transformants on canavanine following *cre*-mediated recombination between the *loxP* sites. Recombination can be induced by replica-plating the Tyr$^+$ Leu$^+$ transformants to medium containing galactose to activate the expression of *cre*. In a second approach, the phenotypic analysis of primary transformants on canavanine before recombination can be skipped. Primary transformants can be replica-plated directly to medium containing galactose to induce recombination and then to plates containing canavanine. Using the latter approach, we identified 389 can^r colonies from the original 7,200 transformants.

Table 1: Amino acid substitutions in NES-A.

Allele	NES-A amino acid sequence	Percent function in NMD*
UPF3	88-LVI RLLPPNL-97	100
upf3-Triple-HA	L88A I90A L93A	25
upf3-nes1	L88V I90V L92G L93V	87
upf3-nes2	L88D I90A L92G L93S	46
upf3-nes3	L88N I90D L93R	nd
upf3-nes4	L88V I90V L93G	nd
upf3-nes5	L88R I90L L92G L93S	37
upf3-nes6	L88G I90V L92P L93K	36
upf3-nes7	L88Q I90R L92V L93G	27

*The percent function was determined using the *CYH2* assay for pre-mRNA accumulation (Materials and Methods and (7)).

The mutations in the pRR2 plasmids from seven of these transformants were analyzed by sequence analysis of PCR-amplified DNA fragments (Table 1, (7)). All seven transformants were chosen based on their robust growth on medium lacking leucine and tyrosine and on medium containing canavanine prior to and after *cre*-induced recombination, respectively. The seven alleles designated *upf3-nes1* through *upf3-nes7* contained three to four amino acid substitutions of the conserved leucine and isoleucine residues in NES-A (Table 1). The amino acid substitutions were analyzed by replacing the wild-type NES in Upf3p with each of the seven NES variants to quantitate their extent of NMD impairment. Quantifying the *CYH2* pre-mRNA/mRNA ratio in transformants carrying the *upf3* alleles revealed that the ability of the mutant proteins to function in NMD ranged from 27% to 87% compared to wild-type Upf3p (Table 1).

Phenotypic analysis of selected mutants

The genetic, molecular, and cytological phenotypes of two alleles, *upf3-nes2* (L88D I90A L92G L93S) and *upf3-nes7* (L88Q I90R L92V L93G) were characterized in the presence or absence of a functional NES at the C-terminus (Table 2). In this analysis we included a previously described allele called *upf3-Triple* (L88A I90A L93A) (7). We reported previously that Upf3p-Triple tagged with the HA epitope was reduced in abundance compared to Upf3p-HA by 50% (6). To increase the abundance of the mutant protein to a level similar to Upf3p-HA, the experiments were done using a plasmid that carries two copies of the *upf-Triple-HA* gene, which results in two-fold over-expression of the mutant protein (6). The *upf3-nes2-HA* and *upf3-nes-HA* alleles produced proteins that were similar in abundance to the wild-type protein when present in a single gene copy.

Table 2: Phenotypic properties of NES-A mutants.

***UPF3* allele**	**NES-A amino acid substitutions**	**Nonsense suppression**	**Percent function in NMD****	**Nuclear export defect*****	**Upf2/Upf3 interaction******
UPF3 (wild-type)	88-LVI RLLPPNL-97	–	100	no	++
upf3-⊿1 (null)	no protein made	+++	0	N/A	N/A
upf3-Triple-HA	L88A I90A L93A	++	25	yes	-
upf3-Triple-HA-Rev		+	46	no	ND
upf3-Triple-HA-M10		++	25	yes	ND
upf3-nes2	L88D I90A L92G L93S	+	46	yes	-
upf3-nes2-Rev		+	46	no	ND

* "+++" represents full suppression of the *leu2-1* and *tyr7-1* nonsense alleles characteristic of the *upf3-⊿1* null allele. "–" indicates the complete lack of suppression characteristic of wild-type *UPF3*. "+" and "++" indicate weaker and stronger levels of suppression intermediate between *upf3-⊿1* and *UPF3*.

