Rescue of the complex temperature-sensitive phenotype of Chinese hamster ovary E36ts20 cells by expression of the human ubiquitin-activating enzyme cDNA

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The ubiquitin conjugation system is a multi-step pathway in which ubiquitin is activated and conjugated to acceptor proteins, one function of which is to target acceptor proteins for rapid degradation within the cell. The conjugation system is involved in many aspects of cellular functions, including the cell cycle. Several cell-cycle arrest mutant cell lines have been characterized and appear to harbour a mutant ubiquitin-activating enzyme, E1, as their primary defect. One such cell line is ts20, which is derived from Chinese hamster ovary E36 cells. This cell line has been used to characterize some of the potential functions of the ubiquitin conjugation system *in vivo*, such as its involvement in

the maturation of autophagic vacuoles. The present study describes the complete rescue of the complex ts20 phenotype following the expression of the cDNA for human E1. Stable transfectants expressing the human E1 cDNA in the CMVneo expression vector were measured for ubiquitin-conjugation activity, protein degradation and growth in culture at the non-permissive temperature. This rescue confirms that the phenotype observed in the ts20 cells is due to a defect in the E1 enzyme. Thus, the ts20 cell line will serve as a useful tool to delineate the functions of the ubiquitin system *in vivo*.

INTRODUCTION

Ubiquitination is a post-translational modification that involves the covalent linkage of ubiquitin (Ub) to the ϵ -amino group of lysine residues in a target protein (Hershko and Ciechanover, 1992). This modification exerts pleiotropic effects on cellular functions, the best characterized of which is the Ub-dependent degradative pathway (Hershko and Ciechanover, 1992; Finley and Chau, 1991). This pathway mediates the degradation of short-lived proteins (Ciechanover et al., 1984), oncoproteins (Scheffner et al., 1990; Ciechanover et al., 1991) and has recently been implicated in the proteolytic processing of major histocompatibility restricted class I antigens (Michalek et al., 1993). In some cases, such as histone and receptor modification, a ubiquitination event is not followed by degradation, and may modulate the function of the target protein (Wu et al., 1981; Yarden et al., 1986; Paolini and Kinet, 1993).

The Ub-activating enzyme, E1, catalyses the activation of Ub as a prerequisite for the Ub conjugation pathway (Haas et al., 1982; Ciechanover et al., 1982). The reactions catalysed by E1 culminate in a high-energy thiolester linkage formed between the E1 and Ub (Haas et al., 1982). In this form, the Ub is available for binding to several E2 (Ub-carrier) enzymes (Hershko et al., 1983). Certain E2 enzymes are able to conjugate Ub directly to target proteins (Pickart and Rose, 1985), while at least one E2 interacts with the Ub-protein ligase (E3), in the conjugation of Ub to acceptors (Pickart and Rose, 1985). The one common step in the pathway, however, is the initial activation of Ub via E1.

Several temperature-sensitive cell lines which harbour a mutant E1 exhibit cell cycle arrest in late S/early G2 phase at the non-permissive temperature. In addition, these cell lines exhibit a

defect in the degradation of short-lived proteins and fail to conjugate radiolabelled Ub to acceptor proteins at the non-permissive temperature (Finley et al., 1984; Kulka et al., 1988; Zacksenhaus and Sheinin, 1990).

Availability of these mutant cell-lines provides a powerful experimental tool to understand the precise cellular functions with which the Ub system is involved. The ts20 cell line, derived from Chinese hamster ovary E36 cells, is one such line (Kulka et al., 1988). Using the ts20 cells, we have demonstrated a novel role for the Ub system in the maturation of autophagic vacuoles (Groppe et al., 1991; Lenk et al., 1992; Schwartz et al., 1992). However, to date, definitive proof that the complex phenotype of the ts20 cells arises from a single defect in the Ub-activating enzyme has been lacking.

