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Rapid turnover of adenovirus E1A is determined through a co-translational mechanism that requires an amino-terminal domain

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The product of the adenovirus E1A 13S mRNA can both stimulate and repress the expression of certain viral and cellular genes. As with several other regulatory proteins, E1A has a short half-life, ~40 min. Although this short half-life is observed in cells expressing the E1A gene, it is not the case with cells injected with E1A protein, where its half-life is very long, generally >15 h. We have sought to reconcile these apparent differences in E1A stability. Using *Xenopus* oocytes, we find that E1A exhibits its characteristic short half-life when it is synthesized from injected mRNA while it has a very long half-life when it is injected as a protein synthesized originally in *Escherichia coli* or reticulocyte lysates. In order to delineate the amino acids responsible for rapid E1A turnover, several deletion mRNAs were constructed, injected into oocytes, and E1A half-life determined. Carboxyl-terminal deletions and an internal deletion of residues 38-86 failed to increase the half-life of E1A. In contrast, amino-terminal deletions of 70 and 14 residues resulted in very stable E1A proteins ($t_{1/2}$ >20 h). Furthermore, deletion of the second amino acid, an arginine, resulted in a stable E1A protein. The amino-terminal region of E1A was able to induce the rapid turnover of a normally stable protein, β-globin, in oocytes injected with an E1A-globin chimeric mRNA. This E1A-induced instability of globin was abolished, however, when the protein was first synthesized in reticulocyte lysates and then injected into oocytes. The amino-terminal region of E1A is also important in governing half-life in adenovirus-infected HeLa cells. These results demonstrate that the half-life of E1A is established co-translationally through a mechanism involving sequences within the amino-terminal 37 residues.

Key words: adenovirus E1A/microinjection/protein turnover/*Xenopus* oocytes

Introduction

A productive infection by the human adenoviruses requires the expression of the viral E1A gene. This gene encodes a single primary transcript which, prior to the onset of viral replication, is alternatively spliced to yield two mRNAs with sedimentation values of 12S and 13S (see Berk, 1986, for review). Since the splicing is in-frame, the 13S mRNA product, containing 289 amino acids, is identical to the 12S mRNA product, containing 243 residues, except for an additional internal 46 amino acids. Products of the E1A gene are capable of both *trans*-activating and repressing transcription of a variety of unrelated viral and cellular genes. *Trans*-activation is predominantly due to the 13S mRNA product, although under certain circumstances activation by the 12S product can also be detected (Borrelli *et al.*, 1984; Leff *et al.*, 1984; Krippl *et al.*, 1985; Richter *et al.*, 1985; Velcich and Ziff, 1985). In addition to transcriptional modulation, E1A can also facilitate cell transformation when acting in conjunction with products of the E1B gene and certain oncogenes (Graham *et al.*, 1977; Houweling *et al.*, 1980; Ruley, 1983). On its own, E1A is capable of extending the growth potential of primary cells, enabling their establishment as permanent cell lines, a property it shares with a number of other oncogenes such as *myc*, *fos*, and *myb*. Additional shared traits between these proteins include nuclear localization and rapid turnover (Curran *et al.*, 1984; Hann and Eisenman, 1984; Boyle *et al.*, 1985).

We have used microinjected *Xenopus* oocytes as a system that is amenable to the study of E1A function. In particular, we have studied the 13S mRNA product that was isolated from *Escherichia coli* expressing the E1A gene (Ferguson *et al.*, 1984). Following oocyte injection, E1A is phosphorylated (Richter *et al.*, 1988), enters the nucleus (Richter *et al.*, 1985), and enhances the expression of either the adenovirus E3 promoter or the human heat shock 70 promoter (Jones *et al.*, 1983; Richter *et al.*, 1987). All of these events also take place in adenovirus infected or E1A transfected mammalian cells (Berk, 1986).

The *E.coli*-expressed protein, when injected into either oocytes (Richter *et al.*, 1985), or monkey cells (Krippl *et al.*, 1984; Ferguson *et al.*, 1985), is very stable. This result is surprising in that E1A is a very unstable protein in adenovirus type 5 infected HeLa cells and transformed 293 cells (Spindler and Berk, 1984; Branton and Rowe, 1985). In an effort to reconcile these apparent stability differences we undertook an investigation of E1A turnover using *Xenopus laevis* oocytes.

E1A, when expressed either in *E.coli* or a rabbit reticulocyte lysate, is very stable in microinjected oocytes This eliminates possible alterations of E1A that might affect stability that are peculiar to bacterial overexpression. However, the normal rapid turnover of E1A is observed if oocytes are injected with E1A mRNA. Thus, there appears to be a causal relationship between *de novo* synthesis of E1A and its half-life. Through the use of deletion mutants, amino acids within the amino-terminal region encompassing residues 1-37 are shown to be involved in co-translational half-life establishment. We propose a model whereby E1A is marked for rapid turnover during its synthesis.

