# The Degradation Sequence of Adenovirus E1A Consists of the Amino-Terminal Tetrapeptide Met-Arg-His-Ile

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**The adenovirus E1A gene product is a potent transcriptional activator and nuclear oncoprotein. Like other regulatory proteins, E1A has a short half-life, in the range of 30 to 120 min. This short half-life, which was measured in cells synthesizing E1A, is not observed in cells injected with E1A protein made in bacteria or in vitro. In these cases, E1A is essentially refractory to degradation. In an attempt to reconcile this apparent paradox, we suggested that E1A was marked for degradation during its synthesis. Furthermore, we showed that a domain in the amino terminus of E1A was required for rapid degradation in cells translating E1A mRNA (J. M. Slavicek, N. C. Jones, and J. D. Richter, EMBO J. 7:3171–3180, 1988). In this study, we have used *Xenopus laevis* oocytes injected with mRNAs encoding altered E1A proteins to show that the amino-terminal tetrapeptide Met-Arg-His-Ile is required for E1A degradation. Even conservative amino acid substitutions in this degradation sequence render it nonfunctional. This degradation sequence can function as a transferable signal, since it induces instability when fused to another normally stable protein. Furthermore, the degradation sequence requires a proximity of no more than six residues from the amino terminus for activity. These data suggest that a *trans*-acting factor recognizes the amino terminus of E1A during the translation of its message to mark the protein for subsequent destruction.**

The E1A gene products from adenovirus have proven to be very useful models for the study of transcriptional control in eucaryotic cells. For example, the 289-amino-acid protein encoded by the E1A 13 S mRNA is required for the transcription of other early viral genes, whose products in turn are essential for a productive infection by the virus (5). Under certain circumstances, E1A activates the expression of some cellular genes as well (34, 48, 54). Conversely, E1A can also repress transcription by interfering with enhancer activity (7, 24, 49). Another activity of E1A is the facilitation of cell transformation, which also requires the product of the adenovirus E1B gene or other oncogenes (20, 26, 42). Another is the establishment of permanent cell lines, which E1A can do independently. In this regard, E1A is similar to other nuclear oncoproteins such as Myc, Myb, and Fos.

While investigating E1A activity several years ago, Harter et al. (23) noted that adenovirus-infected cells contained high levels of E1A mRNA but relatively little E1A protein. Spindler and Berk (47) subsequently demonstrated that this was likely due to a very fast rate of E1A protein degradation; the half-life varied between 35 and 120 min, depending on whether cells were infected with the virus or stably transformed with a portion of the viral genome. In our studies on E1A-induced *trans*-activation, however, we demonstrated that *Escherichia coli*-expressed E1A was very stable in injected *Xenopus laevis* oocytes (37, 38, 40). This same E1A also exhibited remarkable stability in injected monkey cells (15, 29). We subsequently showed that the stability of this protein was not merely a function of bacterial expression, since E1A synthesized in a rabbit reticulocyte lysate from an SP6-derived mRNA was also stable in injected oocytes. When E1A was synthesized in oocytes from injected mRNA, however, it exhibited its characteristic short half-life (44). Thus, the half-life of E1A appeared to be related to the translation of its message. We further showed that an

amino-terminal region of E1A protein that included the penultimate residue, an arginine, was important for its degradation in mRNA-injected oocytes (44).

In this study, we have used *Xenopus* oocytes injected with mRNAs encoding altered forms of E1A proteins to define the complete sequence required for E1A degradation. This sequence is the amino-terminal tetrapeptide Met-Arg-His-Ile. We show that a functional E1A degradation sequence cannot tolerate even conservative amino acid substitutions and that it must reside at most six residues from the amino terminus for degradation to occur. Finally, we demonstrate that this sequence is sufficient to induce instability in another, normally stable, protein. The possible mechanisms by which this sequence marks proteins for degradation are discussed.

