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Genetics

A strategy for the generation of conditional mutations by protein destabilization

(proteolysis/ubiquitin/fusion proteins/mating type)

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ABSTRACT **Conditional mutations such as temperature-sensitive (*ts*) mutations are important for the analysis of protein function but are often difficult, or impossible, to obtain. Here we present a simple method for generating conditional mutations based on the use of a protein-destabilizing genetic element in combination with systems allowing the induction and repression of gene expression. This genetic cassette can be fused to other protein-coding sequences, and once transcription is turned off and synthesis of the gene product ceases, the preexisting protein is rapidly degraded. We have applied this method to the analysis of the yeast *ARD1* gene product, a subunit of an N-terminal acetyltransferase, and show that a complete loss of ARD1 product can be achieved in less than one generation. Despite the rapid loss of ARD1 protein, there is a prolonged delay in the expression of the *ard1* mutant phenotype, suggesting that the acetylated substrates of ARD1 are metabolically stable and/or exert a long-lasting effect on processes such as the repression of the silent mating type cassettes.**

The fusion of protein-coding sequences to promoters regulated by inducing agents such as galactose, metal ions, or heat shock can be sufficient for the generation of conditional mutations if the basal level of expression is substantially lower than that from the natural promoter. Even in such cases, however, decay of activity after transfer to noninducing conditions may take considerably more than one generation if the protein product is stable. Conditional mutations are most useful when the protein product can be rapidly inactivated; for example, the characterization of a cell division cycle (cdc) phenotype requires that cells arrest in the first generation after deinduction.

In the case of the *ARD1* gene of yeast, very low levels of gene product are sufficient to complement a null mutation; the expression obtained from a *GAL10*::*ARD1* promoter fusion under repressing conditions confers a wild-type phenotype on an *ard1v*-carrying strain. To lower the basal level of *ARD1* expression and to ensure rapid elimination of ARD1 protein after transfer to repressing conditions, we used a genetic cassette designed to destabilize the ARD1 protein. The cassette, fused to the N terminus of the *ARD1* gene, encoded ubiquitin, followed by a 31-amino acid segment of the lac repressor (lacI), followed by a 9-amino acid epitope tag (Fig. 1). The lacI segment is sufficient to signal ubiquitin-dependent protein degradation through the N-terminal recognition pathway (1). Ubiquitin fusion proteins are rapidly processed in yeast cells by a deubiquitinating enzyme to yield free ubiquitin plus the remainder of the fusion protein, and this reaction has been used to generate proteins that differ only in their N-terminal amino acid (1, 2). Two versions of the cassette allow the production of proteins beginning in either methionine, a stabilizing amino acid, or arginine, a severely destabilizing amino acid. To further enhance the utility of this cassette, we included a sequence encoding a short segment of hemagglutinin that is strongly and specifically recognized by a mAb (Fig. 1) (4). The epitope tag allows one to easily measure the half-life and abundance of the protein.

MATERIALS AND METHODS

Plasmid Construction. The construction of plasmid pBaBa4 is diagrammed in Fig. 2. A *Bam*HI–*Hinf* I fragment encoding amino acids 318–346 of lacI was ligated to a synthetic *Hinf*I–*Xba* I fragment coding for an epitope (flu) that is recognized by mAb 12CA5 (4). The resulting *Bam*HI–*Xba* I fragment was cloned between the *Bam*HI and *Xba* I sites in the polylinker of plasmid pGem 3Zf(+) to generate plasmid pGem-flu (Fig. 2*A*). A *Hin*cII–*Sph* I fragment containing the entire ARD1 coding sequence [derived from YCp(ARD)18; ref. 5] was then cloned between the *Hin*cII and *Sph* I sites of pGem-flu to generate pGem-flu+ARD. The sequences between the flu epitope and the beginning of the ARD1 coding region were deleted by oligonucleotide-mediated deletion (6) to generate pfluARD. Plasmid pBaBa4 was then generated by cloning the fluARD-containing *Bam*HI–*Sph* I fragment of pfluARD together with an *Eco*RI–*Bam*HI fragment containing the URA3–upstream activating sequence (UAS) gal–ubiquitin–Xaa (where Xaa represents either methionine or arginine) sequences of pUB23 (2) into the yeast shuttle vector pSE362 (provided by Steve Elledge, Baylor College of Medicine, Houston).