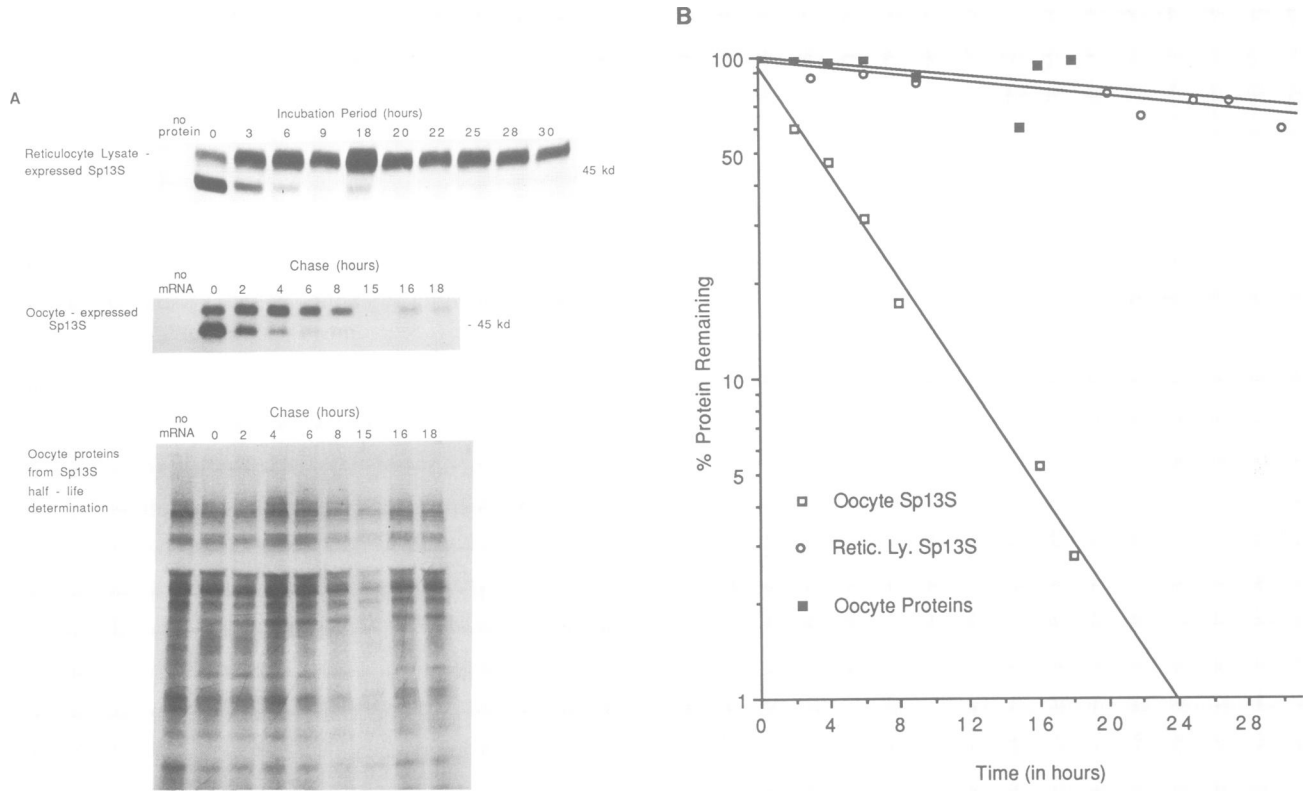

Fig. 1. Half-life determinations of E1A proteins in microinjected *Xenopus* oocytes. (**A**) Stage 6 oocytes were injected with [^{35}S]methionine-labeled reticulocyte lysate-expressed E1A, and incubated for the indicated periods at 19°C. The oocytes were homogenized, the proteins resolved by SDS−PAGE and E1A visualized by autoradiography. Stage 6 oocytes were also injected with SP6 polymerase generated transcripts derived from pSp13S. The oocytes were pulsed with [^{35}S]methionine, and chased with radioinert methionine. After the indicated chase periods E1A was immunoprecipitated from oocyte homogenates, the isolated proteins separated by SDS−PAGE, and E1A visualized by autoradiography. Radiolabeled oocyte proteins, after E1A immunoprecipitation, were also separated by SDS−PAGE and visualized by autoradiography. The autoradiogrpahs of reticulocyte lysate-expressed, and oocyte-expressed E1A shown in part (**A**) were scanned with a densitometer and the amount of E1A present in relation to the zero time points was calculated, and the results graphically depicted by computer generated exponential regression analysis (**B**). Oocyte proteins from the half-life determination of oocyte-expressed Sp13S were precipitated with hot TCA and the radioactivity determined in a liquid scintillation counter. The half-life of bulk oocytes was calculated as described above.

Results

Half-life of E1A in oocytes

The observation that *E.coli*-expressed E1A has an aberrantly long half-life in protein injected cells might suggest that a modification unique to bacterial expression renders E1A refractory to turnover. To assess this possibility the half-life of injected E1A synthesized by reticulocyte lysates was determined. The E1A 13S cDNA was inserted into a vector containing the promoter for SP6 polymerase (pSp13S, Figure 2). Transcripts generated from SP6 polymerase were translated in a reticulocyte lysate in the presence of [^{35}S]methionine. The labeled E1A protein was partially purified on a Sephadex column, concentrated and micro-injected into stage 6 oocytes. Since reticulocyte lysates also phosphorylate E1A, which induces a retarded mobility in SDS gels (Richter *et al.*, 1988), both molecular weight variants of the protein were injected. Figure 1 shows an SDS gel autoradiogram and corresponding densitometric quantitation that reflect the half-life of this protein, which was found to be >20 h. It should be noted that oocytes (as well as HeLa cells) also phosphorylate E1A resulting in the shift of the protein to its higher molecular weight variant over time (Figure 1). In addition, E1A that was generated from a baculovirus vector within cultured insect cells was also stable in microinjected *Xenopus* oocytes (data not shown). These results therefore demonstrate that E1A stability in microinected oocytes is not a consequence of bacterial expression; E1As made in two eukaryotic sytems are also quite stable following injection.

Since E1As from three different sources are very stable in protein injected oocytes, the observed abberant stability is probably not a consequence of the protein's origin. Instead, E1A may be targeted for rapid turnover through a mechanism involving *de novo* synthesis of the protein. To assess this possibility the half-life of E1A expressed in oocytes was determined by pulse−chase methodology as described below using mRNAs generated from pSp13S. Stage 6 oocytes were injected with capped and polyadenylated E1A 13S transcripts and were incubated for 2.5 h during which time the messages were loaded onto polysomes. The oocytes were then injected with [^{35}S]methionine and incubated for a pulse period of 1.5 h and then injected, at a 10 000-fold mole excess, with radioinert methionine (the chase). At several times following the chase injection, the amount of labeled E1A remaining was determined. The oocytes constituting each time point (16) were homogenized and E1A immunoprecipitated with polyclonal antibodies. The isolated proteins were resolved by SDS−PAGE, visualized by autoradiography, and quantitated by scanning densitometry at several exposures. The half-life of oocyte expressed E1A was found to be ~3 h, which markedly contrasts with reticulocyte lysate-

