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In vivo half-life of a protein is a function of its amino-terminal residue

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Science **234**, 179 (1986);
DOI: 10.1126/science.3018930

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48. We thank C. Graham, L. Tomich, and S. Horvath for synthesis of oligonucleotides; C. Prussin for technical assistance; D. Maloney and S. Davis for preparation of sequencing gels; M. DeBruyn for media; C. Katz and C. Elkins for typing; B. Jones for assistance in preparing figures; T. Hunkapiller, B. Arden, R. Barth, M. Kronenberg, E. Meyerowitz, H. Passmore, N. Shastri, G. Siu, L. Smith, and B. Wold for critical discussions during the course of this work and comments on the manuscript. Supported by a postdoctoral fellowship from the Arthritis Foundation (J.A.K.).

2 April 1986; accepted 20 August 1986

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In Vivo Half-Life of a Protein Is a Function of Its Amino-Terminal Residue

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When a chimeric gene encoding a ubiquitin-β-galactosidase fusion protein is expressed in the yeast *Saccharomyces cerevisiae*, ubiquitin is cleaved off the nascent fusion protein, yielding a deubiquitinated β-galactosidase (βgal). With one exception, this cleavage takes place regardless of the nature of the amino acid residue of βgal at the ubiquitin-βgal junction, thereby making it possible to expose different residues at the amino-termini of the otherwise identical βgal proteins. The βgal proteins thus designed have strikingly different half-lives in vivo, from more than 20 hours to less than 3 minutes, depending on the nature of the amino acid at the amino-terminus of βgal. The set of individual amino acids can thus be ordered with respect to the half-lives that they confer on βgal when present at its amino-terminus (the "N-end rule"). The currently known amino-terminal residues in long-lived, noncompartmentalized intracellular proteins from both prokaryotes and eukaryotes belong exclusively to the stabilizing class as predicted by the N-end rule. The function of the previously described posttranslational addition of single amino acids to protein amino-termini may also be accounted for by the N-end rule. Thus the recognition of an amino-terminal residue in a protein may mediate both the metabolic stability of the protein and the potential for regulation of its stability.

In both bacterial and eukaryotic cells, relatively long-lived proteins, whose half-lives are close to or exceed the cell generation time, coexist with proteins whose half-lives can be less than 1 percent of the cell generation time. Rates of intracellular protein degradation are a function of the cell's physiological state, and appear to be controlled differentially for individual proteins (*1*). In particular, damaged and otherwise abnormal proteins are metabolically unstable in vivo (*1*). Although the specific functions of selective protein degradation are in most cases still unknown, it is clear that many regulatory proteins are extremely short-lived in vivo (*1, 2*). Metabolic instability of such proteins allows for rapid adjustment of their intracellular concentrations through regulated changes in rates of their synthesis or degradation. The few instances in which the metabolic instability of an intracellular protein has been shown to be essential for its function include the cII protein of bacteriophage λ and the HO endonuclease of the yeast *Saccharomyces cerevisiae* (*3*).

Most of the selective turnover of intracellular proteins under normal metabolic conditions is adenosine triphosphate (ATP)–dependent and (in eukaryotes) nonlysosomal (*1, 4*). Recent biochemical and genetic evidence indicates that, in eukaryotes, covalent conjugation of ubiquitin to short-lived intracellular proteins is essential for their selective degradation (*1, 5*).

By analogy with the signal sequences that confer on a protein the

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ability to enter distinct cellular compartments (*6*), it could be expected that proteins also contain sets of specific amino acid sequences that alone or in combination would act to determine the half-life of each protein in vivo. A priori, the sequence complexity of such a "half-life rule" might be at least comparable to that of other known signal sequence systems in proteins. Although we still expect the complete half-life rule to be a complex one, we now present evidence that an important component of the rule is startlingly simple.

Rapid in vivo deubiquitination of a nascent ubiquitin-βgal fusion protein. Branched ubiquitin conjugates in which the carboxyl-terminal glycine of ubiquitin moieties is joined via an isopeptide bond to the ε-amino groups of internal lysine residues in proteins apparently comprise the bulk of ubiquitin conjugates in eukaryotic cells (*1, 5, 7*). Joining of ubiquitin to the amino-terminal α-amino groups of target proteins, to yield linear ubiquitin conjugates, may also be chemically feasible (*8*). Whether or not linear ubiquitin-protein fusions are actually synthesized in vivo through posttranslational enzymatic conjugation of ubiquitin to protein amino-termini, such proteins can also be produced by constructing appropriate chimeric genes and expressing them in vivo. Construction of one such gene, which encodes yeast ubiquitin (*9*) linked to βgal of *Escherichia coli*, is shown in Fig. 1. When this gene is expressed in *E. coli*, the resulting βgal-containing protein has an apparent molecular mass which is approximately 6 kD greater than that of the control βgal, a value consistent with the presence of ubiquitin in the protein encoded by the chimeric gene (Fig. 2, lanes a and c). In contrast, when the same gene is expressed in yeast, the corresponding βgal protein is electrophoretically indistinguishable from the control βgal (Fig. 2, lanes a to c). This result is independent of the length of the [^{35}S]methionine labeling period (between 1 and 30 minutes) (legend to Fig. 2). Furthermore, determination of the amino-terminal residue in the putative Met-βgal (half-life, $t_{1/2}$ > 20 hours) by Edman degradation of the in vivo–labeled, gel-purified βgal (Fig. 2, lane d) directly confirmed the presence of the expected Met residue (Fig. 3A and Table 1) at its amino-terminus (*10*). Independent

