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# Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*

(oncogenes/N-myc/c-myc/c-fos/p53/E1A/proteolysis/ATP)

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ABSTRACT **Nuclear oncoproteins are among the most rapidly degraded intracellular proteins. Previous work has implicated the ubiquitin-mediated proteolytic system in the turnover of short-lived intracellular proteins. In the present study, we have evaluated the potential role of the ubiquitin system in the degradation of the specific nuclear oncoproteins encoded by the N-*myc*, c-*myc*, c-*fos*, p53, and *E1A* genes. Each of these nuclear oncoproteins was synthesized *in vitro* by transcription of the appropriate cDNA and translation of the resulting mRNA in the presence of [$^{35}$S]methionine. Degradation of labeled proteins was monitored in the ubiquitin cell-free system. ATP stimulated the degradation of all the proteins between 3- and 10-fold. The degradation was completely inhibited by neutralizing antibody directed against the ubiquitin-activating enzyme, $E_1$, the first enzyme in the ubiquitin-mediated proteolytic cascade. Moreover, degradation in $E_1$-depleted lysates could be restored in each case by the addition of affinity-purified $E_1$. These data suggest that the ubiquitin system mediates the degradation of these oncoproteins *in vitro*. Degradation of other proteins, such as superoxide dismutase, cytochrome *c*, enolase, RNase A, and ornithine decarboxylase, is not mediated by the ubiquitin cell-free system. This suggests that the nuclear oncoproteins studied here possess specific signals that target them for rapid turnover by this proteolytic pathway. Furthermore, the relative sensitivity to degradation of various *E1A* mutants *in vivo* is also maintained in the cell-free system, suggesting that the ubiquitin pathway may play a role in the cellular degradation of these proteins as well.**

The nuclear oncoproteins N-myc, c-myc, c-fos, p53, and E1A are thought to play a role in the regulation of cell growth and differentiation, and they have been implicated in neoplastic transformation. For example, amplification of the N-*myc* gene has been demonstrated in a subset of neuroblastomas. Amplification of this gene is also correlated with advanced stages of disease, rapid tumor progression, and a poor prognosis (1, 2). c-*myc* is activated by translocation or mutation in many B-cell lymphomas (3) or by amplification in other tumor types, such as small cell lung cancer and breast cancer (4, 5). The c-*fos* oncogene product has been implicated in neoplastic transformation as well as in mediating the action of a variety of extracellular stimuli (6, 7). Normal p53 appears to function as a transformation suppressor gene, but mutations have been identified that both stabilize the protein and result in neoplastic transformation (8, 9). Finally, the proteins encoded by early region 1A (*E1A*) of human adenovirus play a central role in the ability of the virus to replicate efficiently and to transform certain primary cells (10).

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A feature common to all five of the nuclear oncoproteins described above is their rapid degradation *in vivo* (11). Rapid degradation of proteins enables the cell to alter the levels of regulatory proteins quickly (12). An interesting feature of intracellular protein degradation in all organisms is its absolute requirement for metabolic energy (reviewed in ref. 13). This ATP dependence apparently reflects mechanisms that endow the proteolytic systems with high specificity toward their protein substrates. An ATP-dependent proteolytic system that consists of several essential components has been characterized recently. The system requires ubiquitin, a 76-residue protein whose covalent conjugation to protein substrates targets them for rapid degradation (reviewed in refs. 13–16). A mammalian mutant cell line harboring a thermolabile ubiquitin-activating enzyme, E1, is grossly deficient in the turnover of short-lived proteins at the nonpermissive temperature (17), suggesting that the system is involved in the degradation of this class of proteins in the cell.

Although the enzymology of the ubiquitin system has been studied in considerable detail, our understanding of the signals that target proteins selectively for rapid turnover is incomplete (reviewed in ref. 16). We describe here a general approach for *in vitro* analysis of degradation of any cellular protein for which a cDNA clone is available. As model substrates we have used N-myc, c-myc, c-fos, p53, and E1A, all of which are rapidly degraded *in vivo*. Using a cell-free system, we demonstrate efficient ubiquitin-dependent degradation of each of these *in vitro*-synthesized nuclear oncoproteins. This approach may permit systematic analysis of structural determinants that render proteins susceptible for ubiquitin conjugation and subsequent degradation.