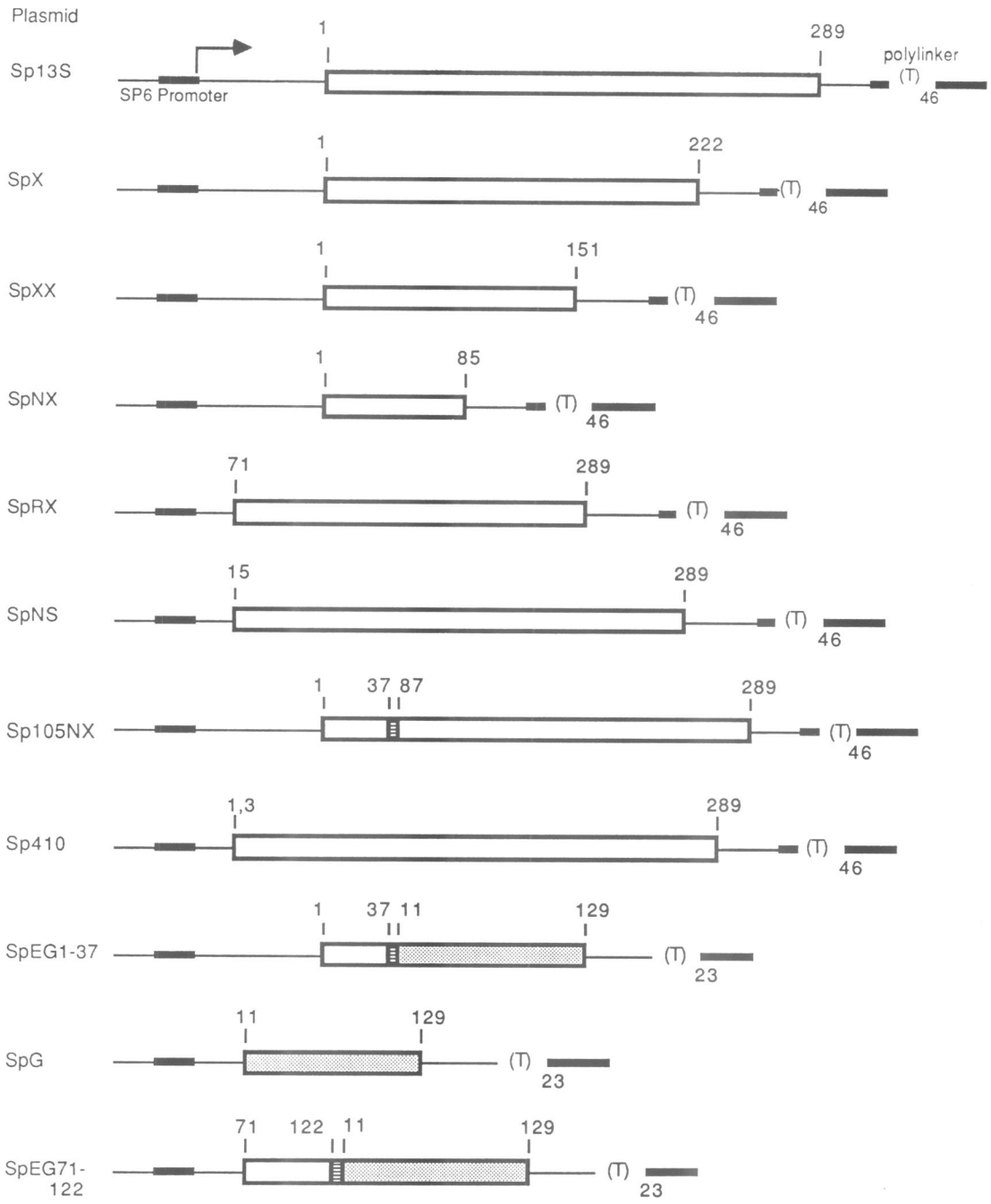

Fig. 2. Description of E1A, E1A mutant and E1A−globin chimeric constructs used in this study. The solid boxes at the left end of the diagrams denote the Sp6 polymerase promoter, the open boxes correspond to E1A coding sequences, the horizontal hatching refers to placement of sequences coding for added amino acids (see Materials and methods), the stippled boxes indicate *Xenopus* β-globin coding sequences, and the solid boxes at the right end of the diagrams denote polylinker sequences. The numbers above the coding regions refer to the amino acid number.

expressed E1A (Figure 1A). The short half-life of oocyte expressed E1A was not a consequence of the batch of oocytes used since in this and all other experiments, the half-life of bulk endogenous oocyte proteins was >20 h (Figure 1B). Therefore, as in mammalian cells, E1A expressed within oocytes exhibits a short half-life. However, the half-life of E1A in HeLa cells, ~40 min (Spindler and Berk, 1984; Branton and Rowe, 1985), is 4-fold less than that observed in oocytes. This difference is probably a consequence of incubation temperatures; HeLa cells are incubated at 37°C while oocytes are incubated at 19°C. If one assumes a Q_{10} value of 2, as has been proposed for half-lives of other proteins (Neff *et al.*, 1979), then a 3.6-fold increase in the rate of E1A turnover would be predicted if oocytes could be incubated at 37°C. Thus, a calculated 50 min E1A half-life in mRNA injected oocytes is very similar to the 37°C value observed in HeLa cells.

Half-lives of E1A deletion proteins

The results described above indicate that a critical determinant of E1A half-life is the site of its synthesis. E1A is rapidly degraded in oocytes expressing the protein *de novo* while it is very stable in oocytes injected with E1A that was synthesized in a heterologous system. These turnover

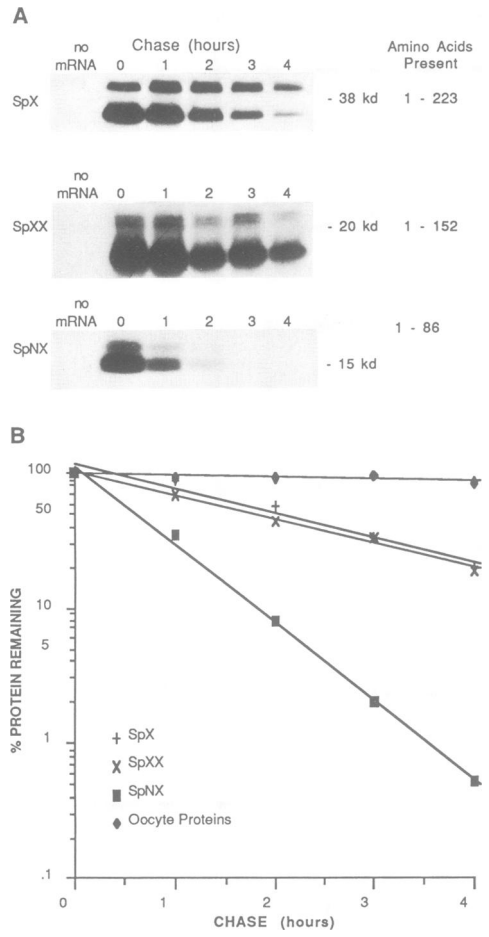

Fig. 3. Half-life determinations of *Xenopus* oocyte-expressed carboxyl-terminal E1A deletion proteins. **(A)** The half-lives of SpX, SpXX, and SpNX were determined as described for Figure 1. Shown are the autoradiographs of representative experiments. **(B)** Graphic depiction of the data presented in (A). The oocyte protein decay values were obtained from the half-life determination of SpX, and were quantitated as described in the legend to Figure 1.