**The percent function was determined using the *CYH2* assay for pre-mRNA accumulation (Materials and Methods and (7)).

***Epitope-tagged Upf3 proteins were localized by immunofluorescence microscopy (Materials and Methods and (7)). Wild-type Upf3p was visualized throughout the cytoplasm with no staining detected in the nucleus (6, 7). A defect in nuclear export was indicated by the increase in nucleolar accumulation (7).

****The ability of the Upf3 proteins to interact with Upf2p was assayed using the yeast two-hybrid system (7). "++" and "-" indicate robust growth and lack of growth, respectively, on selective media. ND = Not Determined.

The *upf3-nes2*, *upf3-nes7*, and *upf3-Triple-HA* alleles all cause suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations, but the efficiency of suppression was less than that of *upf3-⊿1* (Table 2, (6)).The growth tests suggest that all three alleles cause partial impairment of function. When DNA coding for the HIV-1 Rev NES was present following the last codon of each mutant gene, the level of suppression was unchanged for *upf3-nes2* and *upf3-nes7*, but was reduced for *upf3-Triple-HA*. This suggests that the functional impairment caused by the nes2 and nes7 amino acid substitutions was unaltered when a functional NES is added to the C-terminus, whereas the functional impairment caused by the triple alanine substitutions is partially alleviated by the C-terminal addition.

To quantitate the effects of the mutations in the presence and absence of a C-terminal NES, the *CYH2* pre-mRNA/mRNA ratio was determined (Table 2, (6)). The *upf3-nes2* and *upf3-nes7* alleles produce proteins that were 46% and 27% as functional as wild-type Upf3p, respectively (Table 2). The addition of a functional NES at the C-terminus caused no change in the *CYH2* pre-mRNA/mRNA ratio. However, the addition of a C-terminal NES to the Upf3-Triple-HA protein improved function from 25% to 46% (Table 2). To verify that the improved function was due to the addition of a functional C-terminal NES, a control experiment was done using the HIV-1 Rev-M10, which carries a mutation that abolishes the ability of the NES to promote nuclear export. There was no change in the *CYH2* pre-mRNA/mRNA ratio when HIV-1 Rev-M10 was placed at the C-terminus of Upf3-Triple-HA (Table 2).

To examine the effects of the mutations on nuclear export, HA-tagged versions of the mutant proteins were localized by immunofluorescence using HA monoclonal antibodies (Table 2, (6)). In wild-type strains, Upf3p-HA localizes to the cytoplasm without any detectable signal in the nucleus. However, nuclear Upf3p-HA is detected either when the wild-type protein is over-expressed or when mutant Upf3p-HA is impaired in its ability to export from the nucleus. Under these circumstances, the immunofluorescent signal is concentrated in the nucleolus. Since the nucleolus is a sub-region within the nucleus, nucleolar accumulation serves as a marker for impaired nuclear export. HA-tagged versions of *upf3-nes2*, *upf3-nes7,* and *upf3-Triple-HA* produced proteins that accumulate in the nucleolus with some Upf3p still detectable in the cytoplasm. These results are consistent with the hypothesis that the functional impairment of the alleles in NMD is caused at least in part by a defect in nuclear export.

To ask whether the HIV-1 Rev-NES restores nuclear export when added to the C-terminus, we analyzed Upf3p-Triple-HA, Upf3p-Triple-HA-M10 (non-functional NES), and Upf3p-Triple-HA-Rev (functional NES) by immunofluorescence (Table 2, (6)). The nucleolar signal indicative of an export defect was detected in cells carrying the *upf3-Triple-HA* and *upf3-Triple-HA-M10* alleles. However, the nucleolar signal was abolished in cells carrying the *upf3-Triple-HA-Rev* allele. This evidence supports the conclusion that the HIV-1 Rev-NES restores the ability of Upf3p-Triple to export from the nucleus to the cytoplasm. However, the restoration of export results in only a partial restoration of function in NMD as determined by the suppression and mRNA accumulation tests described above.

