The RAD6 (UBC2) gene of *Saccharomyces cerevisiae* which is involved in DNA repair, induced mutagenesis, and sporulation, encodes a ubiquitin-conjugating enzyme (E3). Since the RAD6 gene product can transfer ubiquitin directly to histones in vitro without the participation of a ubiquitin protein ligase (E3), it has been suggested that in vivo it also acts by the unassisted conjugation of ubiquitin to histones or to other target proteins. Here we show that the RAD6 protein can ligate ubiquitin in vitro to a hitherto unknown set of exogenous target proteins (α-, β-, and γ-casein and β-lactoglobulin) when supplemented by a putative ubiquitin protein ligase (E3-R) from *S. cerevisiae*. RAD6 supplemented with E3-R ligates 1 or, sometimes, 2 ubiquitin molecules to the target protein molecule. UBC3 (CDC34) protein in the presence of E3-R has barely detectable activity on the non-histone substrates. Other ubiquitin-conjugating enzymes tested (products of the UBC1 and UBC4 genes) do not cooperate with E3-R in conjugating ubiquitin to the same substrates. Thus, E3-R apparently interacts selectively with RAD6 protein. These findings suggest that some of the in vivo activities of the RAD6 gene may involve E3-R.

The RAD6 (UBC2) gene plays a key role in one of the three DNA repair epistasis groups of *Saccharomyces cerevisiae* (reviewed by Friedberg, 1988). Mutants of this gene are extremely sensitive to UV light, x-rays, and alkylating agents and are impaired in meiotic recombination. Diploid, homozygous rad6 mutants are also defective in sporulation. Another recently documented effect of the rad6 mutation is the stimulation of retrotransposition (Picolglou et al., 1990). These observations suggest that the RAD6 gene product performs multiple functions in the cell. The discovery that the RAD6 gene product encodes a ubiquitin-conjugating enzyme (E3) provides an important clue to its mechanism of action (Jentsch et al., 1987). Since mutation of the ubiquitin acceptor site (Cys48) of RAD6 abolishes its biological functions, it is likely that all of its roles depend on its ubiquitin-conjugating activity (Sung et al., 1990).

The covalent attachment of ubiquitin to protein targets is involved in a variety of functions in eukaryotic cells, including intracellular proteolysis, heat shock response, cell cycle control, and DNA repair (see Ciechanover and Schwartz, 1989; Hershko, 1988; Rechsteiner, 1988 for reviews). Ligation of ubiquitin to cellular proteins is catalyzed by three enzymatic steps. In the first step, ubiquitin is linked via a thioester bond to the ubiquitin activating enzyme E1, with the splitting of a molecule of ATP. The second step is the transfer of ubiquitin to the thiol group of one of a family of ubiquitin-conjugating enzymes (E3) (reviewed by Jentsch et al., 1990). The third step involves one of two alternative mechanisms. 1) Some of the E2 enzymes (such as RAD6 and CDC34 (UBC3) gene products) are able to ligate ubiquitin to target proteins without the participation of additional factors (Pickart and Rose, 1985; Jentsch et al., 1987; Goebl et al., 1988; Haas and Bright, 1988). 2) The conjugation of mult ubiquitin chains by E2 to certain target proteins destined for degradation requires the intervention of an additional class of enzyme known as ubiquitin-protein ligase or E3 (Reiss and Hershko, 1990; Heller and Hershko, 1990; Bartel et al., 1990)

Since the RAD6 (UBC2) gene product can transfer ubiquitin directly to histones in vitro without the participation of an E3, it has been suggested that in vivo it also acts by the unassisted conjugation of ubiquitin to histones or to other target proteins (Jentsch et al., 1987; Sung et al., 1988). Here we show that the RAD6 protein can ligate ubiquitin in vitro to a hitherto unknown set of exogenous target proteins when supplemented by a factor (E3-R) from *S. cerevisiae* which may be a ubiquitin protein ligase (E3).

**EXPERIMENTAL PROCEDURES**

**Materials**

The following materials were obtained from Sigma: albumin (bovine serum), α-casein, β-casein, κ-casein, creatine kinase, β-lactoglobulin, and ubiquitin. Unless stated otherwise, all the proteins were bovine. Pure calf thymus histones H2A, H2B, H3, and H4 were the generous gifts of Lothar Böhmer (University of Stellenbosch Medical School). Histone H2B purchased from Worthington was used unless otherwise stated.

**Yeast Strains**

YG15: MATa, ura3-52, trp1Δ, leu2-2, ade2, cys2. YG 15 (rad6): MATa, ura3-52, trp1Δ, leu2-2, ade2, cys2, rad6. The latter strain is isogenic to YG15 carrying a null rad6 mutation (Kupiec and Simchen, 1984). SUB325: MATa, urbl (null), ard1::HIS3, pep4::URA3, urbl::LEU2, lys2, trp1 (kindly given by Daniel Finley, Harvard Medical School).

**Ubiquitin System Enzymes**

Preparation of Ubiquitin-Sepharose Column Fractions—Extracts of late log-phase cells were prepared essentially as described by Jentsch et al. (1987) except that the cell disruption buffer contained

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50 mM Tris-HCl, pH 7.5, 1 mM DTT (dithiothreitol), 5 mM EDTA, and 0.5 mM PMSF. Fraction II was prepared and chromatographed on ubiquitin-Sepharose columns according to Hershko et al. (1983) with slight modifications (Jentsch et al., 1987). The KCI wash contained most of the E3 and some E1 and E'. The DTT eluate contained most of the E1 and E'. The pH 9 eluate contained some E2 and E'.