## MATERIALS AND METHODS

**Preparation of Labeled Substrates.** cDNA clones of N-*myc* (18), c-*myc* (19), c-*fos* (20), and p53 (21) were kindly provided by L. Stanton and J. Michael Bishop (University of California, San Francisco), Robert Eisenman (Fred Hutchinson Cancer Center, Seattle), Thomas Curran (Roche Institute of Molecular Biology, Nutley, NJ), and Moshe Oren (Weizmann Institute for Science, Rehovot, Israel), respectively. The E1A clones have been described (22). We will refer to the products of these cDNAs as nuclear oncoproteins. Transcription of mRNA from linearized plasmids was carried out *in vitro* using either T7 polymerase (Stratagene; c-*myc*) or SP6 polymerase (Promega; N-*myc*, c-*fos*, p53, and *E1A*) essentially as described by Ramsay *et al.* (18). Single mRNA bands of the appropriate sizes were obtained for all the transcribed genes after formaldehyde/agarose electrophoresis (data not shown).

Translation was carried out in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [$^{35}$S]methionine essentially as suggested by the supplier. After translation, ATP was depleted by incubation for 120 min at 37°C in

the presence of hexokinase and 2-deoxyglucose. Depletion of ATP allowed the removal by isopeptidases of ubiquitin moieties that may have been covalently attached to the substrates during translation (14, 15). Labeled proteins were adsorbed on and eluted from diethylaminoethyl-cellulose (DE52, Whatman) following removal of hemoglobin and ubiquitin as described (23). The proteins were dialyzed extensively to remove excess salt and unincorporated labeled methionine.

**Degradation Assays.** Degradation assays were carried out in reticulocyte lysates as described (23). Samples were processed for SDS/PAGE, and disappearance of labeled proteins was monitored by fluorography. To assess quantitatively the degradation of labeled proteins, release of trichloroacetic acid-soluble material was followed (23). Experiments using anti-$E_1$ IgG were carried out as described (24).

## RESULTS

**ATP Is Required for the Degradation of N-myc, c-myc, c-fos, and p53 in Reticulocyte Lysates.** As shown in Fig. 1 (lanes 1 and 6), the majority of label (>90%) was incorporated into full-length proteins that have molecular masses that are essentially identical to those of the authentic cellular proteins. Minor species were also found that may be either proteins translated from weak alternative initiation sites or degradation products of the authentic proteins. N-*myc* translation yielded two proteins with apparent molecular masses of 58 and 60 kDa, as reported by others (25). Similarly, translation of c-*myc* also yielded two proteins with apparent molecular masses of 56 and 58 kDa, as previously described (19, 26). Translation of c-*fos* yielded a single protein with an apparent molecular mass of ≈57 kDa, very similar to that reported by others (20, 27). Heterogeneity in the apparent molecular masses of N-myc, c-myc, and c-fos is due to both alternative in-frame translation start sites (N-*myc*, ref. 25; c-*myc*, ref. 19), as well as previously documented phosphorylation of each of these proteins (19, 20, 25). The translation of p53 yielded a single protein with an apparent molecular mass of 53 kDa (21). Translation of the E1A mRNAs yielded proteins with the expected molecular masses (ref. 22; data not shown).