Recently, we have cloned and characterized the cDNA encoding the human Ub-activating enzyme (hE1) (Handley et al., 1991), and other laboratories have obtained E1 cDNAs from wheat (Hatfield et al., 1990) and yeast (McGrath et al., 1991). Clones from each source exhibit close homology among the species (McGrath et al., 1991). Furthermore, a partial human cDNA that complements the temperature-sensitive growth-arrest defect in a mouse L-cell mutant, A1S9, has been cloned independently (Zacksenhaus and Sheinin, 1990). Likewise, E1 cDNA has been used to restore the cell-cycle progression of other cell lines mutant for E1, as measured by fluorescent activator cell sorter profiles of cell populations expressing the cDNA following incubation at the non-permissive temperature (Kudo et al., 1991; Nishitani et al., 1992; Ayusawa et al., 1992). The present study presents a rigorous characterization of the phenotypic rescue of the ts20 cell-line through the stable expression of the full length human E1 cDNA. We not only demonstrate correction of the

Abbreviations used: G418, geneticin; E1, ubiquitin-activating enzyme; hE1, human ubiquitin-activating enzyme; E2, ubiquitin-carrier protein; RCMBSA, reduced carboxymethylated BSA; Ub, ubiquitin; DTT, dithiothreitol.

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growth arrest of the ts20 cells, but show restoration of the Ub-conjugation and degradation systems in the transfected cells at the non-permissive temperature. These studies document that the ts20 phenotype arises as a consequence of a defect in E1.

MATERIALS AND METHODS

Translation of active E1 from reticulocyte lysate

Human E1 in pGem7zf+ was linearized using *Hind*III and transcribed using SP6 RNA polymerase. Translation *in vitro* was performed using nuclease-treated rabbit-reticulocyte lysate (Promega) and incorporation of [³⁵S]-methionine (Amersham). The activity of the translated product was measured by its ability to form a specific complex with Ub-sepharose in the presence of ATP. The translation mixture was treated with 2 mM AMP and 0.08 mM pyrophosphate to remove Ub from the active E1. The resultant material was applied to a mini-column of DE52 essentially as described (Ciechanover et al. 1982). 'Fraction II', the high-salt eluate of the DE52 column, was dialysed against 20 mM Tris, pH 7.2/2 mM dithiothreitol (DTT), and concentrated to the original volume of reticulocyte-lysate applied to the column. The reticulocyte lysate fraction II was incubated with Ub-sepharose beads in 20 mM Tris, pH 7.2/5 mM MgCl₂/2 mM ATP/2 mM DTT (plus ATP). Parallel mixtures were incubated in 20 mM Tris, pH 7.2/20 mM 2-deoxyglucose/0.1 mg/ml hexokinase/2 mM DTT (minus ATP). The reacted Ub-sepharose beads were pelleted and the supernatant was removed and prepared for SDS/PAGE by addition of an equal volume of 2 × Laemmli sample buffer containing mercaptoethanol (Laemmli, 1970). Meanwhile, the beads were washed three times in 20 mM Tris, 7.2/2 mM DTT, followed by boiling in 1 × Laemmli sample buffer for 10 min. Ub-sepharose beads were prepared using CH-activated Sepharose 4B (Sigma Chemical Co.) and Ub (Sigma) as described (Ciechanover et al. 1982).

Cell culture and transfection

The Chinese hamster ovary E36 and the corresponding ts20 cell lines were maintained at 30.5 °C (permissive temperature for the ts20 line) in a humidified chamber with 5% CO₂. The cytomegalovirus promoter was used to drive the expression of the cDNA for hE1, which was subcloned into the *Bgl*III and *Hind*III restriction sites of pCMVneo expression vector (a gift from Dr. J. Milbrandt, Washington University School of Medicine) (Crosby et al., 1991). Ts20 cells were transfected using a calcium phosphate coprecipitation method essentially as described (Chen and Okayama 1987). Transfected cells were cultured for 2 weeks in the presence of G418 (Sigma) to select stable transfectants. Visible colonies were isolated and cultured in the continual presence of G418.

Reverse transcriptase PCR

Total RNA from isolated cell-lines was purified by the following method. Briefly, cells were solubilized in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. The solubilized cells were extracted in water-equilibrated phenol and chloroform:isoamyl alcohol (49:1), prior to RNA precipitation with ethanol (Chomczynski and Sacchi 1987). A cDNA synthesis of 2 µg total RNA from each cell line was performed using random primers (Promega) and AMV-reverse transcriptase (Promega). ³²P-dCTP (Amersham) was added as a tracer to quantify the cDNA. PCR reactions were carried out in an Ericomp Twin Block Thermo-

cycler under stringent conditions as follows: 150 ng cDNA, 1.0 mM MgCl₂, 50 µM dNTP, 100 pmol oligonucleotide primers and 2.5 units of Taq DNA Polymerase (Promega), 35 cycles with an annealing temperature of 65 °C. Oligonucleotide primers were synthesized at the Washington University Protein Chemistry Core Facility.