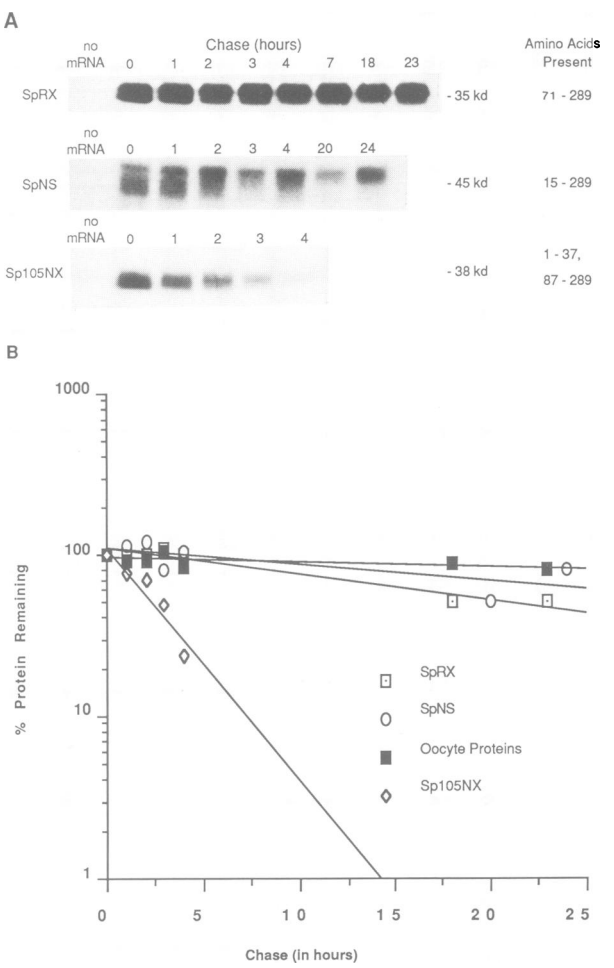

Fig. 4. Half-life determinations of amino-terminal E1A deletion proteins. The half-lives of oocyte-expressed SpRX, SpNS, and Sp105NX were determined as previously described. Shown are autoradiographs **(A)** along with graphic depiction of the data **(B)**. The oocyte protein decay values were from the half-life determination of SpRX.

differences could be a consequence of an *in vivo* cotranslational event that involves a specific region of E1A. In an effort to identify such a region, we have constructed several deletion mutant E1A cDNAs which have been inserted in SP6 promoter-containing vectors; synthesis and expression of the resulting mRNAs would produce truncated E1A proteins whose half-lives could be determined by methionine pulse−chase and immunoprecipitation as described earlier.

The constructs pSpX, pSpXX, and pSpNX have in-frame termination codons and give rise to E1A carboxyl-terminal deletion proteins that contain amino acids 1−222, 1−151 and 1−85, respectively (Figure 2). SP6 polymerase generated transcripts from these mutants were injected into stage 6 oocytes, and the half-lives of the resulting proteins were determined. SpX and SpXX were found to have half-lives of 2.05 and 3.1 h, respectively (Figure 3), which are similar to wild-type E1A (Figure 1). In contrast, SpNX was found to have a half-life significantly shorter, ~30 min (Figure 3). During the immunoprecipitation of SpNX an additional protein of ~16 kd was detected. This protein could be a modified form of SpNX or more likely a cellular protein in association with SpNX that was co-immunoprecipitated. The rapid turnover of these carboxyl-terminal E1A deletion proteins is in contrast to endogenous oocyte proteins which exhibited long half-lives (Figure 3).

The short half-lives of the carboxyl-terminal deletion proteins indicate that residues 86−289 are not necessary for rapid E1A turnover in mRNA injected oocytes. In order to assess the possible role of the amino-terminal residues in E1A turnover, the deletion mutants SpRX and SpNS were constructed (Figure 2). The sequences coding for the amino-terminal 63 residues of E1A were deleted in pSpRX, thereby deleting the first two methionine codons. Translation of pSpRX derived transcripts initiates at the first remaining in-frame AUG codon, which is the third AUG condon in E1A, generating a protein containing residues 71−289. In pSpNS the sequences encoding the initiator methionine were deleted that results in a protein, initiated at the second in-frame AUG codon, containing residues 15−289 . The half-lives of oocyte expressed pSpRX and pSpNS were determined as previously described. In contrast to wild-type E1A (Sp13S) and the carboxyl-terminal deletions, SpRX and SpNS both were very stable, exhibiting half-lives >20 h (Figure 4). These mutants indicate that the amino-terminal region of E1A is necessary for rapid turnover. To further define the 'instability region' the half-life of a deletion mutant

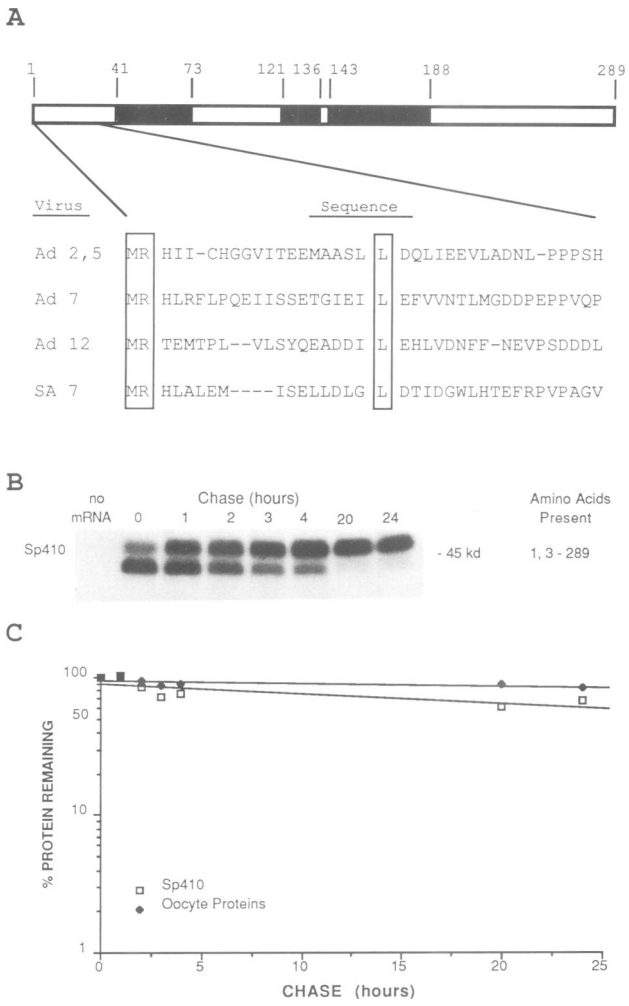

Fig. 5. Comparison of E1A amino-terminal sequences of several adenoviruses, and the half-life determination of Sp410. (**A**) The adenovirus type 5 13S 289 residue E1A protein is diagrammed with the highly conserved regions represented by the shaded boxes. The numbers above the diagram indicate the amino acids bounding the regions. The amino-terminal 37 residues of adenoviruses Ad2, Ad5, Ad7, Ad12 and SA7 are shown below the E1A diagram. (**B**) The half-life of oocyte expressed Sp410, which lacks the second amino-terminal residue, was determined as previously described. Shown is an autoradiograph along with graphic depiction (**C**) of the data. The oocyte protein decay values were obtained from the half-life experiment of Sp410 presented in (**B**).

lacking residues 38−86 was determined (Sp105NX, Figure 2). The half-life of this protein in mRNA injected oocytes was ~3 h (Figure 4). These results indicate that the instability region of E1A is located within the first 37 residues of the amino terminus.

The role of the second amino acid in E1A turnover

As a group the amino-terminal 37 residues of E1A are not highly conserved among the human adenoviruses with the exception of two residues, an arginine and leucine (Figure 5A). These residues are present in human adenoviruses Ad2, Ad5, Ad7 and Ad12 as well as in the simian adenovirus SA7 (Kimelman *et al.*, 1985). To address the importance of the arginine residue in E1A turnover, the single amino acid deletion mutant Sp410 (Figure 2) was constructed that lacks only this residue. The half-life of oocyte-expressed Sp410, determined as described previously, was found to be >20 h (Figure 5). Thus, deletion of this single, conserved arginine residue is sufficient to confer stability to E1A when expressed within oocytes.