## MATERIALS AND METHODS

**Plasmid constructions.** Plasmids E1A Δ6 to 10, E1A Δ9 to 11, PM2.2, PM3.1, PM3.2, PM4.1, and PM5.1 were constructed by oligonucleotide-directed mutagenesis. To construct E1A Δ4 to 25, a *SacI-BamHI* fragment from *dl*1101 (14) (a gift of S. Bayley) was subcloned into the *SacI-BamHI* site of pGEM. Mutant E1A Δ20 to Δ24 was constructed by excising the *EcoRI-PstI* fragment of pSVXL185 (46) (a gift of E. Ziff) and subcloning it into the *EcoRI-SmaI* site of pSP65pA (44). In this particular mutant protein, E1A residues 20 to 22 were substituted by Ser-Ser-Arg that were derived from a *XhoI* linker (TCCTCGAGG). To construct mutant PM2.1, a *SspI-PstI* fragment from PM563 (53) (a gift of E. Harlow) was subcloned into the *SmaI* site of pSP65pA. To link the E1A degradation sequence to globin DNA, oligonucleotides (EG.1, 5′CATGAAACATATTAT and 5′CATGATAATATGTTT; and EG.2, 5′CATGAGACATAT TAT and 5′CATGATAATATGTCT) were annealed in 0.1 M NaCl for 1 h at 55°C and were cloned into the *NcoI* site of *Xenopus* β-globin pSP64XBM (28). To construct the insertion mutants, oligonucleotides (E1AI7.1, 5′CGCCCTATGC AGGCTGCAGGTTTATTAATGCATATTATCATGCA and 5′TGATAATATGCATTAATAAACCTGCAGCCTGCATA
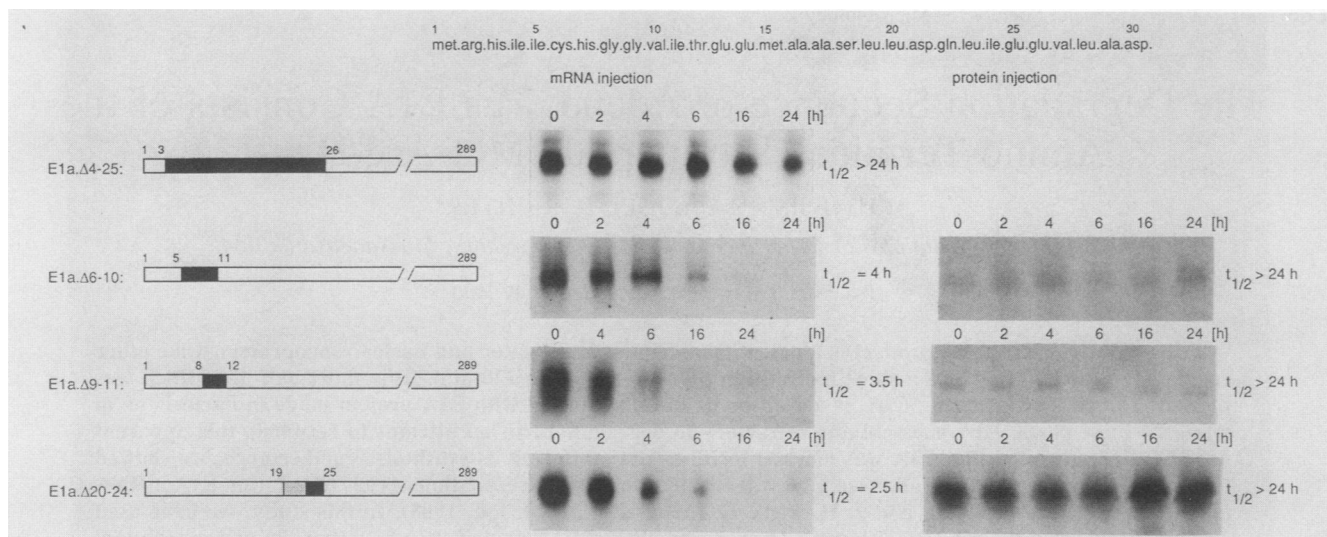
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FIG. 1. Half-life determination of E1A proteins with internal deletions. The top portion of the figure shows the amino acid sequence of the amino-terminal 30 residues of adenovirus type-5 E1A. The left portion shows the schematic representation of the mutant E1A proteins used to define the degradation sequence of E1A. The open boxes refer to the residues that are present and are highlighted by the residue number above the box. The filled boxes refer to deleted residues, and the stippled box refers to substituted residues. The center portion shows autoradiograms of deletion mutant E1A proteins immunoselected from mRNA-injected oocytes. Oocytes were injected with deletion mutant RNAs followed by injection of [$^{35}$S]methionine (pulse) and radioinert methionine (chase). The E1A proteins were then immunoselected at various times following the chase injection and were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The half-lives were determined by scanning densitometry of several exposures of the autoradiograms. The right portion shows autoradiograms of deletion mutant E1A proteins from protein-injected oocytes. mRNAs encoding E1A proteins Δ6 to 10, Δ9 to 11, and Δ20 to 24 were translated in a rabbit reticulocyte lysate supplemented with [$^{35}$S]methionine. The proteins were subjected to chromatography and microconcentration and then injected into oocytes. At several time points following injection, the oocytes were prepared for SDS-polyacrylamide gel electrophoresis and the E1A proteins were detected by autoradiography.