Pulse-Chase Experiments. Wild-type ARD1 protein was expressed from a *GAL10*::*ARD1* promoter fusion; the ARD1 fusion protein containing the destabilizing residue arginine (R–ARD) was expressed from the pBaBa4-R plasmid (Fig. 1). Cells carrying the appropriate plasmid were grown in a medium (7) containing 2% galactose, 0.67% yeast nitrogen base without amino acids (Difco), and amino acids without histidine or methionine until they reached exponential-growth phase. Cultures (10 ml) were harvested and resuspended in 600 μl of the same medium, except that it contained glucose instead of galactose. [^{35}S]Methionine (100 μCi; 1 Ci = 37 GBq) was added, and the cells were incubated at 30°C for 5 min with occasional shaking. After removal of 100 μl of cells, the remainder was harvested by centrifugation and resuspended in 500 μl of the labeling medium containing cycloheximide at 0.5 mg/ml and unlabeled methionine at 0.2 mg/ml. These cultures were incubated at 30°C with occasional shaking, and aliquots (100 μl) were removed at indicated times. Samples were added to 700 μl of IP buffer (150 mM NaCl/50 mM Tris 7.5 (Sigma)/5 mM EDTA/1% Triton) containing pepstatin A (2 μg/ml), aprotonin (10 μg/ml), phosphoramidon (5 μg/ml), chymo-

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Abbreviations: mAb, monoclonal antibody; M–ARD and R–ARD, ARD1 fusion protein containing the stabilizing residue methionine or the destabilizing residue arginine, respectively.

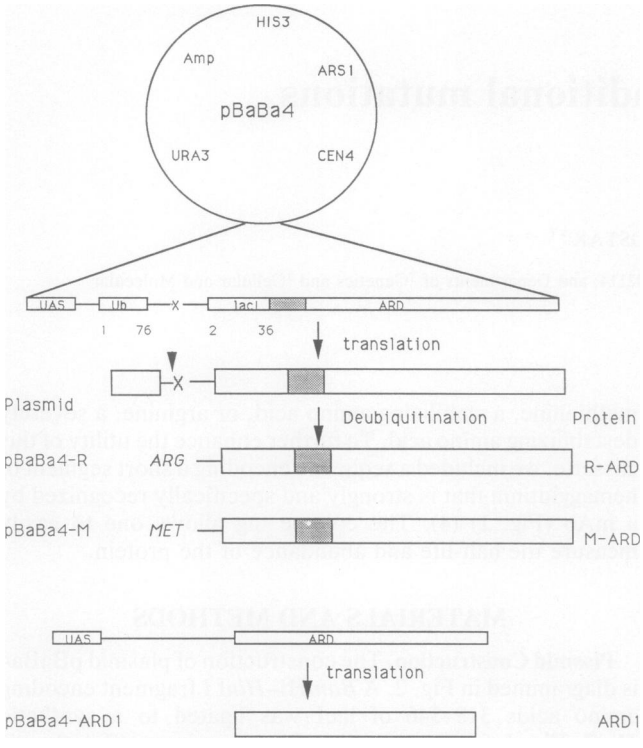


FIG. 1. Schematic representation of plasmid constructs. The arrowhead indicates the site of deubiquitination (1, 2). UAS, upstream activation sequence of the galactose-inducible *GAL10* gene (3); Ub, ubiquitin; X, arginine or methionine. The shaded box represents an epitope from the influenza virus hemagglutinin protein that is recognized by monoclonal antibody (mAb) 12CA5 (4).

statin (20 μg/ml), E-64 (Boehringer Mannheim Biochemicals) (5 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 0.4 ml of glass beads. Cells were disrupted by mixing for 3 min, lysates were centrifuged for 15 min at 12,000 $\times$ *g* at 4°C, and the amount of acid-insoluble ^{35}S cpm in the supernatants was determined. Equal amounts of total acid-insoluble ^{35}S cpm were processed for immunoprecipitation with either anti-ARD1 antibody or the 12CA5 mAb.