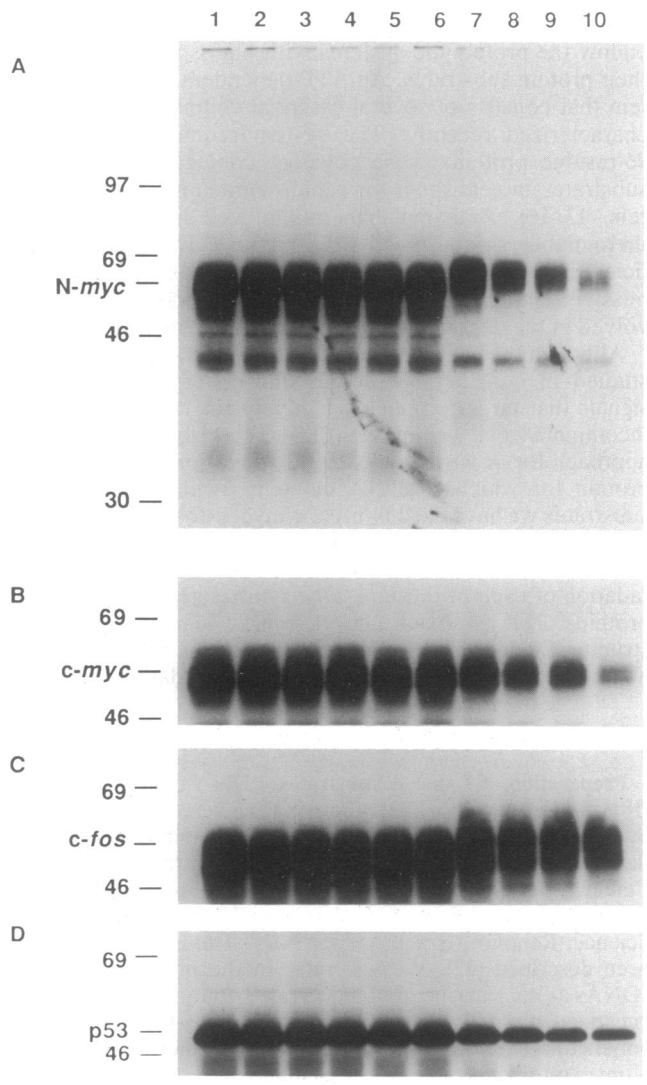


FIG. 1. ATP-dependent degradation of oncoproteins: fluorographic analysis following SDS/PAGE (*A*) N-myc. (*B*) c-myc. (*C*) c-fos. (*D*) p53. Radiolabeled oncoproteins were incubated in reticulocyte lysates without (lanes 1–5) or with (lanes 6–10) ATP. Reactions were terminated after 0 min (lanes 1 and 6), 30 min (lanes 2 and 7), 1 hr (lanes 3 and 8), 2 hr (lanes 4 and 9), and 3 hr (lanes 5 and 10).

Incubation of the labeled proteins for up to 3 hr in the absence of ATP resulted in very little degradation (Fig. 1, lanes 1–5). However, in the presence of ATP, disappearance of a substantial amount of labeled protein was detected (Fig. 1, lanes 6–10). Protein species of lower molecular mass were not detected during the course of incubation in the presence of ATP, suggesting the absence of stable intermediate products of degradation. However, in the cases of N-myc, c-myc, and c-fos, an increase in apparent molecular mass occurred with time in the presence of ATP (Fig. 1, lanes 6–10), which is likely due to phosphorylation of each of these oncoproteins (19, 20, 25). Monitoring the degradation of these four proteins by the release of trichloroacetic acid-soluble material confirmed that there was minimal degradation in the absence of ATP, while the addition of this nucleotide stimulated the degradation 3- to 10-fold (data not shown).

**Degradation of N-myc, c-myc, c-fos, and p53 Requires $E_1$.** To evaluate the potential role of the ubiquitin system in the turnover of these four oncoproteins, neutralizing IgG against $E_1$, the first enzyme in the ubiquitin cascade was used (24). Anti-$E_1$ IgG inhibited ATP-dependent degradation of each of the four oncoproteins in a dose-dependent manner (Fig. 2). By contrast, preimmune IgG was without effect. There was a small amount of ATP-independent degradation that was unaffected by anti-$E_1$ IgG (data not shown), indicating that inhibition is specific for the ATP-dependent component of the degradation. Finally, inhibition of degradation was reversible; after removal of the immune complexes with protein G-Sepharose, ATP-dependent degradation could be restored to normal levels by the addition of affinity-purified $E_1$ (Fig. 2, arrow).

We also monitored the effect of a saturating concentration of anti-$E_1$ IgG on the degradation of the four proteins as determined by SDS/PAGE and fluorography (Fig. 3). Substantial degradation was found in each case only after incubation in the presence of ATP (compare lanes 2 and 3). Although incubation in the presence of preimmune IgG failed to inhibit degradation (lane 4), anti-$E_1$ IgG inhibited degradation completely (lane 5). Finally, degradation could be restored to $E_1$-depleted lysates by the addition of affinity-purified $E_1$ (lane 6).