Western analysis

SDS/PAGE followed by immunoblotting of cell extracts was carried out using a polyclonal antibody to E1 (Handley et al. 1991) and ¹²⁵I-anti-rabbit IgG (Boehringer-Mannheim).

Ubiquitin conjugation activity

Thermal inactivation of the cell lines was performed according to published methods, with some modification (Gropper et al., 1991). Cells were incubated at 43.5 °C for 1 h, or incubated at 30.5 °C. The cell monolayers were lysed in 20 mM Tris, pH 7.2/0.25% Triton-X-100/0.2 mM DTT. The lysates were collected and incubated on ice for 20 min, then centrifuged. Protein concentration of the cleared supernatants was determined using the Biorad protein assay (Biorad). Ub conjugation was measured according to the method of Hershko et al. (1983) with some modification. The reaction mixture contained, in a volume of 50 µl of 50 mM Tris/HCl (pH 7.6)/5 mM MgCl₂/2 mM DTT/20 µg oxidized RNase/50 pmol ¹²⁵I-Ub/1 mM ATP/10 µg creatine phosphokinase/10 mM creatine phosphate. For the minus-ATP condition, parallel reactions were established which contained 2.5 µg hexokinase and 10 mM 2-deoxyglucose. The reactions were incubated at 37 °C for 30 min. The resulting radiolabelled Ub conjugates were visualized with SDS/PAGE and autoradiography.

Protein degradation activity of transfected ts20 cells

The degradation of an exogenous protein substrate, ¹²⁵I-reduced carboxymethylated BSA (¹²⁵I-RCMBSA), was measured in cytosolic extracts (Parker and Topol, 1984) of E36, ts20 and the transfected lines C2, C3 and B11. Briefly, cells were harvested and washed twice in PBS, then swelled in a hypotonic buffer [15 mM KCl/10 mM Hepes (pH 7.6)/2 mM MgCl₂/0.1 mM EDTA/1 mM DTT] and homogenized in a Dounce homogenizer. Following complete breakage (evaluated microscopically), 0.1 volume of hypertonic buffer [1 M KCl/50 mM Hepes, (pH 7.6)/30 mM MgCl₂/0.1 mM EDTA/1 mM DTT] was added and the mixture was centrifuged at 10000 rev. min⁻¹ (*r*_{av.} = 6.98 cm) for 10 min at 4 °C in a Sorvall RC-5B centrifuge using an SS-34 rotor. Glycerol was added to the supernatant to a final concentration of 10%, and following a 15 min centrifugation at 35000 rev. min⁻¹ (*r*_{av.} = 6.57 cm) (using a 70.Ti rotor in a Beckman ultracentrifuge, 4 °C), the cytosolic proteins in the soluble fraction were precipitated in 90% ammonium sulphate. Proteins were resuspended in 25 mM Hepes (pH 7.6)/40 mM KCl/0.1 mM EDTA/1 mM DTT/10% glycerol, and dialysed. The cytosolic extracts were incubated at 39.5 °C for 0, 10, 20 or 40 min and the degradation of ¹²⁵I-RCMBSA was measured. Reaction mixtures contained 50 mM Tris (pH 7.6), 5 mM MgCl₂, 2 mM DTT, ¹²⁵I-RCMBSA (1 × 10⁵ c.p.m./assay) and ATP regenerating or depleting systems, and were incubated at 37 °C for 2 h. ATP regenerating system: 0.5 mM ATP/10 µg creatine phosphokinase/10 mM creatine phosphate. ATP depletion system: 2.5 µg hexokinase/10 mM 2-deoxyglucose. Samples were precipitated with trichloroacetic acid and trichloroacetic acid-soluble radioactivity was counted using a Packard Multiprias γ Counter and expressed as percent of total counts added in the

assay. The small amount of degradation (approx. 5%–7%) measured in the absence of ATP was subtracted from the values presented. Degradation activities as presented were normalized to the percent of the initial degradation activity in the absence of incubation at 39.5 °C.

Growth of E1-transfected cell lines at the non-permissive temperature

Cells were seeded in 24-well dishes at densities of between 1×10^4 and 3×10^4 cells per well in duplicate for each time-point. After approximately 18–24 h in culture at 30.5 °C, the zero time-point was harvested and the remaining cells were cultured at 39.5 °C. Cell counts of viable (Trypan Blue-excluding) cells were performed using a haemocytometer.