Immunoprecipitation. A molar excess of antibody was added to the samples, and they were incubated for 1 hr with rocking at 4°C followed by centrifugation for 10 min at 4°C. The supernatants were incubated with rocking for 30 min with protein A–Trisacryl (Pierce) at 4°C, followed by centrifugation for 1 min. The protein A–Trisacryl pellets were washed three times with IP buffer/0.1% SDS, resuspended in Laemmli sample buffer (8), and heated at 100°C for 3 min; equal portions were subjected to electrophoresis in a 15% discontinuous polyacrylamide/SDS gel with subsequent fluorography.

α-Factor Sensitivity Test. To determine the length of the phenotypic lag for the appearance of α-factor resistance after the loss of ARD1 activity, cultures carrying the BaBa4-R plasmid were grown for two generations after transfer from galactose to glucose, serially diluted 2-fold into yeast extract/peptone/dextrose growth medium, and incubated overnight. The cultures all grew to stationary phase, with each additional 2-fold dilution allowing one or more generation of growth. Optical densities of each culture were measured, and an equal number of cells from each culture were transferred to a 96-well microtiter plate and spotted on plates with a 48-spike prong. The actual number of cell divisions that occurred during incubation was estimated from the optical densities of the starting culture and those after overnight incubation.

RESULTS AND DISCUSSION

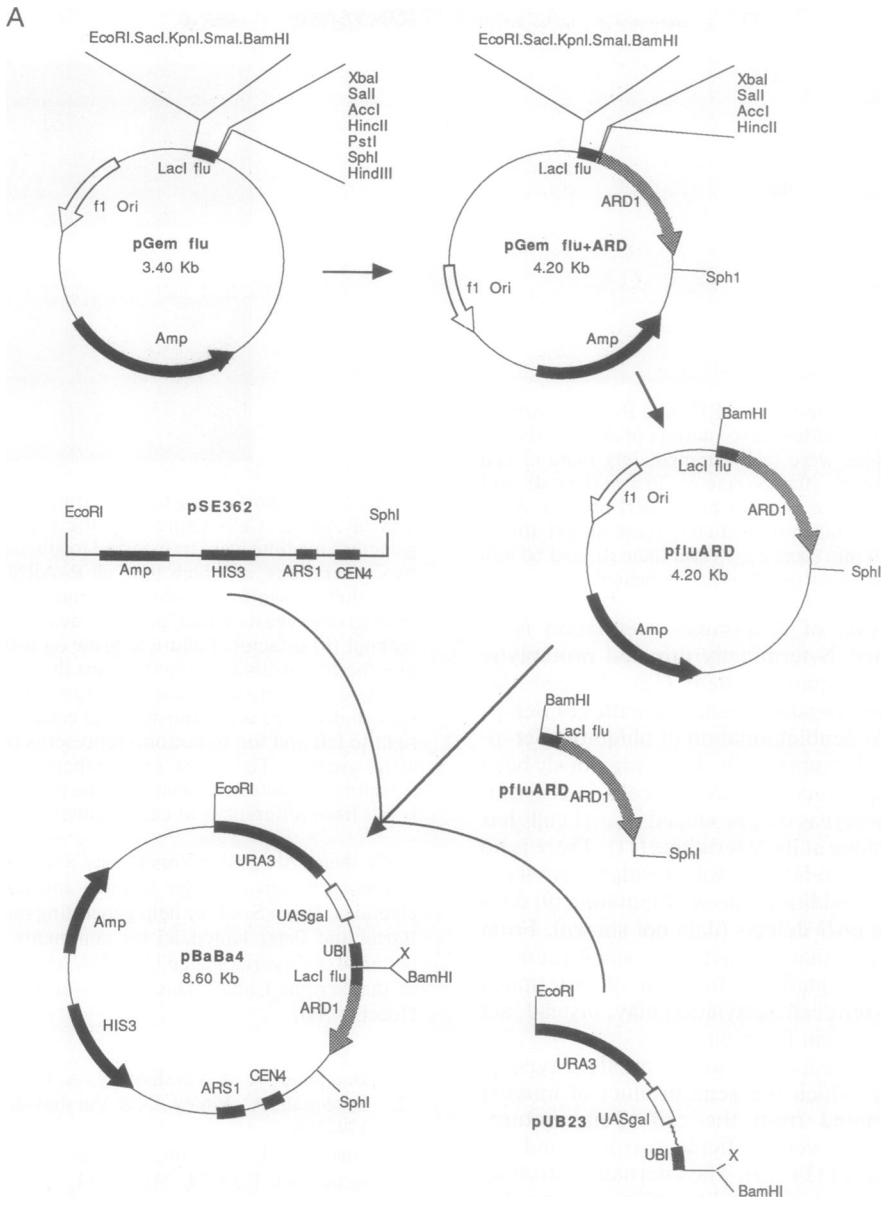
Ubiquitin–lacI–hemagglutinin–ARD1 fusion proteins were made with the cassettes described above (Fig. 1) and expressed in yeast under the control of the *GAL10* promoter (3). The fusion protein N (M-ARD) that has the stabilizing residue methionine (2) at its mature N terminus complements *ard1* mutations both in galactose (inducing conditions) and in glucose (repressing conditions), just as a simple *GAL10*::*ARD1* promoter fusion does. Therefore, the mature fusion protein retains ARD1 activity. In contrast, the fusion protein (R-ARD) that has the destabilizing residue arginine (2) at its mature N terminus complemented the *ard1* defect only in the presence of galactose. This result is consistent with the decreased basal level of expression expected were the mature fusion protein subject to rapid turnover through the N-terminal recognition pathway. Furthermore, when the R–ARD protein is expressed in *ard1 ubr1* double mutant cells, it complemented the *ard1* defect even in the presence of glucose. Because the *ubr1* mutation eliminates all known N-terminal-dependent proteolysis in yeast (9), we infer that the conditional phenotype of the R–ARD fusion is from increased protein turnover.

To demonstrate directly that the conditional phenotype of the R–ARD fusion is from protein destabilization, we carried out a pulse-chase experiment (Fig. 3). The unmodified ARD1 protein is stable over a 30-min chase period (Fig. 3*A*). In contrast, the half-life of the R–ARD protein was found to be <3 min, the earliest time point taken after the chase. These data are consistent with the $\approx$2-min half-life of Arg-β-Gal reported by Bachmair *et al.* (2).

We then determined the half-life of the translatable R-ARD mRNA synthesized during growth in galactose-containing medium. Aliquots were withdrawn at various times after transfer to glucose-containing medium, and cells were pulse-labeled for 5 min. Cell extracts made from these samples were immunoprecipitated with antihemagglutinin antibody and analyzed by SDS/PAGE (Fig. 4). No newly synthesized R–ARD protein was detectable by 30 min after transfer, indicating that both the establishment of glucose repression and mRNA turnover are rapid.