The amino-terminal region of E1A is sufficient to confer instability to a normally stable protein

The results presented above demonstrate that the amino-terminal region of E1A is necessary for it to undergo rapid degradation. We next have asked whether this region is sufficient to confer instability to a normally stable protein. The stable protein employed is *X.laevis* β-globin (XβM), which exhibits a half-life of >20 h in mRNA injected oocytes (data not shown). Figure 2 shows the construction of this chimeric molecule in which sequences encoding E1A residues 1−37 are fused in-frame to sequences encoding XβM. Construction of this chimeric protein necessitated the deletion of the amino-terminal nine residues of XβM and the addition of four residues to yield a chimeric protein consisting of residues 1−37 of adenovirus 5 E1A, additional residues Ser−Ser−Ile−Glu, and residues 11−129 of XβM (SpEg1-37). This chimeric protein is recognized by polyclonal antibodies directed against E1A enabling its immunoprecipitation from oocyte homogenates. An XβM control protein was constructed that contains the initiator methionine, the same four additional residues in SpEG1-37, Ser−Ser−Ile−Glu, and residues 11−129 of β-globin (SpG, Figure 2).

Sp6 polymerase generated transcripts of SpEG1-37 and SpG were microinjected into stage 6 oocytes and the half-lives determined as previously described. The half-lives of oocyte expressed SpEG1-37 and SpG were determined to be 27 min and 5.5 h, respectively, ~12-fold difference (Figure 6). The half-life of SpG is at least several fold less than Sp64−XβM which has a half-life of >20 h. An abnormal conformation of SpG (as a consequence of the deletion of the nine amino-terminal residues) could be responsible for the observed decreased stability of this protein. Nonetheless, the presence of the amino terminal residues of E1A on globin significantly increases its turnover rate.

The attachment of a limited number of residues from one protein onto another protein possibly results in an alteration in the carrier protein's normal conformation. In some cases, this is sufficient to induce instability (Goldberg and St John, 1976). Therefore, the presence of foreign amino acids fused to globin could have a destabilizing effect. In an effort to assess the degree to which this might occur, we have constructed a second E1A−globin hybrid protein for half-life determination.

This chimeric control protein consists of E1A residues 71−122, which were shown not to be part of the region of E1A responsible for rapid turnover (Figures 3 and 4), three additional amino acids, Ser−Ile−Glu, and *Xenopus* β-globin residues 11−129 (SpEG71-122, Figure 2). SpEG71-122 is recognized by polyclonal antibodies directed against E1A, and can be immunoprecipitated from oocyte homogenates. Sp6 generated transcripts were microinjected into stage 6 oocytes and the half-life of SpEG71-122 was determined as previously described. Oocyte expressed SpEG71-122 exhibited a half-life of 1.75 h (Figure 6). This result indicates that the context of a chimeric configuration alone is sufficient to increase the turnover rate of globin since the half-life of SpEG71-122 is ~3-fold less than that of SpG. However, the half-life of SpEG71-122 is ~4-fold greater than the half-

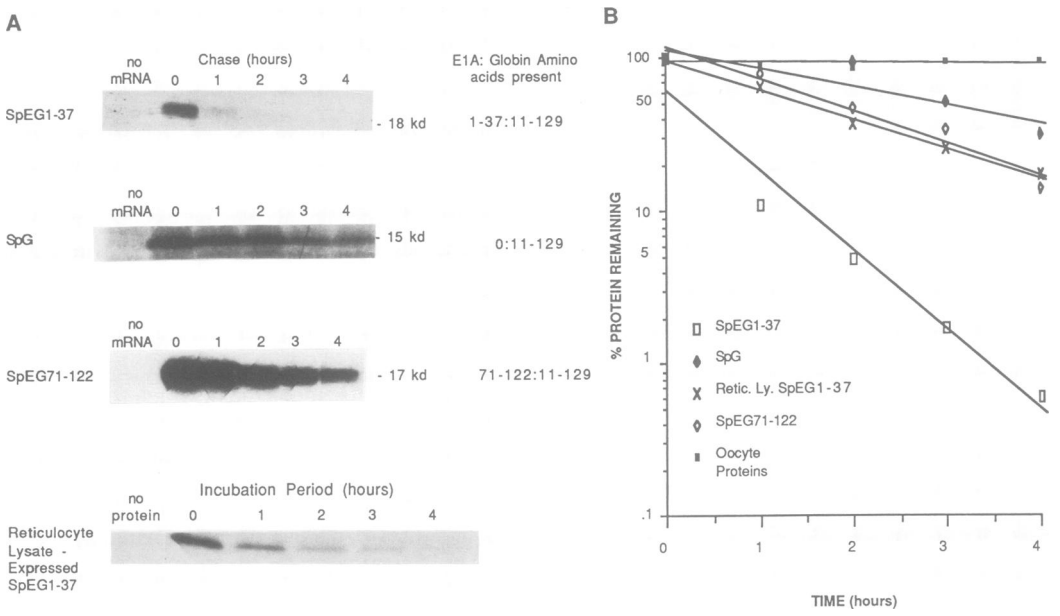

Fig. 6. Half-life determination of chimeric E1A−globin and control proteins. (**A**) The half-lives of oocyte-expressed SpEG1-37, Sp71-122, and reticulocyte lysate-expressed SpEG71-122 in microinjected oocytes were determined as described previously. After SpG mRNA, pulse, and chase injections (as described in the legend to Figure 1) the oocytes were homogenized, and the proteins separated by SDS−PAGE. The labeled proteins were visualized by autoradiography and SpG protein identified by comparison to the control oocytes containing no SpG mRNA (A). (**B**) Graphic depiction of the data presented in (A). The oocyte protein decay values were obtained from the half-life determination of oocyte expressed SpEG1-37.

Table I. Summary of half-life values of E1A in oocytes and HeLa cells

Protein	Origin	E1A amino acids present	Average half-life ± SD (h)	Number of determinations
E1A 410	*E.coli*	1, 3−289	>20	3
Sp13S	Ret. lysate	1−289	>20	3
Sp13S	Oocyte	1−289	3.3 ± 0.9	7
SpX	Oocyte	1−223	2.1 ± 0.2	3
SpXX	Oocyte	1−152	3.1 ± 1.0	3
SpNX	Oocyte	1−86	0.5 ± 0.05	3
SpRX	Oocyte	71−289	>20	3
SpNX	Oocyte	15−289	>20	3
Sp105NX	Oocyte	1−37, 87−289	3.7 ± 0.8	3
Sp410	Oocyte	1, 3−289	>20	3
			Half-life values	Average
SpEG1-37	Oocyte	1−37	0.3, 0.6	0.45
SpG	Oocyte	−	4.5, 6.5	5.5
SpEG71-122	Oocyte	71−122	1.5, 2.0	1.75
SpEG1-37	Ret. lysate	1−37	1.9, 1.5	1.7
E1A 13S	HeLa cell	1−289	0.63, 0.77	0.7
*dl*1504	HeLa cell	15−289	1.3, 2.4	1.9

life of SpEG1-37. We believe that this difference reflects the contribution of the E1A turnover signal to the instability of the chimeric protein, and therefore indicates that turnover as a consequence of the chimeric context is distinct from turnover directed by the E1A instability sequence.