GGGCGAGCT; and E1AI7.2, 5'CGCCCTATGCAGGCTG CAGGTTTATTAATGAGACATATTATCATGCA and 5'T GATAATATGTCTCATTAATAAACCTGCAGCCTGCAT AGGGCGAGCT) were annealed as described above and were inserted into the NsiI site of E1A410 (44). For shorter insertions, oligonucleotides (E1AI4.1/4.2, 5'CATGGGTTT AT and 5'TAATAAACCCATGAGCT; and E1AI2.1/2.2, 5' CTCGAGATGT and 5'TAACATCTCGAGAGCT) were also annealed and ligated to SacI and AseI sites of E1AI7.1/ 7.2.

**Oligonucleotide-directed mutagenesis.** The EcoRI-HindIII fragment from pSP13S (30) were subcloned into the EcoRI-HindIII site of IBI24 and used to transform JM101 cells. Exponentially growing cells were infected with the helper bacteriophage R408, and single-stranded DNA was prepared and purified over a Sepharose CL4B column to avoid random priming by small DNA fragments or RNA. Oligonucleotides that were complementary to regions of the single-stranded DNA designated for mutagenesis were synthesized and purified over a Sephadex G-25 column. The oligonucleotide sequences were: E1A Δ6 to 10, 5'CTGGTCCAAAAG GCTAGCGGCCATTTCTTCGGTAAGATAATATGTCT CAT; E1A Δ9 to 11, 5'ATGAGACATATTATCTGCCACG GTACCGAAGAAATGGCCGCC; PM2.2, 5'ATAATATGT TTCATTTTCAG; PM3.1, 5'GCAGATAATAAGTCTCATT TTC; PM3.2, 5'GCAGATAATACGTCTCATTTTC; PM4.1, 5'GTGGCAGATAAAATGTCTCAT; and PM5.1, 5'TCCG TGGCAGAAAATATGTC. The oligonucleotides were phosphorylated for 1 h at 37°C with polynucleotide kinase, and the enzyme was then heat inactivated at 70°C for 15 min. The oligonucleotides were annealed to the single-stranded DNA for 10 min at 65°C in 20 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–2 mM MgCl$_2$. This was followed by the extension

reaction that consisted of 10 μl of the oligonucleotide annealed to the single-stranded DNA, 1 μl of 10× extension buffer (1× extension buffer is 20 mM Tris hydrochloride [pH 7.5], 20 mM NaCl, 8 mM MgCl$_2$, and 3 mM dithiothreitol), 1 mM deoxynucleoside triphosphate, 2 to 4 U of T4 DNA polymerase, and 2 U of T4 DNA ligase. The mixture was incubated for 5 min at 0°C, 5 min at 20°C, and 90 min at 37°C. The resulting double-stranded DNA was methylated with dam methylase (0.5 to 1 U) and used to transform HB101 cells. Mutant DNAs were determined by double-stranded DNA sequencing by using the dideoxynucleotide method of Sanger et al. (43).