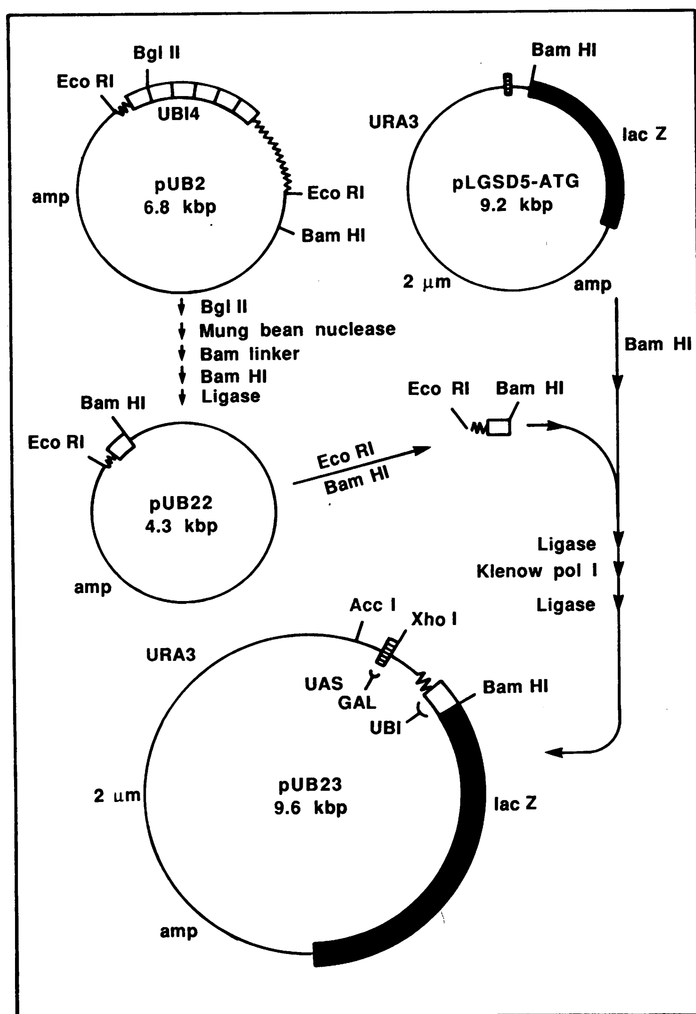

Fig. 1. Construction of a ubiquitin-*lacZ* gene fusion. pUB2, a pBR322-based genomic DNA clone (*9*), contains six repeats of the yeast ubiquitin-coding sequence (open boxes) together with the flanking regions (jagged lines). pUB2 was modified as shown in the diagram by placing a Bam HI site six bases downstream from the first ubiquitin repeat. This allowed the construction of an in-frame fusion (confirmed by nucleotide sequencing) between a single ubiquitin repeat and the *lacZ* gene of the expression vector pLGSD5-ATG [called G2 in (*26*)]. The term "2 μm" denotes a region of the pLGSD-ATG that contains the replication origin and flanking sequences of the yeast plasmid called 2 μm circle (*26*). See Fig. 3B for the amino acid sequence of the fusion protein in the vicinity of the ubiquitin-βgal junction.

Table 1. The N-end rule. In vivo half-lives of βgal proteins in the yeast *S. cerevisiae* were determined either by the pulse-chase technique (for short-lived βgal's, as indicated below) or by measuring the enzymatic activity of βgal (*26*) in crude extracts (*17*). For the measurements of βgal activity, cells growing in a galactose-containing medium (*17*) were transferred to an otherwise identical medium lacking galactose and containing 10 percent glucose. After further growth for at least 5 hours at 30°C, the ratio of total βgal activities before and after shift to glucose was determined for each of the βgal proteins. [*GAL* promoter-driven expression of the fusion genes (Figs. 1 and 3) is repressed in glucose medium (*26*).] For shorter-lived βgal proteins ($t_{1/2}$ <1 hour), the pulse-chase technique was used as well (*14*) (legends to Figs. 2 and 4). Electrophoretic bands of βgal proteins labeled with [^{35}S]methionine in pulse-chase experiments were cut out from scintillant-impregnated dried gels similar to those of Figs. 2 and 4, and ^{35}S in the bands was determined. The in vivo decay of short-lived βgal proteins deviated from first-order kinetics in that the rate of degradation was lower when measured at later (1 hour) time points of the chase, the lower rate reflecting either a time-dependent toxic effect of cycloheximide or intrinsic characteristics of the in vivo degradation process. [Arrest of translation is required for an efficient short-term chase in *S. cerevisiae* because of the amino acid pool equilibration problems related to the presence of vacuoles in this organism (*31*).] The half-life values listed below were determined for the first 10 minutes of chase. We have not yet been able to determine the in vivo half-lives of Cys-βgal, His-βgal, Asn-βgal, Trp-βgal, and Pro-βgal because the first four ubiquitin fusions remain to be constructed, and because ub-Pro-βgal is not significantly deubiquitinated in vivo (see text). Several considerations, including the results of compilation of mature protein amino-termini, suggest that Asn, His, and Trp are destabilizing residues. Furthermore, several lines of evidence (legends to Figs. 4 and 6) suggest that Pro is a stabilizing residue. The listed radii of gyration of amino acids are from (*32*).

Residue X in ub-X-βgal	Radius of gyration of X (Å)	Deubiquitination of ub-X-βgal	$t_{1/2}$ of X-βgal
Met	1.80	+	>20 hours
Ser	1.08	+	
Ala	0.77	+	
Thr	1.24	+	
Val	1.29	+	
Gly	0	+	
Ile	1.56	+	~30 minutes
Glu	1.77	+	
Tyr	2.13	+	~10 minutes
Gln	1.75	+	
Phe	1.90	+	~3 minutes
Leu	1.54	+	
Asp	1.43	+	
Lys	2.08	+	
Arg	2.38	+	~2 minutes
Pro	1.25	−*	~7 minutes

*The rate of in vivo deubiquitination of ub-Pro-βgal is extremely low. The $t_{1/2}$ shown is that of the initial ub-Pro-βgal fusion protein (Fig. 4, lanes j to p).

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evidence that ubiquitin cleavage off the fusion protein occurs immediately after the last Gly residue of ubiquitin is presented below. We conclude that in yeast ubiquitin is efficiently cleaved off the nascent ubiquitin-βgal fusion protein, yielding a deubiquitinated βgal. [The absence of the deubiquitination reaction in *E. coli* (Fig. 2, lane a) is consistent with other lines of evidence indicating that prokaryotes lack both ubiquitin and ubiquitin-specific enzymes (*1, 9*).]

The ubiquitin-βgal junction encoded by the chimeric gene, Gly-Met (Figs. 1 and 3B), is identical to the junctions between adjacent repeats in the polyubiquitin precursor protein (*9*), which is efficiently processed into mature ubiquitin (*9, 11, 12*). Thus it is likely that the same protease, as yet uncharacterized biochemically, is responsible both for the conversion of polyubiquitin into mature ubiquitin and for the deubiquitination of the nascent ubiquitin-βgal protein. If so, one possible way to inhibit the in vivo deubiquitination of the ubiquitin-βgal (and thereby to allow analysis of metabolic consequences of a stable ubiquitin attachment to βgal) would be to convert the Met residue of βgal at the ubiquitin-βgal junction (Fig. 3B) into other amino acid residues (Fig. 3A). The unexpected results of such an approach are described below.

The in vivo half-life of βgal is a function of its amino-terminal residue. The ATG codon that specifies the original Met residue of βgal at the ubiquitin-βgal junction (Fig. 3B) was converted by site-directed mutagenesis (*13–16*) into codons specifying 15 other amino acids (Fig. 3A). These constructions differ exclusively in the first codon of βgal at the ubiquitin-βgal junction (Fig. 3A). After each of the 16 plasmids thus designed was introduced into yeast, analysis of the corresponding βgal proteins pulse-labeled in vivo led to the following results (Figs. 2 and 4 and Table 1):

1) With one exception (see below), the efficient deubiquitination of the nascent ubiquitin-βgal occurs regardless of the nature of the amino acid residue of βgal at the ubiquitin-βgal junction. Thus, the apparently ubiquitin-specific protease that cleaves the original ubiquitin-βgal protein at the Gly-Met junction is generally insensitive to the nature of the first residue of βgal at the junction (Fig. 3A and Table 1). This result, in effect, makes it possible to expose different amino acid residues at the amino-termini of the otherwise identical βgal proteins produced in vivo.