The NES-A region was shown previously to be important but not sufficient for the protein-protein interaction known to occur between Upf2p and Upf3p (7, 25). The ability of Upf3-Triple, Upf3-nes2, and Upf3-nes7 proteins to interact with Upf2p was determined using the yeast two-hybrid system. All three mutant proteins expressed with Upf2p failed to promote growth on selective media indicating a significant decrease in the strength of the interaction (Table 2, (7)).

To summarize, the triple alanine substitutions cause partial loss of function without the C-terminal HIV-1 Rev-NES and partial regain of function when the C-terminal NES is present. The *upf3-nes2* and *upf3-nes7* alleles also cause partial loss of function where *upf3-nes2* is less impaired than *upf3-Triple-HA* and *upf3-nes7*. However, both alleles differ from *upf3-Triple-HA* in that a partial re-gain of protein function does not occur in the presence of a C-terminal NES. All three alleles produce a protein that is impaired for export and for the physical interaction with Upf2p. Overall, the results suggest either that the export of Upf3p occurs by binding to Upf2p or a specific export pathway for Upf3p is necessary to promote an interaction with Upf2p.

Recently, the crystal structures for human Upf3p and Upf2p were reported (26). The amino acid sequence within the NES-A region (residues 87-95 in *S. cerevisiae*) are 78% identical and 89% similar to human *UPF3*, suggested that the folded structure is likely to be similar in both organisms. The NES-A region lies within a three-dimensional structure that resembles an RNA recognition domain, but despite this, human Upf3p fails to bind to RNA. Instead, the NES-A region forms a β-sheet that is critical for the binding of Upf3p and Upf2p. Binding studies revealed that human Upf2p is capable of simultaneous binding to Upf3p and RNA, suggesting that a Upf2p/Upf3p complex may bind to RNA during export.

Our genetic analyses combined with the structural analyses reported in Kadlec *et al.* (26) support a model in which Upf3p exports from the nucleus bound to Upf2p. Amino acid substitutions that impair the export of Upf3p result from an impaired interaction between Upf3p and Upf2p. Thus, NES-A directs nuclear export not by serving as a binding site for an exportin but rather as a binding site for Upf2p. The addition of the HIV-1 Rev-NES promotes export of mutant Upf3 proteins but it fails to restore the activity of the NMD pathway because the essential Upf3p/Upf2p interaction remains impaired.

Adapting the dual selection to study other protein domains

The results of our genetic analyses provide a proof of principle for using a dual selection to analyze the function of protein domains. Modifications could be made to study functional domains in other proteins. The best systems would be those where the crystal structure of a protein is known so that the effects of different amino acid substitutions could be more readily interpreted. The following considerations should guide the design of a dual selection to analyze domains in other proteins:

(1) The domain to be studied must be self-contained, portable, and able to function out of context. The best way to test portability is to attach the domain of interest to a reporter. The C-terminus of a protein is likely the best position for attachment of a portable domain, but this would have to be tested empirically for any given system. Binding domains for DNA, RNA, protein, and small molecules are often self-contained and portable. However, active centers for enzymatic activities or other types of complex domains are often not portable since the active center is the result of protein folding rather than a contiguous amino acid sequence.

(2) Since the selection is conducted *in vivo*, it is most readily adapted to study the products of genes that are dispensable for growth. In our studies, *UPF3* is not essential for growth hence mutations do not reduce viability (5). For essential genes where the mutations of interest are potentially lethal, a condition would be needed where mutants are viable. Such a condition would vary depending on the gene being studied.

(3) The phenotypic assays are critical in terms of the functions they monitor and the ease of scoring. At a minimum, the assays should include (a) a plate test for overall function of the protein that is amenable to mass screening (such as our assays for nonsense suppression), and (b) a specific assay for the function of the domain in which the mutations reside (such as the immunofluorescent localization assay we used for nuclear export and the two hybrid assay for protein-protein interactions).