**In vitro transcription and translation.** DNAs were transcribed in vitro with SP6 or T7 polymerases in the presence of $^7$mGpppG to cap the RNAs (28). Some of the mRNAs were translated in a rabbit reticulocyte lysate supplemented with 50 μCi of [$^{35}$S]methionine. The radiolabeled proteins were centrifuged through a 1-ml Sephadex G-50 column, and the eluate was concentrated with Amicon microconcentrators. About 50 nl of this material was then injected into oocytes and cultured for varying periods of time. The oocytes were homogenized, and the radiolabeled proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography.

**Microinjection of Xenopus oocytes.** Stage 6 Xenopus oocytes were injected with about 50 ng of RNA and cultured for 4 h to allow for polysome loading. The oocytes were injected with about 0.3 pmol of [$^{35}$S]methionine (specific activity, 1,000 Ci/mmol), cultured for 2 h, and then injected with 3 nmol of radioinert methionine. The oocytes were subsequently cultured in Barth's medium supplemented with 1 mM methionine and were frozen in groups of 15 at several time points following the final injection. The oocytes were

homogenized, and E1A was immunoselected and analyzed as described previously (44). The relative amounts of E1A were determined by scanning densitometry of autoradiograms.

## RESULTS

**Identification of the E1A degradation sequence.** In a previous report, we showed that the E1A degradation sequence resided between residues 1 and 37 and that the second residue, an arginine, was part of the sequence (44). We have now determined the carboxy-terminal boundary of the degradation sequence. A series of DNA deletion mutants were constructed and inserted 3' of SP6 or T7 promoters for in vitro RNA synthesis. These mRNAs were injected into *Xenopus* oocytes, followed by a pulse injection of [³⁵S] methionine and a chase injection of radioinert methionine. At several time points, E1A was immunoselected, resolved by SDS-polyacrylamide gel electrophoresis and autoradiography, and quantitated by scanning densitometry. Figure 1 shows the half-lives of several E1A deletion mutant proteins. An E1A protein lacking residues 4 to 25 (E1A Δ4 to 25) had a half-life of >24 h in mRNA-injected oocytes. In contrast, E1A proteins lacking residues 6 to 10 (E1A Δ6 to 10), 9 to 11 (E1A Δ9 to Δ11), or 20 to 24 (E1A Δ20 to 24) all had short half-lives of 4 h or less in mRNA-injected oocytes. Thus, these data suggest that the degradation signal of E1A resides between residues 1 to 5, inclusive, or residues 12 to 19, inclusive. Our previous result demonstrating the importance of residue two for degradation of E1A (44) indicates that the former is more likely to encompass the signal. We also note that all experiments were performed a minimum of three times with similar results among replicates.

The data presented in Fig. 1 and in subsequent figures show that E1A exists in multiple forms that are resolved by SDS-gel electrophoresis. As is the case for several other proteins such as Fos (4), nucleoplasmin (11), Cdc2 (13), polyoma large-T antigen (6), and cyclin (35), E1A is phosphorylated such that an altered electrophoretic mobility is induced (39, 45). The phosphorylation sites that give rise to electrophoretic variants in E1A have been mapped to serine 89 (45) and probably also to a serine(s) residing between residues 224 to 289 (39). Because deletion of these regions does not affect E1A degradation (44), serine phosphorylation, which is the only known posttranslational modification of E1A, is unlikely to be involved in E1A degradation (also, see below).

We showed previously that E1A was refractory to degradation when it was synthesized in vitro and then injected into oocytes. This has been repeated with the E1A deletion mutant proteins described above. The SP6- and T7-derived mRNAs were translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine. The protein was then separated from unincorporated amino acids by gel filtration, concentrated, and injected into oocytes. The amount of E1A remaining at several time points was determined as described above. Figure 1 shows that E1A proteins Δ6 to 10, Δ9 to 11, and Δ20 to 24 were all stable in protein-injected oocytes (i.e., they exhibited half-lives >24 h). The data in which reticulocyte lysate (Fig. 1) and *E. coli* (44)-synthesized E1A were used therefore suggest that this protein is "marked" for rapid degradation while it is being synthesized. It is also important to point out that *trans* activation, one of the major activities of E1A, occurs irrespective of the source of the protein (i.e., oocyte, reticulocyte, or bacteria).