2) The in vivo half-lives of the βgal proteins thus designed vary from more than 20 hours to less than 3 minutes, depending on the nature of the amino acid residue exposed at the amino-terminus of βgal (Figs. 2 and 4 and Table 1). Specifically, deubiquitinated βgal proteins with either Met, Ser, Ala, Thr, Val, or Gly at the amino-terminus have relatively long in vivo half-lives of ~20 hours or more (Fig. 2, lanes d to g, and Table 1), similar to the half-life of a control βgal whose gene had not been fused to that of ubiquitin. In contrast, the βgal proteins with either Arg, Lys, Phe, Leu, or Asp at the amino-terminus have very short half-lives, between approximately 2 minutes for Arg-βgal and approximately 3 minutes for Lys-

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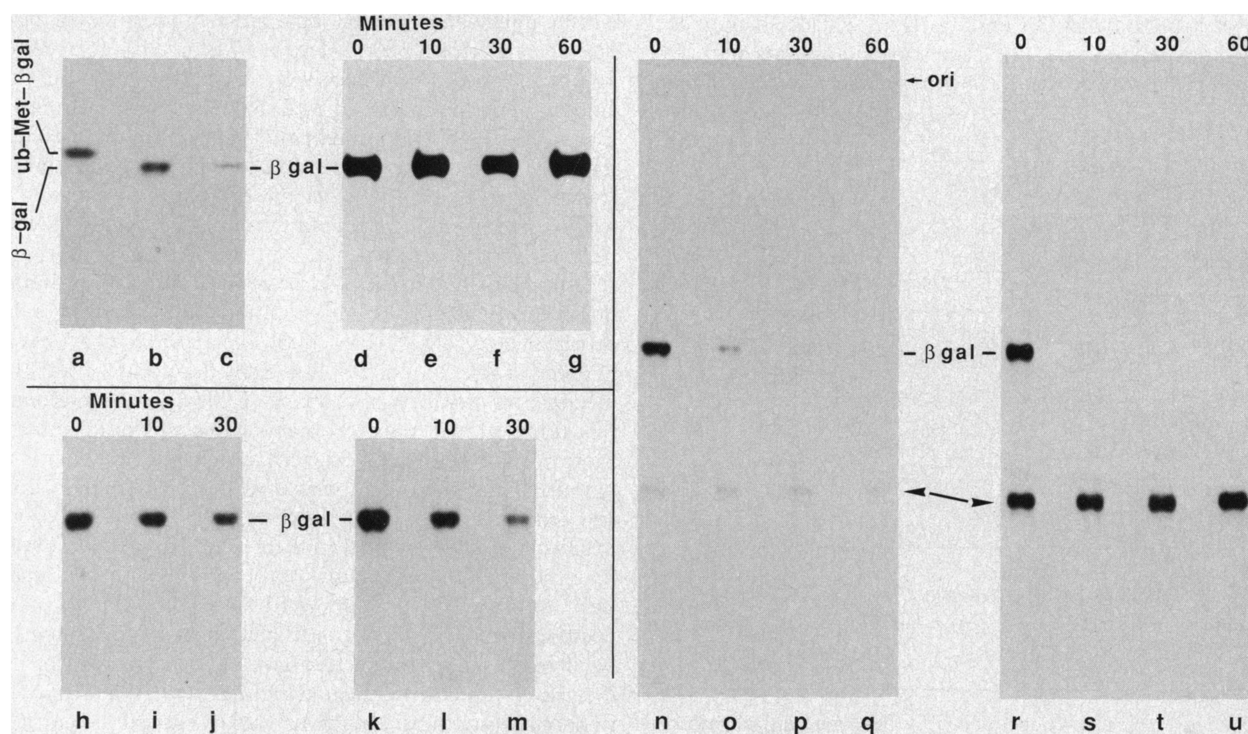

Fig. 2. The in vivo half-life of βgal is a function of its amino-terminal residue. (Lane a) Minicells isolated from an *E. coli* strain carrying pUB23, the initial ub-*lacZ* fusion (Figs. 1 and 3B), were labeled with [^{35}S]methionine for 60 minutes at 36°C, with subsequent analysis of βgal (*27*). The same result was obtained when the labeled minicell SDS extract was combined with an unlabeled yeast SDS extract before immunoprecipitation of βgal. (Lane b) *Saccharomyces cerevisiae* cells carrying pUB23 (Fig. 1), which encodes ub-Met-βgal (Fig. 3B), were labeled with [^{35}S]methionine for 5 minutes at 30°C, with subsequent analysis of βgal (*17*). The same result was obtained with the lengths of the [^{35}S]methionine labeling periods from 1 to 30 minutes, and with yeast extracts produced either by mechanical disruption of cells in the presence of protease inhibitors or by boiling the cells directly in an SDS-containing buffer (*17*). (Lane c) Same as lane a, but with *E. coli* cells carrying the control plasmid pLGSD5 [called G1 in (*26*)], which encodes βgal. (Lanes d to g) *Saccharomyces cerevisiae* cells carrying pUB23 (Fig. 1), which encodes ub-Met-βgal (Fig. 3A), were labeled with [^{35}S]methionine for 5 minutes at 30°C (lane d) followed by a chase in the presence of cycloheximide for 10, 30, and 60 minutes (lanes e to g), extraction, immunoprecipitation, and analysis of βgal (*17*). (Lanes h to j) Same as lanes d to f but with ub-Ile-βgal [see Fig. 3A and (*13*)]. (Lanes k to m) Same as lanes h to j, but with ub-Gln-βgal. (Lanes n to q) Same as lanes d to g, but with ub-Leu-βgal. (Lanes r to u) Same as lanes d to g, but with ub-Arg-βgal. Designations: ori; origin of the separating gel; ub, ubiquitin; βgal, an electrophoretic band of the βgal protein containing a specified amino-terminal residue; in this terminology, the Met-βgal portion of ub-Met-βgal is designated as βgal. Arrowheads denote a metabolically stable, ~90-kD degradation product of βgal, which is formed apparently as the result of an in vivo endoproteolytic cleavage of a proportion of short-lived βgal proteins such as Leu-βgal and Arg-βgal (lanes n to u).

Table 2. Amino-terminal location of an amino acid is essential for its effect on βgal half-life. The insertion mutants were obtained essentially as described in (*13*) for the initial set of mutants, except that a 32-residue oligonucleotide, 5′-CCCGGGGATCCGTGC (G/C/T/) (G/T) CATACCACCTCTTAG was used, containing 14 bases on the 5′ side and 15 bases on the 3′ side of the ambiguous codon inserted behind the Met codon. Bases in parentheses denote ambiguities at the positions 16 and 17 in the sequence. Half-lives of the corresponding βgal proteins were determined as described in the legend to Table 1.