Mutations that cause partial impairment of function can be difficult to distinguish from wild-type, as was the case for *upf3-nes1*, which is impaired only slightly (Table 1). Even for mutations causing greater impairment of function, suppression levels before and after recombination were not readily distinguished by examining the growth of colonies after replica-plating. We resolved this by resorting to more sensitive serial drop tests to assay growth rates (7), but drop tests precluded the ability to screen large numbers of candidate mutants. Despite the fact that our system fell short of the ideal for phenotypic screening as described in Fig. 3, the basic idea behind the dual screen for loss/regain of function could prove useful in analyzing mutations in domains of other proteins using different phenotypic assays.

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PROTOCOLS

Protocols

Protocols are written as performed for the sets of experiments described in the paper. General suggestions are given in brackets. To adapt the methods to study other genes, modifications of restriction sites, PCR conditions, choices of vectors, or other experimental parameters may be required.

Plasmid Construction

Insertion of loxP site after last codon and prior to termination codon of gene

1. Clone *UPF3* into centromeric plasmid pRS314 between restriction sites *Spe*I and *BstE*II.
2. Removal of *Not*I site in the multiple cloning site of pRS314. {These steps can be eliminated if the vector containing the gene of interest does not contain a *Not*I site}.
   a. Digest 5 µg of pLS17 with *Not*I (NEB) in a total volume of 50 µl at 37°C for hours.
   b. Add 1µl of T4 DNA polymerase (NEB) and 100 µM of dNTPs at 16°C for 45 minutes to fill in 5' overhangs. {According to NEB, decreasing temperataure and incubation time to 12°C for 15 minutes reduces the occurrence of recessed ends caused by the 3' → 5' exonuclease activity of T4 DNA polymerase}.
   c. Add 3µl of DNA, 4µl of T4 Ligase Buffer (Invitrogen), 13µl of H_20 and 1µl of T4 Ligase, mix, incubate for 5 hours at 16°C.
   d. Transform 2µl into subcloning competent DH5α cells (Invitrogen) according to manufacturer's instructions. Plate 50, 100 and 200µl of cell suspension following incubation at 37°C for 1 hour on LB plates containing 100 µg/ml ampicillin. Incubate at 37°C for 18 hours.
   e. Pick individual colonies and grow in 1 – 2 ml of LB liquid media containing 100 µg/ml ampicillin.
   f. Isolate DNA by miniprep using Qiagen kit according to the manufacturer's instructions.
   g. Screen colonies for removal of *Not*I site and addition of *Fse*I site. {If plasmids without *Not*I are a small percentage of plasmids recovered, pool colonies isolated in Step d. Isolate the DNA from the pooled colonies, cut with *Not*I and transform into DH5α.}.
3. Insertion of *Not*I site using Chameleon double-stranded site-direected mutagenesis kit (Strategene).
   a. Synthesize an HPLC-purified olgionucleotide containing a 5'–end phosphate to insert a *Not*I site (GCGGCCGC) following the last codon and prior to the termination codon of *UPF3*. Synthesize a second oligonucleotide that changes the unique restriction site within pRS314.
   b. Insert a *Not*I site into *UPF3* using the oligonucleoties described above following manufacturer's instructions.
4. To insert *loxP*, synthesize complementary oligonucleotides that contain a *loxP* site (underlined in the sequence below), generate *Not*I overhangs, and maintain the reading frame after ligation into plasmid generated in Step 3. To aid further cloning the oligonucleotides destroy the *Not*I site upon ligation and generate new restriction sites (*Avr*II, *Cla*I).
   a. 5' - GGC CTA GGA ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TAT CGA TAT
   b. 5' - GGC CAT ATC GAT ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TTC CTA
   c. Combine 250 pmoles of each primer in a total volume of 100µl. Boil for 5 minutes and place immediately on ice.
   d. Digest 1µg of vector generated in Step 3 with *Not*I in a total of 20µl. Clean-up digested vector using QIAquick PCR Purification Kit following manufacturer's instructions.
   e. Combine 2µl of digested vector, 1µl of annealed oligonucleotides diluted 1:10, 4 µl of T4 Ligase Buffer and 1µl T4 Ligase in 20µl total. Mix gently. Maintain at room temperature for 1 hour.
   f. Digest 3µl of the ligation reaction with *Not*I for 2 hours at 37°C.
   g. Transform 2.5µl of the *Not*1-digested ligation into DH5α according to manufacturer's instructions.
   h. Isolate DNA by miniprep using Qiagen kit following manufacturer's instructions.
   i. Screen colonies for removal of *Not*I site, the addition of *Avr*II and *Cla*I restriction sites and by sequence analysis.
   j. Sequence plasmid DNA to determine direction of the *loxP* site. The directional *loxP* sites must be in the same orientation for excision of the intervening sequence to occur after recombination.