**Specificity of Degradation of Proteins by the Ubiquitin Cell-Free System.** The finding that all four proteins are degraded by the ubiquitin cell-free system raises the possibility that the effect is not specific and that any labeled protein can be degraded by this *in vitro* system. However, ubiquitin is conjugated to proteins only after their binding to $E_3$, the ubiquitin-protein ligase. This reaction is highly specific and occurs between unique structural domains of the

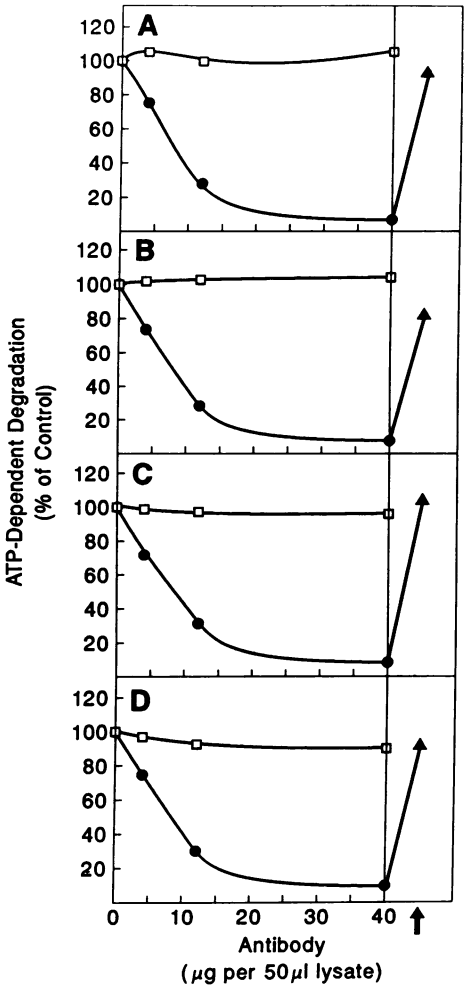


FIG. 2. Inhibition of ATP-dependent oncoprotein degradation by anti-$E_1$ IgG: quantitative analysis of release of radiolabeled trichloroacetic acid-soluble products. (*A*) N-myc. (*B*) c-myc. (*C*) c-fos. (*D*) p53. Oncoproteins were incubated for 3 hr in lysates supplemented with ATP and an ATP-regenerating system after treatment of the lysates with the indicated amounts of preimmune (□) or anti-$E_1$ (●) IgG (24). 100%, Value measured in a system to which no IgG had been added. Values obtained from parallel systems incubated with an ATP-depleting system were subtracted, and the results express ATP-dependent activity. ▲, Degradation in lysates depleted of $E_1$ by precipitation of immune complexes with protein G-Sepharose, followed by addition of 0.5 μg of affinity-purified $E_1$ (24).

substrate and selective binding sites of the enzyme (reviewed in refs. 14 and 16). Other proteins, such as ornithine decarboxylase, are degraded by the cell-free system in a reaction that is ATP dependent but ubiquitin independent (28). In addition, there are proteins that are not degraded at all by the cell-free system. The results presented in Table 1 demonstrate the behavior of various proteins in the *in vitro* system. Although the degradation of bovine serum albumin and lysozyme required ATP (and was also ubiquitin dependent; refs. 14, 23, 24), other proteins, such as superoxide dismutase, cytochrome *c*, enolase, and RNase A, were not degraded by the lysate.

**Correlation Between *in Vivo* and *in Vitro* Degradation of E1A Mutants.** The half-life of the E1A protein in the cell has been determined to be 4 hr, and several mutants of the E1A protein with various half-lives *in vivo* have been constructed (22). These include the NX ($t_{1/2}$ < 30 min), XX ($t_{1/2}$ ≈ 2 hr), and RX ($t_{1/2}$ ≈ 24 hr). We have analyzed the stabilities of the proteins derived from these genes in our cell-free system. As shown in Fig. 4*A*, the relative proteolytic sensitivities of these four