Fusion protein	$t_{1/2}$ of deubiquitinated fusion protein
ub▾ Thr - βgal	>20 hours
ub▾ Met - Thr - βgal	>20 hours
ub▾ Gln - βgal	≈10 minutes
ub▾ Met - Gln - βgal	>20 hours
ub▾ Lys - βgal	≈3 minutes
ub▾ Met - Lys - βgal	>20 hours
ub▾ Arg - βgal	≈2 minutes
ub▾ Met - Arg - βgal	>20 hours
ub - Pro - βgal	≈7 minutes*
ub▾ Met - Pro - βgal	>20 hours

*See note at end of Table 1.

βgal, Phe-βgal, Leu-βgal, and Asp-βgal (Fig. 2, lanes n to u, and Table 1). The half-life of βgal proteins with amino-terminal residues of either Gln or Tyr is approximately 10 minutes (Fig. 2, lanes k to m, and Table 1), while an amino-terminal Ile or Glu confers on βgal a half-life of approximately 30 minutes (Fig. 2, lanes h to j, and Table 1). Both pulse-chase (*17*) and continuous labeling (*18*) techniques were used in these experiments and yielded similar results.

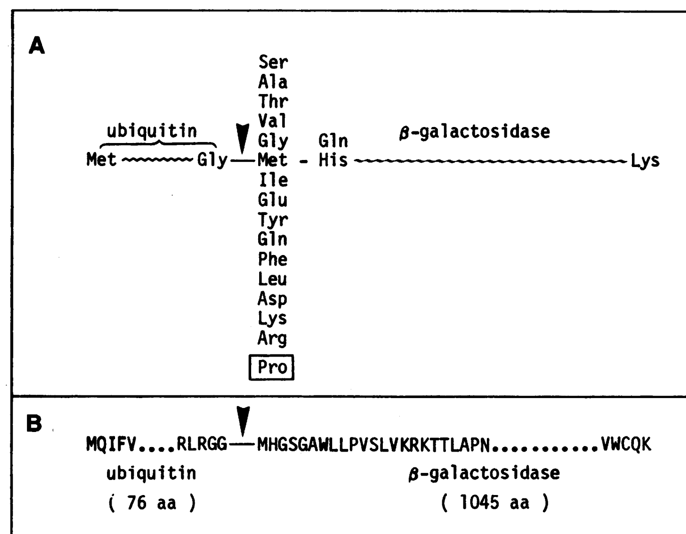

Fig. 3. Changing amino acid residues of βgal at the ubiquitin-βgal junction. (A) The initial plasmid, pUB23 (Fig. 1), which encodes ub-Met-βgal, was mutagenized as described (*13*) to convert the original Met codon ATG at the ub-βgal junction into codons specifying 15 amino acids other than Met. The arrowhead indicates the site of the deubiquitinating in vivo cleavage in the nascent fusion protein that occurs with all of the fusion proteins except ub-Pro-βgal (see text). All of the constructions shown encode His as the second βgal residue. In addition, in some of the constructions (ub-Met-His-Gly-βgal, ub-Met-Gln-Gly-βgal, and ub-Met-Gln-His-Gly-βgal, the last one produced by an insertion mutation; see Table 2), either His or Gln followed Met at the ubiquitin-βgal junction, with indistinguishable consequences for the metabolic stabilities of the corresponding βgal proteins. (B) The amino acid (aa) sequence (in single-letter abbreviations) of ub-Met-βgal, the initial fusion protein (Fig. 1), in the vicinity of the ub-βgal junction. Single-letter amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

We conclude that the set of individual amino acids can be ordered with respect to the half-lives that they confer on βgal when exposed at its amino-terminus. The resulting rule (Table 1) is referred to below as the "N-end rule."

Amino-terminal location of an amino acid is essential for its effect on βgal half-life. We used site-directed mutagenesis to insert a codon specifying a "stabilizing" amino acid (in this experiment, the Met residue) before the first codon of βgal at the ubiquitin-βgal junction (Table 2). Insertion of a stabilizing residue (Met) before either another stabilizing residue (Thr) or a variety of destabilizing residues (Gln, Lys, and Arg) at the ubiquitin-βgal junction invariably results in a long-lived deubiquitinated βgal (Table 2). Furthermore, in contrast to ubiquitin-Pro-βgal, which is not only short-lived but also resistant to deubiquitination (Fig. 4, lanes j to p, and Table 1), ubiquitin-Met-Pro-βgal is efficiently deubiquitinated in vivo to yield a long-lived Met-Pro-βgal (Table 2).

These results show that both the identity of an amino acid residue and its amino-terminal location (presumably the presence of a free α-amino group) are essential for its effect on βgal half-life. In addition, these results (Table 2) further support the expectation that ubiquitin cleavage off the fusion protein occurs immediately after the last Gly residue of ubiquitin (Fig. 3A).

A long-lived cleavage product of βgal is formed during decay of short-lived βgal proteins. The electrophoretic patterns of short-lived (but not of long-lived) βgal proteins invariably contain a specific ~90-kD cleavage product of βgal (Fig. 2, lanes n to u) which, unlike the parental βgal species, accumulates during the postlabeling (chase) period (Fig. 4, lanes m to p). The ~90-kD βgal fragment constitutes a relatively small proportion of the initial amount of the pulse-labeled βgal. Nonetheless, its existence implies that an in vivo endoproteolytic cleavage can rescue a protein fragment from the metabolic fate of its short-lived parental protein. It remains to be seen whether the resulting possibility of multiple half-lives within a single protein species is exploited in the design of naturally short-lived proteins.

Ubiquitin-βgal is short-lived when not deubiquitinated. Ubiquitin-Pro-βgal, the only ubiquitin-βgal fusion that is not deubiquitinated in vivo (Fig. 4, lanes j to p), has a half-life of approximately 7 minutes (Table 1) which is less than 1 percent of the half-life of metabolically stable βgal proteins (Table 1). One interpretation of this result is that a metabolically stable ubiquitin attachment to protein amino-termini is sufficient to signal degradation of acceptor proteins. This interpretation is consistent with earlier biochemical and genetic evidence that ubiquitination of short-lived proteins in a mammalian cell is essential to their degradation (*1, 5*). At the same time, all ubiquitin-βgal fusion proteins other than ubiquitin-Pro-βgal are rapidly deubiquitinated in vivo (Table 1). Thus, the posttranslational amino-terminal ubiquitination of proteins may not be involved in an initial recognition or commitment step that designates proteins for degradation in vivo. Whether posttranslational amino-terminal ubiquitination (if it actually occurs in vivo) is essential for later stages of the degradation pathway remains to be determined. Earlier in vitro experiments indicated that preferential chemical modification of amino-termini of proteolytic substrates inhibits their degradation in an in vitro ubiquitin-dependent proteolytic system (*8*). On the basis of these data, it was proposed that amino-terminal ubiquitination of proteins is essential for their degradation (*8*). An alternative interpretation of the same results is that chemical blocking of proteins' amino-termini prevents the recognition of their amino-terminal residues by the N-end rule pathway whose initial stages are not necessarily ubiquitin-dependent.