Insertion of 2nd loxP site downstream of gene insertion

5. Synthesize complementary oligonucleotides that contain a *loxP* site (underlined in the sequence below) and generate *Kpn*I overhangs (Note: *Kpn*I was chosen because it is a unique restriction site within the vector only). To aid in the cloning of *ADE2*

between the *loxP* sites and the addition of the NES element, destroy the 3' *Kpn*I restriction site and replace with an *Xho*I restriction site (Note: depending on your sequence, it might be necessary to use a different restriction site).
   a. 5' - CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATC TCG AGC GTA C
   b. 5' - GCT CGA GAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATG GTA C
6. Follow steps 4c to 4h (omit Step 4f) as described above.
7. Screen colonies for addition of *loxP* site by PCR, restriction digest and sequence analysis.

Insertion of ADE2 between the loxP sites

8. Synthesize oligonucleotides to amplify *ADE2* and include restriction sites for *BstE*II and *Kpn*I. {We include six random nucleotides (N) at the end of the primer to increase efficiency of digestion of the PCR product by *BstE*II and *Kpn*I.}
   a. 5' - NNN NNN GGT TAC CCA CCA ACA TAA CAC TGA CAT C
   b. 5' - NNN NNN GGT ACC CCT GAC GTA GCG CTA TCC
9. Set up a 50µl PCR reaction containing 1 – 2µl (0.2-0.4 µg) of *S. cerevisiae* genomic DNA, 1x Pfu Buffer, 50 pmoles primers, 200µM dNTPs. Begin cycling at 94°C for 3 minutes. After denaturation, pause cycle and add 1 µl of *Pfu*. Continue with 30 cycles of 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 3 minutes followed by 72°C for 7 minutes. Check 10% of the PCR reaction on a 1% agarose gel to confirm size and purity of the amplified PCR product.
10. Add 50µl of TE(pH 8.0)-buffered-phenol:$CHCl_3$, vortex, centrifuge on max speed for 1 minute, remove aqueous layer, repeat. Add 50µl $CHCl_3$, vortex, centrifuge on max speed for 1 minute, remove aqueous layer. Add 5µl NaOAc, 200µl EtOH to aqueous layer, place on ice for 10 minutes. Centrifuge on max speed for 5 minutes, wash pellet with 70% EtOH. Resuspend pellet in 50µl H_20. {Alternatively, clean up the remaining PCR reaction using QIAquick PCR Purification Kit following manufacturer's instructions.}
11. Digest 1µg plasmid isolated in Step 7 and 4 µl (~0.2 µg) *ADE2* PCR product islolated above with *BstE*II and *Kpn*I. Clean-up digested vector and PCR product using QIAquick PCR Purification Kit following manufacturer's instructions.
12. Combine 5µl of digested vector, 6µl *ADE2* PCR product, 4µl of T4 Ligase Buffer and 1µl T4 Ligase in 20µl total. Maintain at room temperature for 1 hour.
13. Transform 2µl of the ligation reaction into DH5α according to the manufacturer's instructions.
14. Screen colonies for addition of *ADE2* by restriction digest.