The results presented in Figure 1 show that E1A turnover is related to translation, and as such we would predict that the half-life of reticulocyte expressed SpEG1-37 in microinjected oocytes would be comparable to that of oocyte-expressed SpEG71-122. Protein instability within the oocyte as a consequence of a chimeric context should be independent of the synthetic origin of the protein. Therefore, a transcript encoding the chimeric protein SpEG1-37 was expressed in reticulocyte lysates containing [^{35}S]methionine, the labeled protein was partially purified, injected into stage 6 oocytes, and the half-life determined as previously described. The half-life of reticulocyte expressed SpEG1-37 within oocytes was determined to be ~1.7 h (Figure 6). This value is ~4-fold greater than the observed half-life of SpEG1-37 when expressed within oocytes, and is essentially identical to that of oocyte-expressed SpEG71-122. Taken together, the results of the chimeric E1A-globin experiments

indicate that the E1A instability sequence is sufficient to increase the turnover rate of a foreign protein.

Turnover of wild-type E1A and dl1504 within HeLa cells

We have determined whether the amino terminal region of E1A is important for its stability in HeLa cells. Mutant *dl*1504 contains a deletion from −44 to +63 relative to the transcription initiation site of the E1A gene (Osborne *et al.*, 1982). Translation of the encoded mRNA initiates at the second AUG codon at residue 15, thereby generating a protein containing amino acids 15−289, which is identical to SpNS. Resolution of the E1A proteins derived from 13S and 12S messages (Richter *et al.*, 1985) allows for the selective determination of the half-lives of the 13S mRNA-specific proteins. In good agreement with previous determinations (Spindler and Berk, 1984; Branton and Rowe, 1985) adenovirus type 5 13S mRNA derived wild type E1A exhibited a half-life of ~45 min (Figure 7). In contrast, the half-life of 13S mRNA-derived E1A from *dl*1504 was found to be ~2.2 h. Thus, deletion of the 14 amino-terminal residues of E1A decreased its turnover rate 3-fold when expressed in HeLa cells.

The 3-fold difference in stability between wild-type E1A and *dl*1504 E1A expressed in HeLa cells is several fold less than that observed between Sp13S and SpNS expressed in oocytes. These half-life differences could be temperature dependent as discussed earlier. In addition, the tolerance of HeLa cells and oocytes to proteins with abnormal conformations may differ. The turnover of abnormal proteins is known to be several fold greater than that of their normal counterparts in mammalian cells (Goldberg and St John, 1976). Therefore, within HeLa cells the stabilizing effect of the deletion of the amino-terminal 14 residues of *dl*1504 may be in part offset by the destabilizing effect of an altered conformation as a consequence of the loss of these residues.

Table I summarizes all of the E1A half-life determinations in both oocytes and HeLa cells. These data underscore the remarkable stability of E1A in protein injected oocytes as well as in mRNA injected oocytes in which E1A sequences encoding amino terminal residues have been deleted. Also, this table notes that variation in half-life from different experiments (and different batches of oocytes) is low.

Discussion

Following injection into *Xenopus* oocytes, adenovirus-5 E1A is phosphorylated (Richter *et al.*, 1988), transported to the nucleus (Richter *et al.*, 1985; Richter and Jones, 1986) and stimulates the transcription of injected genes (Ferguson *et al.*, 1984; Richter *et al.*, 1985, 1987). These same events occur in both E1A-injected mammalian cells and in mammalian cells expressing the E1A gene. One difference between E1A-injected cells and cells expressing the E1A gene, however, is E1A stability. The protein appears to be stable when injected into cells while it degrades with rapid kinetics in cells expressing the gene (Ferguson *et al.*, 1984; Spindler and Berk, 1984; Branton and Rowe, 1985; Richter *et al.*, 1985). This observation has prompted us to investigate the mechanisms that govern E1A stability.

Since the initial protein injection experiments utilized E1A synthesized in *E.coli*, our initial concern was that some modifiation of E1A unique to bacterial expression could have made it refractory to turnover. Alternatively, perhaps synthesis of E1A in a heterologous system somehow influences stability in protein injected cells. To distinguish between these possibilities, the half-life of E1A expressed by reticulocyte lysates in injected oocytes was determined. Similar to *E.coli* expressed E1A, reticulocyte lysate generated E1A exhibited a long half-life within oocytes. When E1A is synthesized *de novo* from injected mRNA however, it has a very short half-life. These results indicate that the half-life of E1A is determined co-translationally. This should not be confused with co-translational turnover (Maicas *et al.*, 1988), however, since the E1A peptide chain is completed before degradation begins.

The importance of the amino-terminal region of E1A for instability

In order to identify a region of E1A that is critical for instability, we determined the half-lives of a series of E1A deletion proteins expressed within oocytes. Deletion of carboxy-terminal residues 86−289, and residues 38−86 did not result in a protein with increased stability. In contrast, deletion of amino-terminal residues 1−70 resulted in a stable E1A protein. The deletion analysis indicates that the instability region is within the amino-terminal 37 residues (Table I). With the exception of the initiator methionine, only two of the amino-terminal 37 E1A residues, an arginine and leucine, are highly conserved among human adenoviruses (Kimelman *et al.*, 1985). Deletion of the conserved arginine residue results in an E1A with greater stability in mRNA injected oocytes (Table I). These results indicate that this arginine residue is part of the instability region. Work is currently in progress to define this region of E1A.

That the amino-terminal region of E1A is sufficient to induce protein turnover was demonstrated by the observation that an E1A−globin hybrid protein (SpEG1-37) rapidly degrades in mRNA-injected oocytes (Figure 7). This same protein, when synthesized in reticulocytes and subsequently injected into oocytes, exhibits increased stability (by ~4-fold). This underscores the results presented in Figure 1 which show that only E1A proteins synthesized *de novo* are marked for turnover.