Short-lived βgal proteins are multiply ubiquitinated in vivo. Overexposures of the pulse-chase fluorograms (Fig. 2) reveal that

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the major band of a deubiquitinated, short-lived βgal protein coexists with a "ladder" of larger molecular mass, βgal-containing bands irregularly spaced at 4- to 7-kD intervals (Fig. 4, lanes c to g). No such larger species appear when the fluorograms of long-lived βgal proteins are similarly overexposed (Fig. 4, lanes a and b). Immunological analysis with both antibodies to βgal and antibodies to ubiquitin demonstrates that the ladder βgal species contain ubiquitin (Fig. 5).

A model for the selective degradation pathway. With the exception of natural or engineered ubiquitin fusion proteins (*9, 11*) (Fig. 1 and Table 1), nascent proteins apparently lack ubiquitin moieties. The in vivo amino-terminal processing of nascent noncompartmentalized proteins generates their mature amino-termini via the action of amino-terminal peptidases whose substrate specificities have been partially characterized (*19*). We suggest that the amino-termini thus generated are recognized by an "N-end–reading" enzyme. One specific model is that a commitment to degrade a protein molecule is made as a result of the recognition of its amino-terminal residue by a stochastically operating enzyme whose probability of "clamping" at the target's amino-terminus is determined by the N-end rule (Table 1). Once the commitment is made, it is followed by a highly processive ubiquitination (possibly from the amino-terminal toward the carboxyl-terminal region) of the target protein which in the case of βgal is conjugated to more than 15 ubiquitin moieties per molecule of βgal (Fig. 4, lanes c to g, and Fig. 5). The multiply ubiquitinated target protein is then degraded by a "downstream" enzyme (*1*) for which the ubiquitin moieties of the target serve as either recognition signals or denaturation (unfolding) devices, or both.

The ubiquitin-containing ladder βgal species (Fig. 4, lanes c to l, and Fig. 5) are apparently branched ubiquitin conjugates (*1, 5, 7*) in which the carboxyl-terminal glycine of ubiquitin moieties is joined to the ϵ-amino groups of internal lysine residues in βgal. Surprisingly, the ladder βgal species derived from ubiquitin-Pro-βgal are electrophoretically indistinguishable from the analogous species of βgal whose amino-terminal ubiquitin is cleaved off the nascent fusion protein (Fig. 4, lanes j to l, and Fig. 5). If the electrophoretically indistinguishable ubiquitinated βgal species are indeed structurally homologous, these results would be compatible with two alternative models in which, immediately after the first ubiquitins are branch-conjugated to βgal, either a branch-ubiquitinated ubiquitin-Pro-βgal undergoes amino-terminal deubiquitination or, alternatively, an analogous βgal species lacking the amino-terminal ubiquitin moiety reacquires it. Experimental resolution of this ambiguity may establish whether the posttranslational amino-terminal ubiquitination of proteins (if it occurs in vivo) plays a role in the selective protein turnover.

Although both prokaryotic and eukaryotic proteins appear to follow the N-end rule (see below), bacteria apparently lack the ubiquitin system. Thus it is possible that the hypothetical N-end–recognizing protein is more strongly conserved between prokaryotes and eukaryotes than is the rest of the selective degradation pathway. Interestingly, the properties of a mammalian protein E3 whose presence is required for ubiquitination of proteolytic substrates by ubiquitin-conjugating enzymes in vitro (*1, 7, 8*) are consistent with its being a component of the N-end–recognizing protein.

The N-end rule and the known amino-termini of intracellular proteins. The unblocked amino-terminal residues in metabolically

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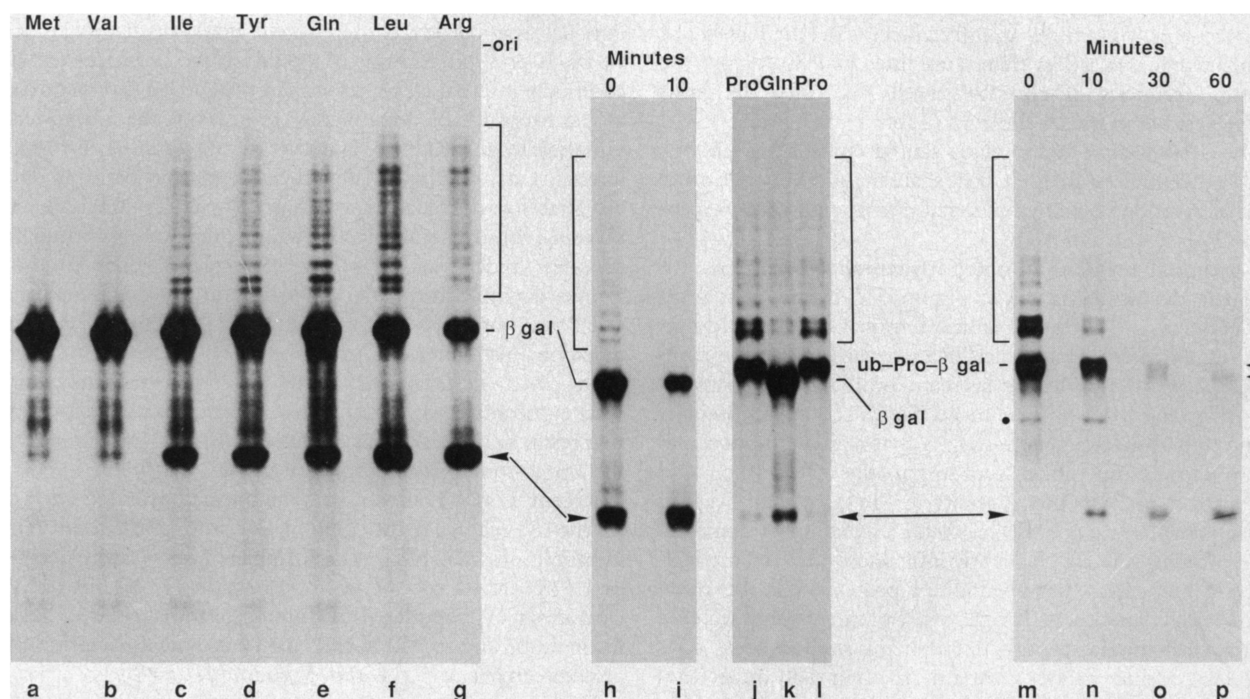