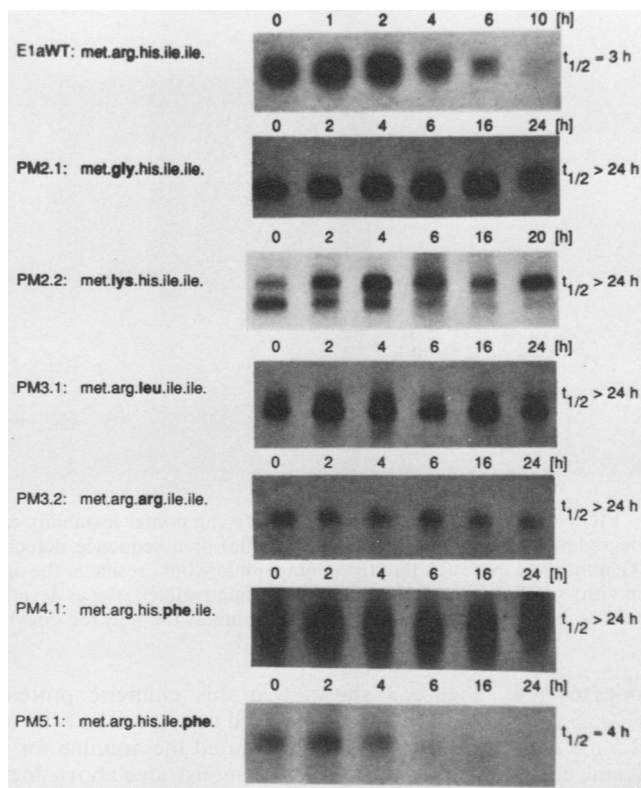Oligonucleotide-directed mutagenesis was used to pre-



FIG. 2. Delineation of the E1A degradation sequence by single amino acid substitutions. The half-lives of mutant E1A proteins containing single amino acid changes in mRNA-injected oocytes were determined as described in Fig. 1. The left portion of the figure shows the sequence of the five amino-terminal residues, and the residue in bold type denotes the mutation. The two molecular weight variants of E1A that are occasionally observed are due to differential phosphorylation.

cisely identify the important residues for E1A degradation (Fig. 2). For reference, the half-life of wild-type E1A is shown and was determined to be 3 h in mRNA-injected oocytes. Alteration of the second residue, an arginine, to either a glycine (PM2.1) or a lysine (PM2.2) resulted in a stable E1A that had a half-life of >24 h. This confirms our earlier report that the arginine was required for degradation (44). We note that protein 2.2 consists of two forms that, as mentioned above, are due to differential phosphorylation. We emphasize that the faster-migrating (nonphosphorylated) form does not degrade but instead is converted (phosphorylated) to the slower-migrating form (see also references 39 and 45). Densitometric quantitation of the autoradiogram revealed that the phosphorylated form increased while the nonphosphorylated form decreased (data not shown). Mutation of the third residue, a histidine, to either a leucine (PM3.1) or an arginine (PM3.2) also conferred stability to E1A. When the fourth residue, an isoleucine, was mutated to a phenylalanine (PM4.1), E1A again was stable. However, mutation of the isoleucine at position five to a phenylalanine (PM5.1) did not affect the rapid degradation of E1A. Therefore, the E1A degradation sequence is Met-Arg-His-Ile.