RESULTS

Previously, the human E1 cDNA was transcribed, and translated *in vitro*, to yield an immunoreactive protein product with the predicted molecular mass of E1 (Handley et al., 1991). We determined the activity of the translated product *in vitro* by examining its ability to specifically bind to Ub-sepharose in an ATP-dependent manner. In the absence of ATP, all of the translated material remained in the soluble fraction (Figure 1, lane 1), while no radioactive material associated with the Ub-sepharose (Figure 1, lane 2). In the presence of ATP, however, the majority of the translated product associated with the Ub-sepharose (Figure 1, lane 4), indicating an enzymically active E1 translation-product.

To facilitate rescue studies, the cDNA for hE1 was subcloned into the pCMVneo expression vector. Ts20 cells were transfected with the E1 expression-plasmid, then selected in the presence of G418. We obtained 11 stable, G418-resistant lines.

These lines were screened for expression of hE1 mRNA by reverse-transcriptase PCR. Northern analysis could not be used to assess hE1 expression because of strong cross-reactivity between hE1 cDNA and E36 cell mRNA (Handley et al., 1991). Therefore, an oligonucleotide primer pair specific for the hE1 sequence was identified and used to analyse first-strand cDNA

Figure 1 Activity of *in vitro* translated human E1

E1 was transcribed, translated and partially purified from reticulocyte extracts as described in the Materials and methods section. The material was incubated with Ub-sepharose in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of ATP. Lanes 1 and 3 represent unbound material and lanes 2 and 4 represent material bound to Ub-sepharose.

Figure 2 Reverse transcriptase PCR assay of E1 expression in ts20 transfected cells

Total RNA was isolated from cell lines and used as a template for cDNA synthesis, as described in the Materials and methods section. A hE1-specific oligonucleotide pair was used to prime PCR reactions. The lanes are labelled with the cell lines from which the RNA was isolated. Gel Marker 1 (Research Genetics) was used as a molecular mass marker (1000, 700, 500, 400, 300, 200, 100 and 50 bases).

Figure 3 Western-blot analysis of E1 expression in ts20 cell lines

Cell extracts of the ts20 cell lines were prepared as described in the Materials and methods section. Total protein (30 µg) was loaded in each lane for SDS/PAGE, then blotted onto nitrocellulose. Anti-hE1 polyclonal antibody (JJJ), was followed by 125 I-anti-(rabbit IgG) to visualize E1. Individual lanes are labelled with the cell lines. For details of (a) and (b) see text.

synthesized from the total RNA of the eleven G418-resistant lines (Figure 2). Seven of the stable lines: A17, B3, B18, C2, C3, D3 and D7 were positive for expression of the human-specific cDNA product (Figure 2). On the other hand, lines A16, B4, B11 and D8 did not express hE1 mRNA (Figure 2).

Immunoblots of cell extracts from the various ts20 transfected lines were performed using anti-hE1 polyclonal antibody. An

Figure 4 Ub-conjugation activities of transfected ts20 cell lines

Cells were incubated for 60 min at 43.5 °C, or maintained at 30.5 °C, then lysed (see Materials and methods section) and assayed for ^{125}I -Ub conjugation activity. The conjugation reactions were carried out in the presence or absence of ATP and visualized by SDS/PAGE and autoradiography. Data from each cell line is presented as four lanes: 30.5 °C plus and minus ATP, followed by 43.5 °C plus and minus ATP. The cell lines are labelled below the gels.

increase in the relative amount of E1 protein over untransfected control was observed in certain ts20 transfected lines (Figure 3a: A17, B3, B18; Figure 3b: C2, C3, D3, D7). These lines correspond to those expressing hE1 mRNA (Figure 2).

Previous studies have shown that E1 purified from red blood cell lysate migrates as a doublet of approximately 110 kDa/115 kDa on SDS/PAGE (Ciechanover et al. 1982). We found that the stably transfected ts20 cells also exhibit a 110 kDa/115 kDa E1 doublet by immunoblotting (Figure 3). Various exposure times (data not shown) have revealed the presence of both bands of the E1 doublet in each transfected cell line, indicating that both bands of the doublet arise from the single hE1 cDNA.