Fig. 4. Ubiquitin-βgal is short-lived if not deubiquitinated. (Lanes a to g) *Saccharomyces cerevisiae* cells carrying plasmids encoding ub-X-βgal fusion proteins in which X is the residue indicated at the top of each lane were labeled for 5 minutes at 30°C with [^{35}S]methionine, with subsequent extraction, immunoprecipitation, and analysis of βgal (*17*). Fluorographic exposures for these lanes were several times longer than those for similar patterns in Fig. 2 to reveal the multiple ubiquitination of short-lived βgal proteins. (Lanes h and i) Fluorographic overexposure of lanes n and o in Fig. 2 reveals the ladder of multiply ubiquitinated Leu-βgal proteins in a pulse-chase experiment (zero and 10 minutes chase, respectively). (Lane j) Same as lanes a to g, but with ub-Pro-βgal. (Lane k) Same as lane j but with ub-Gln-βgal. (Lane l) Same as lane j. (Lanes m to p) *Saccharomyces cerevisiae* cells carrying a plasmid encoding ub-Pro-βgal were labeled for 5 minutes at 30°C with [^{35}S]methionine (lane m) followed by a chase in the presence of cycloheximide for 10, 30, and 60 minutes (lanes n to p) (*17*). The upper small arrow to the right of lane p denotes ub-Pro-βgal, a small proportion of which is still present after a 1-hour chase. The lower small arrow indicates an apparently deubiquitinated Pro-βgal that slowly accumulates during chase and is metabolically stable. The dot to the left of lane m denotes an endogenous yeast protein that is precipitated in some experiments by the antibody used (*17*). Square brackets denote the multiply ubiquitinated β-gal species (see Fig. 5). Other designations are as in Fig. 2.

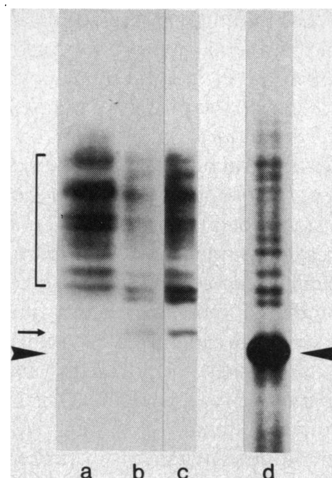

Fig. 5. The "ladder" βgal species contain ubiquitin. (Lane a) *Saccharomyces cerevisiae* cells carrying a plasmid which encodes ub-Gln-βgal (*13*) were grown and disrupted, and the extracts processed for isolation of βgal proteins by affinity chromatography on a column with immobilized antibody to βgal (*28*). The βgal proteins thus obtained were subjected to electrophoresis in a polyacrylamide-SDS gel, transferred to nitrocellulose, and probed with an antibody to ubiquitin (*28, 29*). (Lane b) Same as lane a but with ub-Pro-βgal. (Lane c) Same as lane b but a longer autoradiographic exposure. (Lane d) *Saccharomyces cerevisiae* cells carrying a plasmid that encodes ub-Leu-βgal (*13*) were labeled with [^{35}S]methionine for 5 minutes, with subsequent extraction, immunoprecipitation, and electrophoresis of βgal (*17*) (the same sample as in Fig. 4, lane f). Square brackets denote the multiply ubiquitinated Gln-βgal species detected with antibody to ubiquitin. The arrow indicates the band of ub-Pro-βgal, the initial fusion protein seen in lanes b and c. The arrowheads indicate the position of the band of deubiquitinated βgal (detectable by either Coomassie staining or metabolic labeling but not by antibody to ubiquitin) derived from the ub-Gln-βgal fusion protein.

stable, noncompartmentalized proteins from both prokaryotes and eukaryotes are exclusively (Fig. 6A) of the stabilizing class (Met, Ser, Ala, Gly, Thr, Val), that is, the class that confers long in vivo half-lives on βgal (Table 1). The one short-lived intracellular protein for which the mature amino-terminus is known is the cII protein of phage λ, the central component of a trigger that determines whether λ grows lytically or lysogenizes an infected cell (*3*). The half-life of cII in λ-infected *E. coli* is less than 3 minutes (*3*). Strikingly, the mature amino-terminus of cII starts with Arg (*20*), the most destabilizing residue in the N-end rule (Table 1).

While the destabilizing amino acids can be either hydrophobic, uncharged hydrophilic or charged, they share the property of having larger radii of gyration than any of the stabilizing amino acids except Met (Table 1) [see also (*19*)].

Amino-terminal residues in compartmentalized proteins are largely of the destabilizing class. Figure 6 illustrates a striking difference between the choice of amino-terminal residues in long-lived, noncompartmentalized intracellular proteins (A) and in compartmentalized proteins, such as secreted proteins (B), many of which are also long-lived in their respective extracellular compartments. One implication of this finding is that a single intracellular degradation pathway operating according to the N-end rule could be responsible both for the diversity of in vivo half-lives of intracellular proteins and for the selective destruction of compartmentalized proteins that are aberrantly introduced into the intracellular space. Some miscompartmentalized proteins may be more harmful to the cell than others. It is therefore of interest that secreted eukaryotic toxins contain strongly destabilizing residues (Arg, Lys, Leu, Phe, Asp) at their amino-termini more often than the general population of secreted proteins (Fig. 6, B to D).

The above considerations also suggest that, if the topological outside of a cell, such as lumens of the endoplasmic reticulum and Golgi, and the extracellular space, were to have degradation pathways analogous to the N-end rule pathway, they could be based on inverted versions of the N-end rule in which the amino-terminal residues that are destabilizing inside the cell are now the stabilizing ones and vice versa.

Possible role of the N-end rule pathway in the turnover of long-lived proteins. Long-lived intracellular proteins with destabilizing (Table 1) penultimate residues generally retain their initial amino-terminal methionine (*19*). As is discussed above (Fig. 6A), amino-terminal residues in long-lived intracellular proteins that do undergo amino-terminal processing are invariably of the stabilizing class (Table 1). An interesting possibility that would involve the N-end rule pathway in the turnover of long-lived proteins is that the rate-limiting step in the in vivo degradation of long-lived proteins may be a slow aminopeptidase cleavage that exposes a destabilizing residue, followed by rapid degradation via the N-end rule pathway. Fine-tuning of the rate of degradation may in this case be a function of the rate of aminopeptidase cleavage that exposes a destabilizing residue, rather than a function of the residue's destabilizing capacity according to the N-end rule.

The N-end rule and selective degradation of short-lived and damaged proteins. The recognition of polypeptide chain folding patterns or of local chemical features that target an otherwise long-lived but damaged protein for selective degradation in vivo is unlikely to be mediated directly by the N-end rule pathway. Instead, we suggest that specific proteases (analogous in function to nucleases that recognize specific lesions in DNA) cleave a targeted protein so as to expose a destabilizing residue at the amino-terminus of one of the two products of a cut. One testable prediction of this model is that the initial cleavage products of the degradation pathway should bear destabilizing residues at the amino-termini. The preferential exposure of destabilizing residues at the amino-termini of products of the initial protein cleavages may be due either to intrinsic specificities of the proteases involved or simply to the fact that most of the amino acids belong to the destabilizing class (Table 1). Furthermore, initial cleavages of a protein would be expected to destabilize aspects of its original conformation, thus increasing the probability of further internal cuts. Whether the initial cleavage products of a protein would be degraded exclusively via the N-end rule pathway or would have to be processed further by additional internal cleavages should depend on several factors, such as the exposure of destabilizing residues at the amino-termini of initial cleavage products, and the relative rates of introduction of internal cuts. In this model, the N-end rule pathway should be essential for degradation of most of the metabolically unstable proteins, from chemically damaged, prematurely terminated, improperly folded, and miscompartmentalized ones to those that cannot assemble into native multisubunit aggregates, and finally to otherwise normal proteins that are short-lived in vivo. Thus, the metabolic instability of a protein may be mediated not only by the exposure of a destabilizing residue at its amino-terminus, but also by local conformational and chemical features of its polypeptide chain that result in proteolytic cleavages exposing destabilizing residues at the amino-termini of cleavage products.