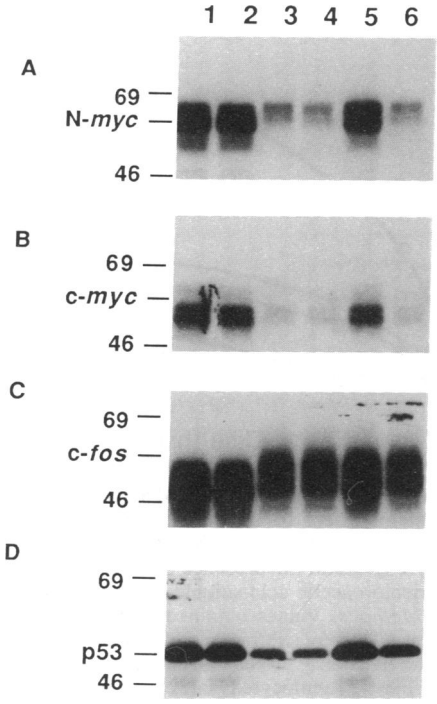


FIG. 3. Inhibition of degradation of oncoproteins by anti-$E_1$ IgG as determined by SDS/PAGE followed by fluorography. (*A*) N-myc. (*B*) c-myc. (*C*) c-fos. (*D*) p53. Oncoproteins were incubated in lysates for 0 min (lane 1) or 3 hr (lanes 2–6). Lanes: 2, with the ATP-depleting system; 3, with ATP and the ATP-regenerating system; 4, as for lane 3 but with 40 μg of preimmune IgG; 5, as for lane 3, but with 40 μg of anti-$E_1$ IgG; 6, as for lane 5 except that 0.5 μg of affinity-purified $E_1$ was added to the lysate after the removal of $E_1$-immune complexes with immobilized protein G.

proteins were also maintained *in vitro*. Also, the degradation of all these proteins was ATP and ubiquitin dependent (Fig. 4*B*). However, the half-lives of the proteins *in vivo* and *in vitro* cannot be compared precisely, in part because the components of the *in vitro* degradation system are not stable during long periods of incubation.

## DISCUSSION

In the present study, we have shown that ATP stimulates *in vitro* degradation of the nuclear oncoproteins N-myc, c-myc, c-fos, and p53, between 3- and 10-fold. ATP-dependent degradation was completely inhibited by neutralizing antibody directed against $E_1$. Moreover, degradation in the $E_1$-depleted lysate could be restored in each case by the addition of affinity-purified $E_1$. Because $E_1$ is required for both ubiquitin conjugation and protein degradation (17, 23),

Table 1. ATP-dependent degradation of $^{125}$I-labeled lysozyme, bovine serum albumin, superoxide dismutase, cytochrome *c*, enolase, and RNase A

| $^{125}$I-labeled substrate | Degradation, % | |
|---|---|---|
| | − ATP | + ATP |
| Lysozyme | 7 | 31 |
| Bovine serum albumin | 1 | 29 |
| Superoxide dismutase | 3 | 4 |
| Cytochrome *c* | 2 | 2 |
| Enolase | 4 | 5 |
| RNase A | 6 | 7 |

Degradation of the labeled substrates (iodinated as described in ref. 29) was determined by measuring the release of trichloroacetic acid-soluble products as described in *Materials and Methods*.

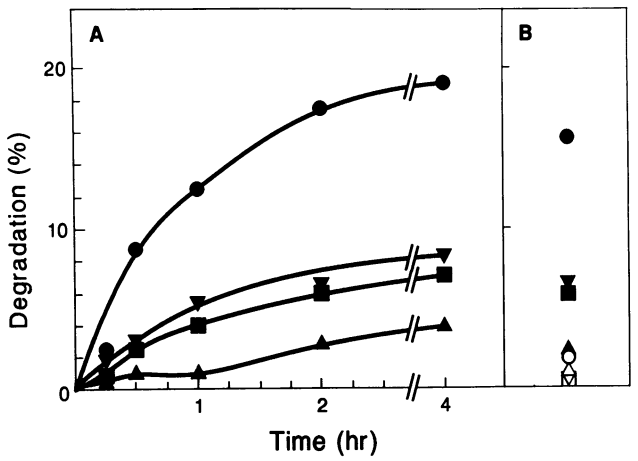


FIG. 4. Degradation of wild-type and mutant E1A proteins by the cell-free system: effect of inhibition of $E_1$. (*A*) Degradation of wild-type (■), NX (●), RX (▲), and XX (▼) E1A proteins was assayed in reticulocyte lysate in the presence of ATP and monitored by the release of trichloroacetic acid-soluble material as described in *Materials and Methods*. Values obtained from parallel systems incubated without ATP were subtracted, and the results are expressed as percent of total labeled protein degraded in an energy-dependent manner. (*B*) First, reticulocyte lysates were incubated with affinity-purified anti-$E_1$ (42 μg) and the immune complexes were removed as described in the legends to Figs. 2 and 3 and in ref. 24. Then, the labeled substrates were added, and the incubation was continued for 4 hr in the absence (open symbols) or presence (closed symbols) of affinity-purified $E_1$ as described (24).