Because the ability of the ts20 cell extracts to support Ub conjugations is abolished at the non-permissive temperature (Kulka et al. 1988), we undertook to measure the Ub-conjugation activity in the rescued and non-rescued cell lines. The conjugation activities from extracts of cells that were incubated at 43.5 °C for

60 min compared with extracts of cells maintained at 30.5 °C are shown in Figure 4. The conjugation activity of ts20 cell extracts incubated at 43.5 °C is markedly diminished compared with that seen in extracts of cells incubated at 30.5 °C (the permissive temperature for the ts20 cell line). On the other hand, incubation at the elevated temperature had little effect on the Ub-conjugation activity in E36 cell extracts. Both E36 and ts20 cell lines transfected with the pCMVneo vector alone exhibited conjugation activity identical with that of the wild-type cells following incubation at the non-permissive temperature (results not shown). Conjugation activity comparable to that observed for the wild-type E36 cells was detected in seven of the transfected cell lines following incubation at 43.5 °C (D3, B3, A17, C2, D7, B18, C3). Consistent with all other findings, these seven cell lines are the same lines which express hE1 mRNA. The four lines which do not express hE1 mRNA (D8, B11, A16, B4) show conjugation activities characteristic of non-transfected ts20 cells following incubation at 43.5 °C.

Figure 5 Degradation of ^{125}I -BSA in cell extracts at 39.5 °C

Cytosolic extracts of E36 (●), ts20 (○), C3 (△), C2 (□) or B11 (◇) cell lines were prepared and assayed as described in the Materials and methods section. Degradation activities have been normalized to 100%.

In addition to the defect in the Ub conjugation system (Kulka et al., 1988 and Figure 4), a defect in protein-degradation activity has been demonstrated in the ts20 cell line (Gropper et al., 1991). Ub-conjugation activity in cell lysates of ts85 cells (a cell line derived from mouse mammary carcinoma FM3A cells which harbours a thermolabile E1 enzyme) is inhibited within 20 min at 39.5 °C (Mayer et al., 1989). Therefore, protein-degradation activity was measured in cytosolic extracts from E1-transfected ts20 cell lines following incubation at 39.5 °C. Figure 5 demonstrates that incubation at 39.5 °C for 0–40 min did not inhibit the ability of cytosolic extracts from transfected lines C2 or C3 to degrade an exogenously added, ^{125}I -labelled protein substrate. The degradation activities of the C2 and C3 lines were within experimental error of the degradation activity measured for the wild-type E36 cells. However, the activity of non-transfected ts20 cell extract was markedly diminished within 20 min of temperature shift to 39.5 °C. Similarly, a cell extract of the B11 transfected line, which does not express hE1 mRNA and does not overproduce E1 protein, exhibited markedly less degradation activity upon temperature shift to 39.5 °C (Figure 5).

Figure 6 Growth of E1-transfected ts20 cells at 39.5 °C

Cells were seeded at starting densities of 1×10^4 – 3×10^4 cells/well. At zero time, the cells were shifted to 39.5 °C and maintained at that temperature. Cells were counted at 24 h intervals. Only viable cells were included in the counts, as determined by Trypan Blue exclusion. For details of (a) and (b) see text.

The ts20 cell line exhibits rapid arrest of cell proliferation in culture upon temperature shift to 39.5 °C (Kulka et al., 1988). Therefore, an important measure of the rescue of the ts20 cell line by wild-type hE1 was the ability of the transfected cell lines to grow at the non-permissive temperature. Each G418-resistant cell line was cultured at 39.5 °C parallel to both E36 wild-type and ts20 non-transfected cells. The results are shown in Figure 6. Seven of the G418 resistant cell lines (Figure 6a: D7, B18, A17, B3; Figure 6b: C2, D, C3) exhibited growth characteristics similar to those of E36 cells. On the other hand, the 39.5 °C culture conditions were fatal to the remaining four cell lines as well as the ts20 non-transfected cells. These same four lines failed to express hE1 mRNA (Figure 2) and failed to conjugate ^{125}I -Ub to acceptor proteins following temperature shift (Figure 4). E36 and ts20 cells mock-transfected with pCMVneo alone showed identical growth characteristics as the E36 and ts20 cell lines which were untransfected (data not shown).

DISCUSSION

We have effectively demonstrated the activity of the hE1 cDNA product both *in vitro* and *in vivo*. Previous results have indicated that an immunospecific E1 protein product of the correct molecular mass was translated in a reticulocyte lysate system using the hE1 cDNA as a template (Handley et al. 1991). We now demonstrate that this translated protein product is enzymically active and thus able to bind specifically to Ub in the presence of ATP. Additionally, the hE1 cDNA is able to mediate the phenotypic rescue of the ts20 cell line *in vivo*, providing definitive confirmation that the only defect in these cells is due to a defect of endogenous E1. Of eleven G418 resistant cell lines produced from transfecting hE1 cDNA into ts20 cells, seven expressed hE1 mRNA and were phenotypically rescued.