**The E1A degradation sequence is sufficient to confer protein instability.** To assess whether the E1A degradation sequence can induce instability in another protein, we have fused it to the amino terminus of *Xenopus* β-globin, which has been shown previously to be a stable protein in mRNA-injected
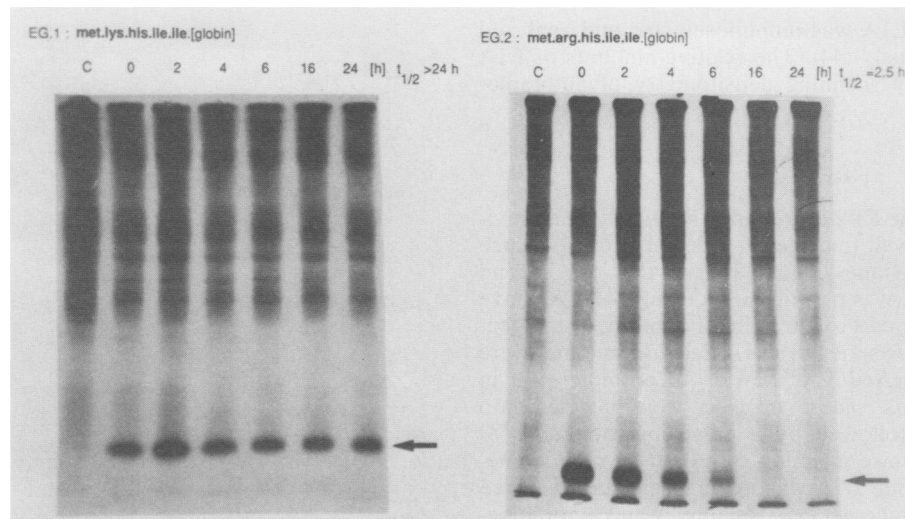
FIG. 3. The E1A degradation sequence can confer instability to a normally stable protein. Oligonucleotides encoding the wild-type E1A degradation sequence (Met-Arg-His-Ile-Ile) or a sequence defective for degradation (Met-Lys-His-Ile-Ile) was fused to DNA coding for *Xenopus* β-globin such that the pentapeptide would reside at the amino terminus of the derived chimeric protein. Oocytes were injected with in vitro-synthesized mRNA, and the labeling regimen was as described in Materials and Methods. Control (C) oocytes were not injected with mRNA but were otherwise treated the same as the injected oocytes. The arrows denote the chimeric E1A-globin proteins.

oocytes (44). Figure 3 shows that this chimeric protein (EG.2) was unstable in mRNA-injected oocytes (a half-life of 2.5 h). As a control, we have substituted the arginine for a lysine at position 2, which we have demonstrated above does not induce E1A degradation. This chimeric protein (EG.1) was stable in mRNA-injected oocytes. Thus, the E1A degradation signal can act independently on the remainder of the protein.

**An E1A degradation sequence requires close proximity to the amino terminus.** In a final series of experiments, we have determined whether the E1A degradation sequence must reside at or near the amino terminus. To do this, we have constructed six mutant proteins, three of which consisted of a random two, four, or seven residues followed by an E1A protein that does not normally degrade (i.e., with the amino-terminal sequence Met-His-Ile-Ile; cf., reference 44). Figure 4 shows that these chimeric proteins (E1aI2.1, E1aI4.1, and E1aI7.1) had half-lives >24 h and therefore were stable in mRNA-injected oocytes. The second three proteins were composed of the same two, four, or seven residues linked to an E1A protein that contains the wild-type degradation sequence (E1aI2.2, E1aI4.2, and E1aI7.2). The proteins with
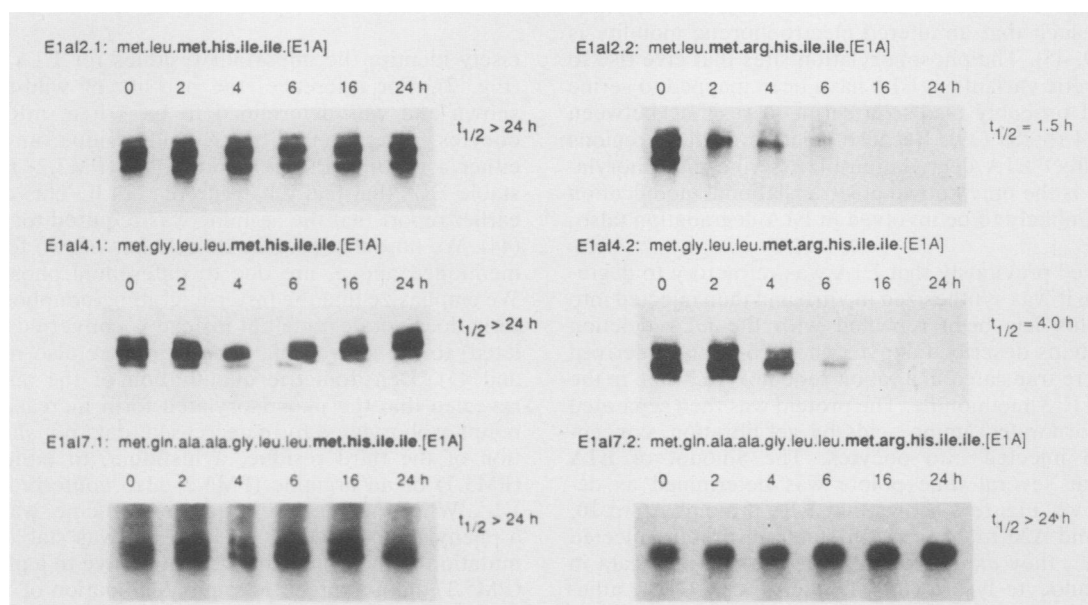