Having established that the R–ARD protein is rapidly destroyed after transfer to glucose-containing medium, we used the R–ARD construct to follow the kinetics of appearance of the α-factor-resistant phenotype of the *ard1* mutation. Whiteway *et al.* (5) have shown that the α-factor resistance of *ard1*-containing cells is a result of the inappropriate expression of the silent mating type locus *HML* in mutant cells. We grew cells expressing R–ARD in galactose-containing medium to exponential-growth phase, then transferred the cells to a glucose-containing medium. The cells were grown in glucose for an additional two generations, and 2-fold serial dilutions were then grown overnight. Cultures that had grown less than approximately nine divisions after transfer remained α-factor sensitive (Fig. 5). Thus, although the ARD1 protein disappears rapidly (less than one generation) after transfer to glucose medium, significant α-factor resistance was seen only after prolonged delay (nine generations).

Manifestation of the α-factor-resistant phenotype requires a certain number of cell divisions, rather than simply a given incubation time. The same pattern of growth retardation by α-factor was obtained when the cultures described above were incubated for another 24 hr at 30°C, showing that the agents responsible for the α-factor sensitivity are metabolically stable. These data indicate that the ARD1 protein is not directly involved in the maintenance of repression of the silent mating type locus because the repressed state is maintained long after the R–ARD protein has been degraded. ARD1 protein is a subunit of an N-terminal acetyltransferase (refs. 10 and 11; unpublished work, E.-C.P and J.W.S.) Therefore, the simplest explanation of the phenotypic lag is that acetylated ARD1 substrates are required for repression of the silent mating-type locus *HML*.



B LacI flu

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      BamHI
      /
 1    GGATCCGGAGCTTGGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCC   60
      CCTAGGCCTCGAACCGACAACGGGCAGAGTGACCACTTTTCTTTTTGGTGGGACCGCGGG
      G  S  G  A  W  L  L  P  V  S  L  V  K  R  K  T  T  L  A  P
                                          HinfI
                                          /
61    AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCCTACCCATACGACGTTCCAGAC   120
      TTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGGATGGGTATGCTGCAAGGTCTG
      N  T  Q  T  A  S  P  R  A  L  A  D  S  Y  P  Y  D  V  P  D
                        XbaI
                        /
121   TACGCTTCTTTGGGTGGTTCTAGA  144
      ATGCGAAGAAACCCACCAAGATCT
      Y  A  S  L  G  G  S  R
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fluARD

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TACCCATACGACGTTCCAGACTACGCTTCTTTGGGTGGTATGCCTATTAATATT
ATGGGTATGCTGCAAGGTCTGATGCGAAGAAACCCACCATACGGATAATTATAA
Y  P  Y  D  V  P  D  Y  A  S  L  G  G  M  P  I  N  I
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FIG. 2. Plasmid pBaBa4. (*A*) Schematic diagram of the construction of pBaBa4. (*B*) (*Upper*) Nucleotide sequence of the *Bam*HI–*Xba* I fragment encoding the lacI-flu segment used in the construction of pBaBa4. (*Lower*) Nucleotide and amino acid sequences of the flu–ARD junction. Amino acids recognized by mAb 12CA5 (4) are underlined, and those of the ARD1-coding region are italicized. UASgal, upstream activation sequence of the galactose-inducible *GAL10* gene; UBI, ubiquitin; ARS, autonomously replicating sequence; Amp, ampicillin resistant.

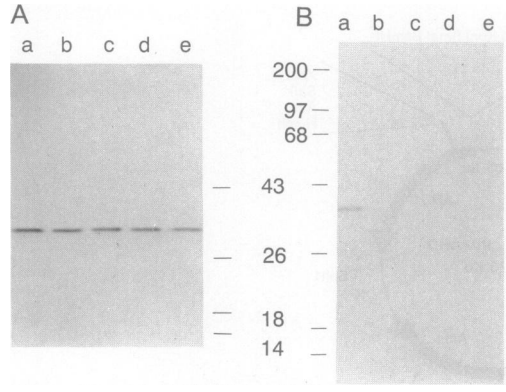

FIG. 3. Determination of stability of ARD1 and R–ARD proteins by pulse-chase analyses. (*A*) Pulse-chase analysis of ARD1 protein. After pulse-labeling samples were taken immediately (lane a) and after a chase of 5 min (lane b), 10 min (lane c), 30 min (lane d), and 60 min (lane e). (*B*) Pulse-chase analysis of R–ARD protein. After pulse-labeling samples were taken immediately (lane a) and after a chase of 3 min (lane b), 10 min (lane c), 30 min (lane d), and 60 min (lane e). Molecular size markers (in kDa) are indicated.