Human red blood cell E1 appears as a doublet of approximately 110 kDa/115 kDa upon SDS/PAGE and immunoblotting (Handley et al., 1991). E1 purified from rabbit reticulocytes migrates at approximately 210 kDa protein on gel filtration and as a doublet of about 105 kDa/107 kDa by SDS/PAGE (Ciechanover et al., 1982). The molecular mass difference observed between the gel-filtration analysis (210 kDa) and SDS/PAGE (approx. 105 kDa) suggests that E1 exists as a dimer (Ciechanover et al., 1982). Whether E1 is a homodimer, composed of two polypeptides from a single messenger RNA, or a heterodimer arising from the association of two different proteins, was previously unknown. The Western data presented in Figure 3 indicate that both bands of the E1 doublet are produced following expression of the single E1 cDNA in transfected cells. This result indicates that both bands of the doublet are the products of a single mRNA. Thus, E1 is most likely a homodimer, and the difference in electrophoretic mobility between the doublet bands may be due to post-translational modification. For example, E1 is heavily phosphorylated (Kong and Chock, 1992, and A. L. Schwartz, unpublished work).

The initiator methionine residue for E1 translation has not been identified directly. The cDNA hE1 consists of a single open reading frame, predicting a protein of 1058 amino acids, with a corresponding molecular mass of 117715 Da. Although this figure is within reasonable agreement with the apparent molecular mass of purified E1 (Handley et al. 1991), the second and third in-frame methionine residues predict proteins whose molecular masses are within the same size range (112946 Da and 109629 Da, respectively). The human cDNA product, when expressed in the ts20 cells, approximately comigrates with the endogenous hamster E1 (Figure 3). Thus, the translational start-

site and post-translational processing of the E1 protein product may be conserved between species.

The phenotype of the ts20 cells was complemented by the wild-type hE1 cDNA. The Ub-conjugation activity was restored in transfected cells when examined at the non-permissive temperature (Figure 4). Proteolytic activity was also restored in extracts of cells transfected with hE1 (Figure 5). Ub conjugation is involved in both proteolytic and non-proteolytic processes within the cell (reviewed in Hershko and Ciechanover, 1992). Although the primary defect in the ts20 cells is a temperature-sensitive E1 enzyme, it is not known exactly which processes involving ubiquitination lead to the presentation of the complex phenotype of the ts20 cells.

The hE1 cDNA supports the growth of the ts20 cells at the non-permissive temperature (Figure 6). The rescue of the growth-arrest phenotype in the ts20 cells may be a reflection of the restoration of one or more distinct functions of Ub conjugation in the cell. Ub-dependent proteolysis is the most thoroughly characterized function for the Ub-conjugation system. The mitotic cyclins have been identified as substrates for the Ub system. Ubiquitination precedes the rapid degradation of cyclin B which triggers exit from M phase in *Xenopus* oocyte extracts (Glotzer et al., 1991). Hershko et al. demonstrated that methylated Ub, which cannot support the formation of Ub-branched chain structures necessary for proteolytic targeting, inhibited the degradation of both A and B type cyclins in clam embryo extracts (Hershko et al. 1991). These studies, combined with the known cell-cycle arrest of the E1 mutant cell lines, provide additional evidence for the involvement of the Ub system in the cell cycle. Non-proteolytic functions of the Ub system may also be involved in the regulation of the cell cycle. For example, chromatin structure may be regulated in part by the ubiquitination of histones (Bonner et al., 1988).

That a temperature-sensitive Ub-activating enzyme is the sole defect in the ts20 mutant cell line is definitively demonstrated by the phenotypic rescue of the ts20 cells with the cDNA encoding wild-type hE1. Because revertant cell lines have not been isolated for the ts20 cells, the evidence presented here is the first that conclusively demonstrates a single defect in this cell. Whether the phenotype of the ts20 cells is mediated through proteolytic and/or non-proteolytic roles for the Ub system is unclear. Thus, the ts20 cell will serve as a useful tool in future studies directed toward understanding the specific roles played by the Ub system in cell-cycle regulation.

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