FIG. 4. A functional E1A degradation sequence requires close proximity to the amino terminus. Oligonucleotides encoding two, four, or seven randomly chosen amino acids were inserted immediately 5' of sequences encoding either a mutant E1A that lacks the second residue, an arginine (E1AI2.1, E1AI4.1, or E1AI7.1), or wild-type E1A (E1AI2.2, E1AI4.2, or E1AI7.2). In vitro-synthesized mRNAs were injected into oocytes, and the chimeric E1A proteins were analyzed as described in Materials and Methods. Above the figures are the amino-terminal sequences of the proteins with the two, four, or seven random residues in lightface type and wild-type or mutant E1A degradation sequences in bold type.

two or four residues preceeding the degradation sequence had short half-lives ($\leq 4$ h), and therefore degradation occurred normally. In contrast, the protein with seven residues preceding the degradation sequence had an abnormally long half-life ($>24$ h). We therefore infer from such experiments that a functional E1A degradation sequence requires a proximity to the amino terminus of no more than six residues.

## DISCUSSION

This study demonstrates that the E1A 13 S mRNA product from adenovirus type 5 contains a defined amino acid sequence at its amino terminus that is required for rapid degradation. Location of this sequence, Met-Arg-His-Ile, is important for function, since its placement seven residues, but not two or four residues, from the amino terminus destroys activity. Furthermore, this sequence acts as a transferable signal because its fusion to globin induces the rapid degradation of this normally stable protein in mRNA-injected oocytes.

**Specificity of the E1A degradation sequence.** We have made several amino acid substitutions in the degradation sequence to determine the specificity of the signal. Interestingly, a functional E1A degradation signal cannot tolerate even conservative amino acid substitutions. For example, substitution of a lysine for the arginine at position two or an arginine for the histidine at position three abolishes the ability of E1A to degrade. This remarkable specificity might suggest that other proteins with short half-lives would have an identical sequence at their amino termini. Although a comparison of E1A proteins from other adenovirus serotypes shows a conserved region at the amino terminus, it is not absolute (27). E1A protein from adenovirus type 2 does contain the same degradation sequence as that from type 5; E1A from type 7, however, has the sequence Met-Arg-His-Leu and that from type 12 has the sequence Met-Arg-Thr-Glu. Whether types 7 and 12 E1A proteins have analogous degradation sequences or even whether they have half-lives as short as the type 2 or 5 protein is not known.