these data suggest that the ubiquitin system mediates the ATP-dependent degradation of these oncoproteins *in vitro*.

Several lines of evidence support the conclusion that ubiquitin-dependent degradation of the nuclear oncoproteins studied here is specific. We have shown that several other proteins, such as RNase A, superoxide dismutase, enolase, and cytochrome *c*, are stable when incubated *in vitro*. In addition, Bercovich *et al.* (28) have shown that ornithine decarboxylase is degraded in reticulocyte lysate in an ATP-dependent but ubiquitin-independent manner. These findings support the hypothesis that only proteins harboring specific structural recognition markers are recognized by the ubiquitin-conjugating machinery and degraded.

Our data demonstrate a role for the ubiquitin system in degrading these nuclear oncoproteins *in vitro*. In addition, analysis of the E1A mutants demonstrates a correlation between the relative stabilities of these proteins *in vitro* and *in vivo*. Data from other laboratories also support this correlation. Rote *et al.* (30) have found a strong correlation between the stabilities of 22 proteins microinjected into human cells and their rates of degradation in reticulocyte lysates. Gonda *et al.* (31) have shown that the N-end rule, according to which the *in vivo* stability of certain proteins is determined by their $NH_2$-terminal residue, is preserved in the reticulocyte cell-free system as well. Finally, it has been shown that ornithine decarboxylase is degraded both *in vivo* (32) and in reticulocyte lysate (28) in an ATP-dependent but ubiquitin-independent mode. Thus, the ubiquitin system may play a role in the degradation of the nuclear oncoproteins studied here *in vivo* as well.

Assessment of the involvement of the ubiquitin system in the degradation of specific proteins *in vitro* has traditionally involved the depletion of ubiquitin from crude lysates and the demonstration of reconstitution of ATP-dependent degradation by addition of purified ubiquitin (13). However, it has recently been shown that, for certain proteins, fractionation of crude lysate inactivates or removes a factor(s) necessary for ubiquitin-dependent degradation (24). The nuclear oncoproteins studied here apparently fall into this class of substrates, since we could not observe substantial ATP-dependent degradation in ubiquitin-depletion lysate on addition of exogenous ubiquitin (unpublished results). Another approach to establish the involvement of the ubiquitin system in protein turnover has been to identify high molecular weight ubiquitin conjugates of the protein substrate (13–15, 23). The absence of detectable ubiquitin conjugates has led others (33) to conclude that the ubiquitin system is not involved in the degradation of c-myc *in vivo*. We have not been able to detect high molecular weight ubiquitin conjugates of the nuclear oncoproteins in this study. Detection of such conjugates may be possible *only* in cases where their steady-state level and specific activities are relatively high. For example, the *in vitro* degradation of bovine serum albumin, which is clearly ubiquitin dependent, is *not* accompanied by the appearance of detectable ubiquitin conjugates (29).

The signals that target specific proteins for rapid degradation by the ubiquitin system *in vitro* are not completely understood (14, 16). One feature that affects recognition by the ubiquitin system is the identity of the $NH_2$-terminal residue of the protein (31). However, other findings suggest that this is not the only signal and most probably not the predominant one (14, 16, 24). An advantage of the system utilized in our studies is the ability to alter the primary sequence of protein substrates. Thus, mutation of the five substrates identified here should facilitate dissection of structural features of these rapidly degraded proteins that are important for their recognition by the ubiquitin system. In fact, the E1A mutants provide a powerful tool in the study of such recognition markers.