Insertion of HIV-1 Rev NES 3' of 2nd loxP site

15. Synthesize complementary oligonucleotides that contain the HIV-1 Rev NES sequence (underlined in sequence below) with overhangs compatible with *Xho*I, generate a *Not*I restriction site and maintain the reading frame after *loxP* recombination.
   a. 5' – TCG AGT GCC ATT GCA ATT ACC ACC ATT GGA AAG ATT GAC TTT AGA TAG C
   b. 5' – GGC CGC TAT CTA AAG TCA ATC TTT CCA ATG GTG GTA ATT GCA ATG GCA C
16. Follow steps 4c-4h (omit step 4f) as describe above.
17. Screen colonies for addition of domain by PCR, restriction digest and sequence analysis.

Insertion of the 3'-UTR of choice downstream of the domain of choice

18. Synthesize oligonucleotides to amplify the *UPF3* stop codon and 3'-UTR and include restriction sites for *Not*I.
   a. 5' - NNN NGC GGC CGC GTC GAC TAG TAA AAG CTC ATG GCT TCT TAT AT
   b. 5' - NNN NGC GGC CGC GTA CCG TTT TTT AGT TCT ATG GTA ACC GTC TC
19. Set up a 50µl PCR reaction containing 1–2µl (0.2-0.4 µg) of *S. cerevisiae* genomic DNA, 1x Pfu Buffer, 50 pmoles primers, 200µM dNTPs. Begin cycling at 94°C for 3 minutes. After denaturation pause cycle and add 1µl of *Pfu*. Continue with 30 cycles of 94°C 45 seconds, 50°C 45 seconds, 72°C 2 minutes followed by 72°C for 3 minutes.
20. Run 10% of the reaction on a 1% gel to confirm the size. Clean-up the remaining PCR reaction using QIAquick PCR Purification Kit following manufacturer's instructions. Elute PCR product in 30µl H_20. {Alternatively, use the clean-up procedure described in step 10 to purify PCR fragment}.
21. Digest 15µl of the PCR product with *Not*I for 2 hours at 37°C. Clean-up digested PCR product using QIAquick PCR Purification Kit following manufacturer's instructions. Elute the PCR product in 30µl H_20.
22. Digest 1µg of the vector with 1 unit of *Not*I for 2 hours at 37°C. After digestion add 1 unit of calf intestinal alkaline phosphatase (CIP). Incubate at 37°C for 15 minutes. Clean-up digested plasmid using QIAquick PCR Purification Kit following manufacturer's instructions. Elute the vector in 30µl H_20.
23. Combine 4µl of digested vector, 12µl 3'-UTR PCR product, 4µl of T4 Ligase Buffer and 1µl T4 Ligase in 20µl total. Maintain at room temperature for 1 hour.
24. Transform 3µl of the ligation reaction into *E. coli* DH5α following manufacturer's instructions.
25. Screen colonies for addition of 3'UTR by restriction digest and sequence analysis.