One proposed function of N-terminal acetylation is to stabilize proteins against N-terminally directed proteolytic systems, such as the ubiquitin system (12). To test this hypothesis, we compared β-galactosidase activities of Ser–β-Gal produced by *in vivo* deubiquitination of ubiquitin–Ser–β-Gal (2) in *ARD1*$^+$ and *ard1* mutant cells. It has previously been shown that Ser–β-Gal produced in *ARD1*$^+$ cells is N-terminally blocked (11, 13), whereas that produced in *ard1* cells has an unblocked serine residue at its N terminus (11). There is no significant difference in the relative β-galactosidase activity in *ard1* and *ARD1*$^+$ cells. In addition, the *ubr1* mutation (9) does not suppress any of the *ard1* defects (data not shown). From these results, we conclude that the metabolic stabilization of target proteins is not a significant function of N-terminal acetylation in yeast. N-terminal acetylation may, instead, act as a direct modifier of protein function.

We have used protein destabilization to create a type of conditional mutation in which the gene product of interest can be rapidly eliminated from the cell. Temperature-sensitive mutations can be very difficult to isolate and are often too leaky to be useful (14, 15). The alternative strategy that we have described is free of these limitations and may for most genes be the method of choice for obtaining conditional mutations.

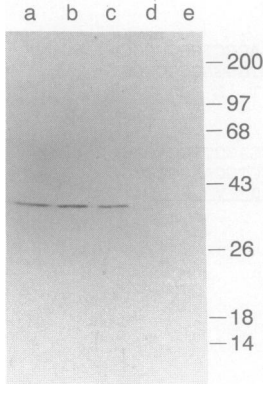

FIG. 4. Determination of translatable mRNA after transfer of cells from galactose to glucose medium. Samples were taken immediately (lane a), 5 min (lane b), 10 min (lane c), 30 min (lane d), and 60 min (lane e) after transfer and pulse-labeled for 5 min. Extracts were prepared and immunoprecipitated as described.

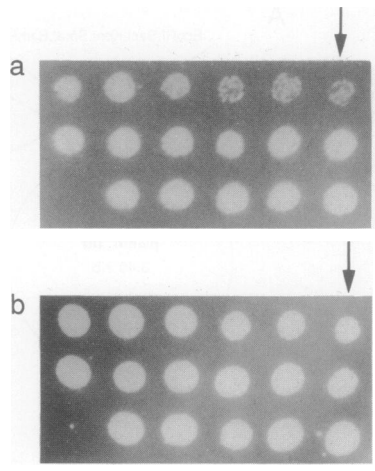

FIG. 5. Phenotypic lag for appearance of α-factor resistance after loss of ARD1. Cells expressing the rapidly degraded R–ARD in galactose medium were transferred to glucose medium and grown for two generations, at which time all R–ARD had decayed. The cells were then grown for an additional number of generations and then spotted onto yeast extract/peptone/dextrose plates either with (*a*) or without (*b*) α-factor. Failure to grow on α-factor indicates retention of α-factor sensitivity; growth means that cells have become α-factor resistant. The arrow indicates a culture estimated to have undergone six cell divisions after transfer, and each spot after the arrow (from right to left and top to bottom) represents one additional generation after transfer. The yeast extract/peptone/dextrose plate lacking α-factor is a control showing that the growth retardation on α-factor is not from differences in cell numbers plated.

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