Regulation of degradation of nuclear oncoproteins clearly plays an important role in determining steady-state levels of protein expression. This in turn is of interest from the standpoint of oncogene activation. Impaired turnover of nuclear oncoproteins *in vivo* would result in an increase in protein expression without requiring a change in transcription or translation. Mutations in protein coding sequences that alter signals for recognition by protein degradation systems represent a potential mechanism by which oncogene activation might occur.

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1. Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. & Bishop, J. M. (1984) *Science* **224**, 1121–1124.
2. Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y. & Hammond, D. (1985) *N. Engl. J. Med.* **313**, 1111–1116.
3. Leder, P., Battey, J., Lenoir, G., Moulding, C., Murphy, W., Potter, H., Stewart, T. & Taub, R. (1985) *Science* **232**, 765–771.
4. Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F. & Minna, J. D. (1983) *Nature (London)* **306**, 194–196.
5. Kozbor, D. & Croce, C. M. (1984) *Cancer Res.* **44**, 438–441.
6. Jenuwein, T., Muller, D., Curran, T. & Muller, R. (1985) *Cell* **41**, 629–635.
7. Kruijer, W., Cooper, J. A., Hunter, T. & Verma, I. M. (1984) *Nature (London)* **312**, 711–716.
8. Finlay, C. A., Hinds, P. W. & Levine, A. J. (1989) *Cell* **57**, 1083–1093.
9. Reich, N. C., Oren, M. & Levine, A. J. (1983) *Mol. Cell. Biol.* **3**, 2143–2150.

10. Ruley, H. E. (1983) *Nature (London)* **304,** 602–606.
11. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234,** 364–368.
12. Schimke, R. T. (1973) *Adv. Enzymol.* **37,** 135–187.
13. Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51,** 335–364.
14. Hershko, A. (1988) *J. Biol. Chem.* **263,** 15237–15240.
15. Rechsteiner, M., ed. (1988) *Ubiquitin* (Plenum, New York).
16. Ciechanover, A. & Schwartz, A. L. (1989) *Trends Biochem. Sci.* **14,** 483–488.
17. Ciechanover, A., Finley, D. & Varshavsky, A. (1984) *Cell* **37,** 57–66.
18. Ramsay, G., Stanton, L., Schwab, M. & Bishop, M. J. (1986) *Mol. Cell. Biol.* **6,** 4450–4457.
19. Hann, S. R., King, M. W., Bentley, D. C., Anderson, C. W. & Eisenman, R. N. (1988) *Cell* **52,** 185–195.
20. Curran, T., Gordon, M. B., Rubino, K. L. & Sambucetti, L. C. (1987) *Oncogene* **2,** 79–84.
21. Oren, M., Binez, B., Givol, D., Rechavi, G. & Zakut, R. (1983) *EMBO J.* **2,** 1633–1639.
22. Slavicek, J. M., Jones, N. C. & Richter, J. D. (1988) *EMBO J.* **7,** 3171–3180.
23. Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983) *J. Biol. Chem.* **258,** 8206–8214.
24. Mayer, A., Siegel, N. R., Schwartz, A. L. & Ciechanover, A. (1989) *Science* **244,** 1480–1483.
25. Makela, T. P., Saksela, K. & Alitalo, K. (1989) *Mol. Cell. Biol.* **9,** 1545–1552.
26. Ramsay, G., Evan, G. I. & Bishop, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7742–7746.
27. Curran, T., Miller, A. D., Zokas, L. & Verma, I. M. (1984) *Cell* **36,** 259–268.
28. Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. & Kahana, C. (1989) *J. Biol. Chem.* **264,** 15949–15952.
29. Ferber, S. & Ciechanover, A. (1986) *J. Biol. Chem.* **261,** 3128–3134.
30. Rote, K., Rogers, S., Pratt, G. & Rechsteiner, M. (1989) *J. Biol. Chem.* **264,** 9772–9779.
31. Gonda, D. K., Bachmair, A., Wünning, I., Tobias, J. W., Lane, W. S. & Varshavsky, A. (1989) *J. Biol. Chem.* **264,** 16700–16712.
32. Rosenberg-Hasson, Y., Bercovich, Z., Ciechanover, A. & Kahana, C. (1990) *Eur. J. Biochem.* **185,** 469–474.
33. Luscher, B. & Eisenman, R. N. (1988) *Mol. Cell. Biol.* **8,** 2504–2512.