The role of the amino-terminal 14 residues in E1A turnover within HeLa cells was also assessed. The half-life of E1A derived from *dl*1504, which generates an E1A containing residues 15−289, exhibited greater stability than wild-type E1A (Table I). This result, and experiments with adenovirus-12 E1A suggest that the same mechanisms governing E1A turnover in mRNA-injected oocytes also apply to mammalian cells. The adenovirus 12 E1A degrades with rapid kinetics in cells expressing the gene (Lucher *et al.*, 1986). When this E1A is expressed in *E.coli* and then injected into monkey (Vero) cells, it is stable (Krippl *et al.*, 1986). Recently, adenovirus type 12 E1A (the 12S mRNA product) that has been modified at the amino terminus was found to be stable in KB cells (Lucher *et al.*, 1986). The nature of the amino-terminal modification of this E1A is unknown. However, the methionine residues at positions 1 and 5 were not detected after N-terminal sequencing, and the modified protein exhibited a mol. wt of 39 kd whereas the unmodified 12S protein was 43 kd. This suggests that these residues, which contain the region we have identified as being important for E1A instabilty, were deleted.

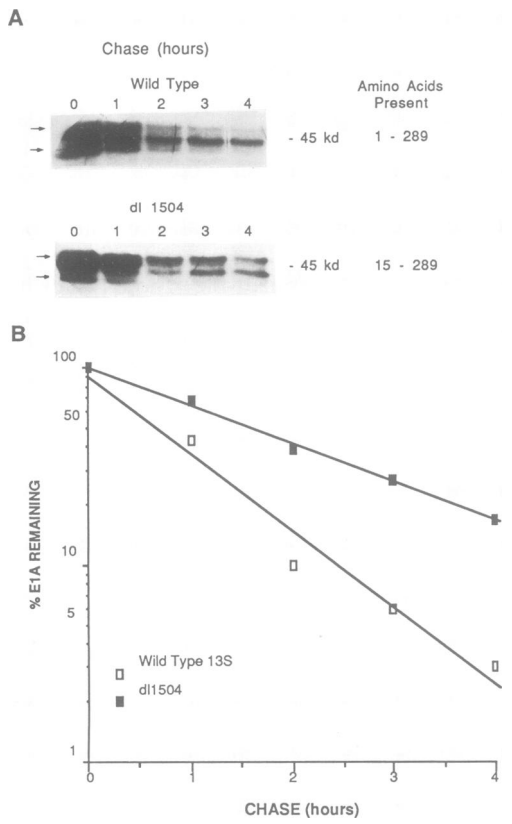

Fig. 7. Half-life determination of wild-type 13S E1A and *dl*1504 in HeLa cells. (**A**). HeLa cells were infected with wild-type adenovirus type 5 or *dl*1504 virus, pulsed with [^{35}S]methionine, and chased with radioinert methionine. E1A proteins were immunoprecipitated with rabbit anti-E1A polyclonal antibodies, the proteins separated by SDS−PAGE, and E1A proteins visualized by autoradiography. The arrows denote the proteins encoded by the 13S transcript. Shown are the autoradiographs (**A**) and densitometric quantitation (**B**).

Therefore, the data in the present communication are entirely consistent with these observations.

Mechanisms of protein turnover

Recent work on intracellular proteolysis has taken into account the role of protein primary structure in the determination of protein turnover (Hershko and Ciechanover, 1982; Dice, 1987; Rechsteiner *et al.*, 1987, for reviews). Several studies have searched for conserved regions among both long and short lived proteins in order to discern possible underlying proteolytic control mechanisms. Studies by Bachmair *et al.* (1986) have implicated the amino terminal residue as the one that governs protein half-life. Methionine is one residue which, according to this 'N-end rule', stabilizes proteins. We do not know whether the amino-terminal methionine residue remains on E1A in mRNA injected oocytes. However, the initiator methionine remains attached to E1A when it is expressed by reticulocyte lysates (Downey *et al.*, 1984). According to the N-end rule, reticulocyte lysate expressed E1A would be expected to be stable within oocytes, which is in agreement with our results (Table I). However, methionine has been determined to be the amino-terminal residue of adenovirus type 12 E1A derived from infected KB cells, and this E1A exhibits a short half-life (Lucher *et al.*, 1986). It is therefore clear that the amino-terminal residue is not the sole determinant of E1A stability.

A correlation between the presence of peptide regions rich in proline, glutamate, serine and threonine (PEST regions) and rapid protein turnover has recently been suggested (Rogers *et al.*, 1986). Adenovirus type 5 E1A contains four PEST regions encompassing residues 44−94 (region 1), 125−149 (region 2), 177−202 (region 3) and 223−244 (region 4). To address the contribution of these regions to protein instability experimentally through the use of deletion proteins is difficult due to the possible alteration of conformation which could be destabilizing. However, in mutants SpX and SpXX, PEST regions 4 and 3−4, respectively, were deleted and these deletions did not result in an increase (or decrease) in protein stability. In addition, SpRX and SpNS, which contain PEST regions 2−4 and 1−4, respectively are stable in oocytes. *dl*1504 (containing all four PEST regions) exhibited increased stability in HeLa cells when compared to wild-type E1A (Table I). Thus, we cannot ascribe an 'instability' function to any of the PEST regions in E1A.

Ubiquitin has been shown to be involved in the turnover of proteins with short half-lives (Rechsteiner, 1987, for review). We do not know if E1A is degraded by a ubiquitin mediated proteolysis pathway. However, we do not observe high mol. wt E1A species which might possibly be E1A−ubiquitin conjugates, as found by Bachmair *et al.* (1986). In addition, ubiquitin conjugation to protein occurs through covalent attachment to the ϵ amino group of lysine residues. There are three lysine residues within E1A, although none are located within the amino-terminal region. Furthermore, the half-life of deletion E1A SpXX, which contains no lysine residues, is the same as wild-type E1A (Table I).

Can the prolonged stability of E1A account for transactivation by the 12S product in protein infected cells?

Previous studies have shown that *E.coli*-expressed E1A derived from the 12S message *trans*-activates the early viral E3 promoter in both microinjected oocytes and Vero cells. In addition, the first exon of E1A was shown to be sufficient for *trans*-activation (Ferguson *et al.*, 1985; Richter *et al.*, 1985; Krippl *et al.*, 1985). These results have been difficult to reconcile with the findings that localize the *trans*-activation function of E1A to a conserved region that is almost exclusively comprised of the unique residues encoded by the 13S mRNA (Berk, 1986, for review; Lillie *et al.*, 1987). The findings of this study may explain these apparent contradictions. Perhaps the protein encoded by the 12S mRNA is capable of *trans*-activation but the concentration is insufficient for transcriptional stimulation in cells expressing this protein. In protein injected cells, however, the relatively large amounts that are injected, coupled with its prolonged stability, would allow its effective concentration to be very much higher, which might then be sufficient to stimulate transcription.

A model for E1A turnover

The results of this study show that E1A is marked for rapid degradation while it is being synthesized. Since residue(s) at the amino terminus are required for turnover, we envision that it is this region of the protein that is recognized by a cellular factor following its emergence from the ribosome.