Recent work has shown that the purified protease La [a product of the *E. coli lon* gene (*3*)] cleaves the purified protein N [a metabolically unstable protein of phage λ, and an in vivo substrate of La (*3*)] at six specific positions within N. All but one of these cleavages yield peptides whose amino-terminal residues are destabilizing according to the N-end rule of the present work, in remarkable agreement with the above model (*21*).

For any given protein, a variety of factors in addition to the N-end rule may combine to modulate its half-life in vivo. Among such factors may be the flexibility and accessibility of the protein's amino-terminus (*22*), the presence of chemically blocking amino-terminal groups such as the acetyl group, the distribution of ubiquitinatable lysine residues near the amino-terminus, and other variables, such as the structure of the carboxyl-terminus. Since amino-terminal regions of multisubunit proteins are commonly involved in the interfaces between subunits (*22*), quaternary structure of proteins is yet another parameter that is expected to modulate the impact of the N-

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end rule pathway on protein half-lives in vivo. Finally, as suggested above, the N-end rule pathway may also be essential for the degradation of proteins whose initial recognition as targets for degradation is independent of the structures at their amino-termini.

Functional significance of posttranslational addition of amino acids to amino-termini of proteins. It has been known for many years that in both bacteria and eukaryotes there exists an unusual class of enzymes, aminoacyl-transfer RNA-protein transferases, which catalyze posttranslational conjugation of specific amino acids to the mature amino-termini of acceptor proteins in vitro (*23*). The posttranslational addition of amino acids to proteins in vivo dramatically accelerates in a stressed or regenerating tissue, for example, after physical injury to axons of nerve cells (*24*). The N-end rule provides an explanation for this phenomenon. We suggest that selective changes in metabolic stability of otherwise undamaged, long-lived proteins that may be required by a changed physiological state of the cell are brought about by posttranslational addition of destabilizing amino acids to the amino-termini of target proteins in vivo. Strikingly, the known reactions of posttranslational addition of amino acids to proteins (*23, 24*) involve largely those amino acids (Arg, Lys, Leu, Phe, and Tyr) that are destabilizing according to the N-end rule (Table 1). Physiological states in which addition of destabilizing amino acids to proteins could be expected to occur include entry to and exit from the cell cycle, responses to chemical or physical stress, and specific differentiation events, such as erythroid differentiation and spermatogenesis, in which a proportion of preexisting, otherwise long-lived intracellular proteins is selectively degraded.

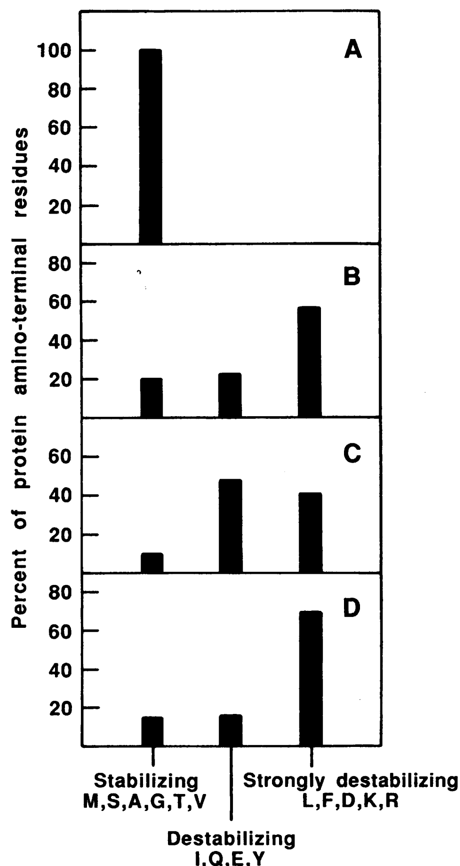

Fig. 6. Both prokaryotic and eukaryotic long-lived intracellular proteins have stabilizing amino acid residues at their amino-termini, whereas secreted proteins exhibit a complementary bias. (A) Long-lived, directly sequenced, intracellular (noncompartmentalized) proteins with unblocked amino-termini from both prokaryotes (77 proteins) and eukaryotes (131 proteins) were distributed into three groups according to the nature of their amino-terminal residues as defined by the N-end rule (Table 1). All 208 of the long-lived intracellular proteins examined bear exclusively stabilizing residues at their amino-termini. (B to D) Analogous diagrams are presented for 243 secreted eukaryotic proteins (B), for 37 light and heavy immunoglobulin chains (C), and for 94 secreted eukaryotic toxins (D). Entries in C and D are subsets of entries in B. For proteins in B to D, the amino-termini compiled correspond, whenever the assignment is possible, to the most processed form of a protein that is still located within a secreting cell. The data in A to D were manually compiled from the entire set of complete protein sequences available before 1981 (*30*). The same conclusions have been reached after a more detailed and extensive, computer-assisted tabulation of protein amino-termini with the use of the current National Biomedical Research Foundation database. The amino-terminal residues of Asn, Cys, His, and Trp were excluded from the compilation because in vivo half-lives of the corresponding βgal proteins are still unknown (see, however, the legend to Table 1). Although the amino-terminal Pro was also excluded from the compilation, Pro appears to be a stabilizing residue for βgal (legends to Fig. 4 and Table 1), consistent with the frequent presence of Pro at the amino-termini of long-lived noncompartmentalized proteins (*30*). The single-letter amino acid abbreviations are given in the legend to Fig. 3.

The in vitro degradation of some proteolytic substrates in a ubiquitin-dependent system from mammalian reticulocytes has been shown to depend on the presence of certain aminoacyl-tRNA's (*25*). We suggest that this phenomenon also reflects a requirement for posttranslational addition of specific destabilizing amino acids to the amino-termini of proteolytic substrates. The initial proteolytic substrates in question (*25*) have amino-terminal residues of Asp or Glu, both of which are destabilizing according to the N-end rule (Table 1). This raises an interesting and testable possibility that certain amino-terminal residues in proteins may not be directly destabilizing as such but only through their ability to be conjugated to other destabilizing residues.

In summary, the recognition of an amino-terminal residue in a protein appears to mediate both the metabolic stability of the protein and the potential for regulation of its stability. These insights have a number of functional and practical implications, some of which have been discussed above.