Mutagenesis/library construction

1. Synthesize mutagenic oligonucleotide inserting random nucleotides within the four codons of *UPF3* NES-A (see Materials and Methods and Fig. 2 for additional details on NES-A mutagenesis).
   a. 5' – CAA CCG AGA ATG AAG GAT TTA AG(A,C,G) (A,C,T,G)(A,C)G TT(A,C,G) (A,C,T,G)(A,C)A GA(A,C,G) (A,C,T,G)(A,C)(A,C,G) (A,C,T,G) (A,C)C CTC CAA ATT TGA CTG CAG ATG
2. Synthesize second oligonucleotide downstream of NES-A and unique restriction site *Sna*BI.
   a. 5' – CCA GTT CCA ACC AGC TCT G
3. Set up a 50µl PCR reaction containing 0.05µg plasmid generated in Step 25, 1x *Pfu* Buffer, 50 pmoles primers, 200µM dNTP mix. Begin cycling at 94°C for 3 minutes. After denaturation pause cycle and add 1µl of *Pfu*. Continue with 30 cycles of 94°C 45 seconds, 47°C 45 seconds, 72°C 1 minute followed by 72°C for 3 minutes (PCR Round 1 in Fig. 2).
4. Run 10% of the PCR reaction on a 1% agarose gel to confirm PCR product. Clean-up the remaining PCR reaction using Qiagen QIAquick PCR Purification Kit following manufacturer's instructions. Include an additional wash with 750µl of 35% guanidine hydrochloride prior to the Buffer PE wash step. Elute DNA in 50µl of H_20. PCR fragment will be used as a megaprimer in the subsequent PCR reaction.
5. Synthesize T7 oligonucleotide upstream of NES-A and unique restriction site *Sac*II.
   a. 5' – TAA TAC GAC TCA CTA TAG GG
6. Set up a 50µl PCR reaction containing 0.05µg plasmid generated in Step 25, 1x *Pfu* Buffer, 50 pmoles oligonucleotide, 25 - 50µl of megaprimer, 200µM dNTP mix. Begin cycling conditions of 94°C for 3 minutes. After denaturation pause cycle and add 1µl of *Pfu*. Continue with 30 cycles of 94°C 45 seconds, 47°C 45 seconds, 72°C 1 minute followed by 72°C for 3 minutes (PCR Round 2 in Fig. 2).
7. Run 10% of the PCR reaction on a 1% agarose gel to confirm PCR product. Clean-up the remaining PCR reaction using Qiagen QIAquick PCR Purification Kit following manufacturer's instructions. Elute DNA in 50µl of H_20.
8. Amplify PCR fragment using outside oligonucleotides from steps 2 and 5. Set up a 50µl PCR reaction containing 2µl PCR product, 1x Pfu Buffer, 50 pmoles oligonucleotides, 200µM dNTP mix. Begin cycling conditions of 94°C for 3 minutes. After denaturation pause cycle and add 1µl of *Pfu*. Continue with 30 cycles of 94°C 45 seconds, 60°C 45 seconds, 72°C 2 minutes 30 seconds followed by 72°C for 3 minutes.
9. Digest 5µg of PCR fragment containing the mutated sequence in 1x restriction enzyme buffer and 5 units of *Sac*II and *Sna*BI in 100µl total volume at 37°C for 3 hours. Repeat with 5µg of plasmid generated above.
10. Run the digested products on a 1% agarose gel and gel purify the fragments using Qiagen QIAquick Gel Purification Kit following manufacturer's instructions. Elute PCR product and digested vector in 50µl of H_20.
11. Combine 4µl of digested vector, 4µl PCR product, 4µl of T4 Ligase Buffer and 1µl T4 Ligase in 20µl total. Maintain at 15°C overnight. As a control set up the same ligation reaction substituting H_2O for the PCR product.
12. Transform 2µl of ligation reaction in XL-10 Gold Ultracompetent Cells (Stratagene) following manufacturer's instructions. Plate 200µl of the cell suspension onto each of 5 LB plates containing 100µg/ml ampicillin. Grow for 18 hours at 37°C.
13. Pool the cells on the plates by adding 1-2ml of LB containing 100µg/ml ampicillin to all the plates and gently removing the cells using a rounded-off end of a glass pipet.
14. Combine the cells from all plates and isolate plasmid DNA.

Transformation of yeast

To transform yeast by electroporation, use the Genepulser Electoporation apparatus from Biorad. Prepare cells according to (17) as follows:

1. Grow a fresh patch of the strain to be transformed on solid YEPD (yeast extract/dextrose) medium. Cells can be used for about one month.
2. Scrape cells from the patch with a toothpick and suspend in 20µl of 1M sorbitol plus 20mM HEPES.
3. Wash 2x in 1 mM sorbitol plus 20mM HEPES.
4. Pellet and resuspend in a suitable volume of 1M sorbitol plus 20mM HEPES. Each transformation requires a 40 µl cell suspension.
5. Add up to 1 µg of plasmid DNA in no more than 5µl to each transformation and transfer to a 0.2 cm electroporation cuvette.
6. Electroporate with the Biorad Genepulser set to 1.4 kV, 200Ω, 25µF.
7. Immediately add 0.25 ml of 1M sorbitol. Remove from the electroporation cuvette and plate on selective medium.