Such a cellular factor could either be polysomal or freely soluble. If it is freely soluble, however, then it cannot recognize E1A when it has already assumed its three-dimensional configuration. Thus, it is possible that the amino end of E1A is buried within the remainder of the protein, and is not accessible for factor interaction. Such a factor could actually be a protease, a protein involved in proteolysis that associates with E1A, or an enzyme that modifies E1A so that it can be recognized by a protease. With any of these scenarios, it would be important not only to identify the residues, in addition to the second arginine residue, that are required for turnover but also the cellular proteins that might bind to this region. Such experiments are presently underway.

Materials and methods

Plasmids and viruses

The constructions of plasmids pSp13S, pSpX, pSpXX, pSpNX, pSpRX, pSp105NX (Richter *et al.*, 1988) and pAS1 E1A 410 (Ferguson *et al.*, 1984) have been described previously. *dl*1504 (Osborne *et al.*, 1982) was a gift from A.J.Berk. pSp410 was generated by isolating an *Nde*I–*Hpa*I fragment containing the E1A coding region from pRX3, (which contains sequences 0–1880 from pAS1 and the adenovirus type 5 sequences coding of the E1A and E1B genes in pBR327) filling in with DNA polymerase I (Klenow), and ligating into the *Sma*I site of pSP65pA. pSP65pA is a modified form of pSP65 containing a 46 bp A–T tract inserted into the polylinker *Pst*I site. pSpNS was constructed by treating the *Nde*I–*Hpa*I fragment from pRX3 with *Bal*31 and ligating into pSP65pA. This removed the sequences coding for the initiator methionine residue so that translation would begin at the second AUG. pSpEG1-37 was constructed from E1A sequences isolated from pSp105NX (Richter *et al.*, 1988), and *X.laevis* β-globin sequences from pSP64–XβM (Krieg and Melton, 1984). pSp105NX, which contains a linker with *Xho*I and *Bgl* II sites inserted at nucleotide position 815, was digested with *Pvu*I and *Bgl* II, and pSP64-XβM was digested with *Pvu*I and *Bcl*I. THe *Pvu*I–*Bgl* II fragment containing the amino-terminal 37 residues of E1A was ligated to the *Pvu*I–*Bcl*I fragment from pSP64-XβM containing residues 11–129 of β-globin to yield pSpP105EG. To bring the E1A and globin sequences in-frame, pSp105EG was digested with *Xho*I, filled in with Klenow enzyme, and ligated to give pSpEG1-37, which contains the four added residues Ser–Ser–Ile–Glu between E1A and globin. pSpG was constructed by digesting pSP64-XβM with *Nco*I and *Bcl*I, removing the internal fragment, and ligating after addition of a *Nco*I–*Bcl*I linker (15mer). This linker adds the four amino acids Ser–Ser–Ile–Glu between the initiator methionine and the isoleucine residue at position 11. pSpEG71-122 was constructed from pSpRX and pSP64-XβM. The *Pvu*I–*Cla*I fragment containing the sequences coding for the amino-terminal 51 residues of SpRX was ligated to the *Pvu*I–*Bcl*I fragment of pSP64-XβM containing sequences coding for residues 11–129 after addition of a *Cla*I–*Bcl* I linker (9mer) to yield pSpEG71-122. This linker adds residues Ile and Glu between residues 71–122 of E1A and 11–129 of β-globin.

In vitro transcription, translation and protein purification

Capped and polyadenylated mRNAs for *in viro* translation and oocyte injections were generated by Sp6 polymerase from *Hin*dIII or *Pst*I digested plasmids essentially as described by Melton *et al.* (1984) as detailed by Richter *et al.* (1988). Plasmid templates were prepared as described by Slavicek and Krider (1987).

In vitro translation was by micrococcal nuclease treated reticulocyte lysates (Promega Biotech). Each reaction contained 35 μl of lysate in a total volume of 50 μl with 50 μCi [^{35}S]methionine (sp. act. >1000 Ci/mmol). After incubation for 45 min at 30°C, the equivalent of two to four reactions were applied to a G100–120 Sephadex column and 1 ml fractions were collected using a running buffer of 50 mM Tris (pH 7.5), 0.1 mM DTT, 1 mM EDTA and 20 mM NaCl. Fractions containing E1A proteins were identified after SDS–PAGE of aliquots by autoradiography, pooled and concentrated using Centricon miroconcentrators (Amicon).

Half-life determination of in vitro expressed E1A within oocytes

Partially purified reticulocyte lysate expressed E1As were microinjected into stage 6 oocytes (30 nl of protein concentrated to 25–50 c.p.m./nl) in groups of 10 oocytes. The oocytes were incubated for various times, homogenized in Barth's saline containing 0.1% Triton X-100, centrifuged to remove yolk proteins, and the components of the supernatants were separated by SDS–PAGE. The gels were treated with an autoradiographic enhancer (Amplify, NEN), dried and E1A proteins were visualized by autoradiography and quantitiated by scanning densitometry at several different exposures of the autoradiogram.

Half-life determinations of oocyte expressed E1As

Manually defolliculated stage 6 oocytes were each injected with ~30 ng of SP6 polymerase generated mRNAs in groups of 16 oocytes. The oocytes were incubated at 19°C for 2.5 h to allow the loading of mRNA onto polysomes. The oocytes were then injected with ~0.3 pmol [^{35}S]methionine (sp. act. >1000 Ci/mmol), incubated at 19°C for 1.5 h, and injected with 3 nmol radioinert methionine. After 1 h incubation in Barth's saline containing 1 mM radioinert methionine, the zero time point was taken. Subsequent time points were taken after 1–25 h of additional incubation. The oocytes were homogenized in Barth's medium with 0.1% Triton X-100; the homogenates were clarified of yolk proteins by centrifugation, and the supernatants were adjusted to 1 × RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 1.0% deoxycholate, 0.1% SDS). The samples (except for the half-life determinations of SpNX) were placed in a boiling water bath for 10 min and clarified by centrifugation. Polyclonal antibodies directed against E1A were added to the supernatants which were incubated at room temperature for 2 h. Protein A Sepharose was then added to select the E1A–antibody complexes. The immunoprecipitated proteins were separated by SDS–PAGE, the labeled proteins visualized by autoradiography and quantitated by scanning densitometry.

Half-life determinations in HeLa cells

The half-lives of E1A from adenovirus type 5 and *dl*1504 in HeLa cells were determined as essentially described by Branton and Rowe (1985). HeLa cells were pulsed for 45 min in radioinert methionine free DME medium with [^{35}S]methionine, 50 μCi/3 × 10^5 cells (sp. act. >1000 Ci/mmol) 16 h after infection (at a m.o.i. of ~40). The cells were chased with DME containing radioinert methionine, collected and prepared for immunoprecipitation by E1A polyclonal antibodies as described by Branton and Rowe (1985). The immunoprecipitated proteins were separated by SDS–PAGE, the labeled proteins visualized by autoradiography, and quantitated by scanning densitometry.

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Note added in proof

Mutant SpRX (Figure 4A) contains residues 71–289.