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10. *Saccharomyces cerevisiae* cells carrying pUB23 (Fig. 1), which encodes ub-Met-βgal (Fig. 3A), were labeled with [^{35}S]methionine; the cells were extracted, and βgal was purified by immunoprecipitation, and electrophoresis as described (*17*). The wet polyacrylamide gel was subjected to autoradiography, the band of βgal was excised, and the electroeluted βgal was subjected to six cycles of radiochemical sequencing by Edman degradation. (The sequencing was carried out by W. Lane at the MicroChem Facility of Harvard University.)
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13. pUB23 (Fig. 1) was treated sequentially with Acc I, the Klenow fragment of pol I, and Bam HI. A fragment containing the Xho I site was purified and inserted between a filled-in Hind III site and a Bam HI site of the M13mp9 phage DNA (*14*). Site-directed mutagenesis (*15*) was carried out as described in (*16*) (with minor modifications), with the use of a synthetic 25-residue oligodeoxyribonucleotide containing 10 bases on the 5′ side and 12 bases on the 3′ side of the Met codon of βgal. All four bases were allowed to occur at the original Met codon positions during synthesis. Primary phage plaques were screened by hybridization (*33*), with the use of a 12-residue oligonucleotide probe spanning the region of codon changes and hybridizing to the original sequence. Nonhybridizing plaques containing inserts of the expected size were sequenced by the chain termination method (*34*). To transfer the desired constructs into the pUB23 background, we treated the replicative form DNA of mutant phages with Xho I and Bam HI, and added it to the same digest of the plasmid pLGSD5-ATG (Fig. 1) (*26*). The ligated mixture was used to transform the *E. coli* strain MC1061 (*35*). Colonies containing plasmids of interest (in which the open reading frame of βgal had been restored) were recognized by their light blue color on X-gal plates.
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17. *Saccharomyces cerevisiae* cells of the strain BWG-9a-1 (*MATα his4 ura3 ade6*), transformed (*31*) with plasmids of interest were grown at 30°C to A_{600} of approximately 5 in a medium of 2 percent galactose, 0.67 percent Yeast Nitrogen Base without amino acids (Difco), adenine (10 μg/ml) and amino acids including methionine (*31*). Typically, cells from a 5-ml culture were harvested by filtration through the well of a Millipore microtiter filtration plate, washed several times on the filter with the same medium lacking methionine, and resuspended in 0.3 ml of 1 percent galactose, 40 m*M* potassium phosphate buffer (*p*H 7.4). [^{35}S]Methionine (50 to 100 μCi) was then added, and the cells were held for 5 minutes at 30°C; the cells were collected by filtration and resuspended in 0.4 ml of the growth medium containing cycloheximide at 0.5 mg/ml. Samples (0.1 ml) were withdrawn at indicated times, and added to 0.75 ml of cold buffer A (*27*) containing leupeptin, pepstatin A, antipain, aprotinin, and chymostatin (Sigma) (each at 20 μg/ml), in addition to 0.4 ml of glass beads. Immediately thereafter, the cells were disrupted by vortexing for approximately 3 minutes at 4°C; the extracts were centrifuged at 12,000*g* for 3 minutes and the radioactivity of acid-insoluble ^{35}S in the supernatants was determined. Portions of the supernatants containing equal amounts of the total acid-insoluble ^{35}S were processed for immunoprecipitation with a monoclonal antibody to βgal (*36*). Ascitic fluid containing a molar excess of the antibody (at least tenfold) was added to each portion, with subsequent incubation at 4°C for 2 hours; protein A–Sepharose (Pharmacia) was then added, the suspension was incubated with rocking at 4°C for 30 minutes, and centrifuged at 12,000*g* for 1 minute. The protein A–Sepharose pellets were washed three times in buffer A (*27*) containing 0.1 percent sodium dodecyl sulfate (SDS), resuspended in an SDS-dithiothreitol (DTT)–containing electrophoretic sample buffer (*37*), heated at 100°C for 3 minutes, and centrifuged at 12,000*g* for 1 minute. Equal portions of the supernatants were subjected to electrophoresis in a 7 percent discontinuous polyacrylamide-SDS gel (15 by 15 by 0.15 cm) with subsequent fluorography. In some experiments, the above protocol was not used but the extracts were prepared by boiling cells directly in the presence of SDS [see (*27*)] with essentially the same results.
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27. Plasmid pUB23 (Figs. 1 and 3) was introduced into DS410, a minicell-producing *E. coli* strain (*38*). Minicells were prepared and labeled during a 60-minute period at 36°C with [^{35}S]methionine (>600 Ci/mmol, Amersham) as described (*38*). Labeled minicells were centrifuged, resuspended in 2 percent SDS, 10 m*M* DTT, 10 m*M* Na-Hepes (*p*H 7.5) and heated at 100°C for 3 minutes. After centrifugation at 12,000*g* for 1 minute, the supernatant was diluted 20-fold with buffer A [1 percent Triton X-100, 0.15*M* NaCl, 5 m*M* EDTA (sodium salt), 50 m*M* Hepes (sodium salt), *p*H 7.5], and phenylmethylsulfonyl fluoride (PMSF) and *N*-ethylmaleimide were added to 0.5 m*M* and 10 m*M*, respectively. After 4 hours at 4°C, the sample was dialyzed against buffer A containing 0.5 m*M* PMSF overnight at 4°C, and processed for immunoprecipitation (*17*).
28. *Saccharomyces cerevisiae* cells carrying plasmids of interest were grown in 800 ml of a uracil-deficient medium (*17*), harvested, and then disrupted with glass beads in buffer A (*17*) containing leupeptin, pepstatin A, antipain, aprotinin, and chymostatin (each at 3 μg/ml). The extract was centrifuged at 12,000*g* for 3 minutes. Saturated ammonium sulfate was added to the supernatant to a final concentration of 57 percent. After overnight incubation at 4°C, the precipitated protein was collected by centrifugation at 23,000*g* for 30 minutes. The pellet was redissolved in buffer A containing protease inhibitors. After clarification at 12,000*g* for 3 minutes, the sample was passed through an affinity column that had been prepared by cross-linking an immunoglobulin G (IgG) fraction from an ascitic fluid [containing a monoclonal antibody to βgal (*17*)] to Affi-Gel 10 (Bio-Rad). The IgG fraction used for cross-linking had been purified from the ascitic fluid by affinity chromatography on protein A–Sepharose. After washing with buffer A lacking Triton X-100, the antibody-bound proteins were eluted with 0.25*M* glycine-HCl (*p*H 2.6). The eluate was immediately adjusted to *p*H 7.5 with 1*M* Hepes (sodium salt) (*p*H 8.5), and thereafter made 0.1 percent in SDS. The sample was concentrated by ultrafiltration in Centricon 30 (Amicon), and subjected to electrophoresis in a 7 percent discontinuous polyacrylamide-SDS gel (*37*). Electroblotting of proteins to nitrocellulose, and immunoblot analysis with a peptide-mediated antibody to ubiquitin were performed as described (*29*). The same results were obtained with a different antibody to ubiquitin obtained from A. Haas (Medical College of Wisconsin, Milwaukee).
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23 July 1986; accepted 8 September 1986

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