A search of the NBRF-PIR data base (Version 23) did not reveal other proteins with an E1A-like degradation sequence at their amino termini. Other proteins that are known to degrade rapidly, such as the cyclins (31, 33), c-Myc (32), c-Myb (32), c-Fos (12), p53 (21), and ornithine decarboxylase (17) also do not contain an amino-terminal E1A-like degradation sequence. However, the regions that are important for the degradation of two of these proteins have been mapped. The degradation of sea urchin cyclin B requires residues 13 to 90 (33) and the degradation of mammalian ornithine decarboxylase requires the carboxy-terminal 37 residues (17). Although it is not presently known whether the putative degradation regions of these proteins are sufficient to induce degradation when fused to other proteins, one might speculate that they, too, could function as independent degradation signals. If true, this would suggest that other proteins also have discrete signals that regulate their destruction. Thus, degradation signals could be the basis by which cells degrade specific proteins rapidly at some times and not others, as is the case with cyclin (31, 33), while maintaining a set rate of destruction of other proteins by a more general mechanism, such as the ubiquitin system (8, 9, 25, 36).

**Models for protein degradation.** Several proteins that contain regions rich in proline, glutamate, serine, and threonine (PEST) have short half-lives, and it has been suggested that

these PEST sequences are important for degradation (41). Therefore, alteration or removal of these PEST regions might be expected to increase the stability of a protein. E1A contains four PEST regions that reside between residues 44 to 94, 125 to 149, 177 to 202, and 223 to 244; data presented previously had shown that removal of the PEST sequences does not increase the stability of E1A (44). Moreover, the present study demonstrates that conservative amino acid substitutions in a region outside of the PEST sequences have profound effects on E1A degradation. Therefore, we feel that PEST sequences are unlikely to play a role in E1A degradation.

The amino-terminal amino acid has been shown to be an important determinant for protein degradation (1–3, 19). To assess whether this could also play an important role in E1A degradation, we performed amino-terminal sequencing of E1A proteins from mRNA-injected oocytes. The initiation methionine was present on both wild-type E1A and an E1A that does not degrade (i.e., an E1A that carries a deletion of the arginine at position two) (44). Thus, the amino-terminal amino acid does not appear to influence E1A degradation.

We have also attempted to determine whether E1A degrades via a ubiquitin-mediated pathway. By using antiubiquitin antibodies, we have been unable to immunoselect E1A-ubiquitin conjugates. In addition, the lysine residues of E1A to which ubiquitin might be conjugated reside at positions 208, 253, and 285. This carboxy-terminal region can be deleted without affecting rapid E1A degradation (44), suggesting that ubiquitin does not mediate the destruction of E1A.

By what mechanism, then, does E1A degrade? We suggest that a factor recognizes the amino-terminal tetrapeptide soon after it emerges from the ribosome, which is when it should be readily accessible for factor interaction. Such a factor would then mark E1A for subsequent degradation by a protease. This could explain why E1A does not degrade in protein-injected cells or in mRNA-injected oocytes when the degradation sequence is placed at a distance from the amino terminus. In these instances, one would envisage that the signal is hidden by protein conformation. This proposal has some similarities to two other models, that for signal recognition particle binding to a nascent peptide chain for protein secretion (50) and for the autogenous regulation of tubulin mRNA destruction. In the latter case, tubulin monomers are thought to bind the nascent tubulin peptide as it emerges from the ribosome, which then induces mRNA degradation (10). It is interesting to note that the amino-terminal sequence of tubulin that is required for mRNA degradation is Met-Arg-Glu-Ile (14, 16, 55), which is very similar to that of E1A.

In attempting to define E1A functions in molecular terms, several laboratories have shown that many cellular proteins coimmunoprecipitate with E1A (14, 18, 22, 51). In one case, a 300-kDa protein from HeLa cells that binds wild-type E1A fails to do so when a glycine is substituted for the arginine at position two (52, 53). In addition, this mutant E1A is transformation defective and accumulates to a greater extent than wild-type E1A (52). This same mutation leads to stability of E1A in mRNA-injected oocytes (Fig. 2). Whether the 300-kDa protein is important for E1A degradation is unknown, although one might speculate that its homolog or an analogous protein binds E1A in mRNA-injected oocytes. We are attempting to determine whether such is the case.

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