The assay mixture contained in a volume of 12.5 μl: 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 2.5 μg of creatine kinase, 1.5 pmol of [¹²⁵I]-ubiquitin (2 × 10⁵ cpm), 10 μg of protein substrate, and 10 μg/ml each of the following peptide protease inhibitors: leupeptin, pepstatin, chymostatin, bestatin, and antipain. Purified E1, E2, and E3 were added as indicated. Alternatively in experiments with crude ubiquitin-Sepharose fractions, KCI eluate, DTT eluate, and pH 9 fraction, each containing about 0.5 μg of protein, were added as indicated. After incubation for 1 h at 30°C, the reaction was stopped with SDS electrophoresis sample buffer and samples were heated for 3 min in a boiling water bath. Samples were subjected to electrophoresis on 18% SDS-polyacrylamide gels (Thomas and Kornberg, 1975) followed by autoradiography.

**RESULTS**

**A Factor (E₃-R) Required for the Ubiquitination of Certain Proteins by the RAD6 Gene Product**—We have partially purified a factor from yeast (referred to hereafter as E₃-R) which is required, together with E₁ and RAD6 protein (E₃), for the ubiquitination of certain exogenous substrates. Fig. 1 shows the ubiquitination of several protein substrates by various combinations of purified yeast E₁, E₃ (RAD6 protein), and E₃-R. The ubiquitination of α-, β-, and κ-casein and β-lactoglobulin required the presence of E₃-R in addition to E₁ and E₃. In contrast, the ubiquitination of ubiquitin to histone H2B required only E₁ and E₃, as has previously been reported (Jentsch et al., 1987; Sung et al., 1988). The molecular weights of the ubiquitinated products indicated that a single ubiquitin molecule had been ligated to the substrate molecule. An exception was α-casein which formed a double band apparently corresponding to mono- and diubiquitinated products of α-casein (Fig. 2). In contrast, the ubiquitination of histone H2B was similar to that of histone H2B without E₃-R (Fig. 3A). The ubiquitination of histone H2B was also similar (RAD6: H2B > H2A > H3 = H4; CDC34: H2B > H2A = H3 > H4). UBC1 protein plus E₃ had appreciable activity on core histones (not shown). In contrast, UBC4 protein supplemented with E₃ had no detectable activity on core histones (not shown).

The activities of RAD6 and CDC34 proteins on substrates requiring E₃-R for ubiquitination were markedly different (Fig. 3, A and B). RAD6 protein plus E₃-R rapidly ubiquitinated α- and β-casein and β-lactoglobulin, to an extent similar to that of H2B without E₃-R (Fig. 3A). CDC34 protein plus E₃-R, on the other hand, had barely detectable activity on the substrate histones (Fig. 3B).

The system containing CDC34 protein, ubiquitinated endogenous substrates to form high molecular weight (>66 kDa) conjugates, and the process was apparently enhanced by E₃-R (Fig. 3B). UBC4 and UBC1 proteins had no activity on the three test substrates with or without E₃-R (not shown). Thus, E₃-R seems to cooperate selectively with RAD6 protein in the ubiquitination of α- and β-casein and β-lactoglobulin. These findings imply that *in vivo* E₃-R participates in the ubiquitin-
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FIG. 1. Effect of omitting E₁, E₃, or E₅-R on the ubiquitination of various substrates. The conjugation system was supplemented with: histone H2B (lanes 1 and 18–22), α-casein (lanes 2–5), β-casein (lanes 6–9), β-lactoglobulin (lanes 10–13), or κ-casein (lanes 14–17). The complete system contained E₁ (0.06 pmol), E₃ (RAD6 protein, 0.66 pmol), and E₅-R (4.5 nanounits). Lanes 5, 9, 13, 17, 21, complete system; lanes 2, 6, 10, 14, 20, E₅-R omitted; lanes 3, 7, 11, 15, 22, E₄ omitted; lanes 4, 8, 12, 16, E, omitted; lane 18, E₂ and E₅-R omitted; lane 19, E₁ and E₅-R omitted.

FIG. 2. Ubiquitination of core histones by RAD6 (UBC2) and CDC34 (UBC3) proteins. The conjugation system contained E₁ (0.06 pmol) and RAD6 protein (0.33 pmol) (A) or CDC34 protein (0.33 pmol) (B). Substrates were histones H2A (lane 1), H2B (lane 2), H3 (lane 3), or H4 (lane 4). Pure calf thymus histones given by Lothar Böhm were used in this experiment.

Activity of unknown targets by the RAD6 gene product.

Lack of Detectable Activity on the Test Substrates in a rad6 Null Mutant—Fig. 4 shows that substrates requiring E₆-R were not ubiquitinated by ubiquitin system enzymes prepared from a rad6 null mutant (–). Controls (+) showed that the same substrates were ubiquitinated by ubiquitin system enzymes from an isogenic RAD6 strain. When RAD6 protein was added to rad6 mutant extracts, strong ubiquitination of all the test substrates was observed (not shown). Thus, E₆-R was present in the rad6 mutant extract but was not detectable unless exogenous RAD6 protein was added. This indicates that E₆-R has a preference for the RAD6 gene product and does not interact detectably with other major E₆ species present in growing cells.

Activity on the Test Substrates in a ubr1 Null Mutant—The question arose whether E₆-R is identical with the product of the UBRI gene which encodes the E₆ involved in “N-end rule” selection (Bartel et al., 1990). Three of the substrates requiring E₆-R have N-terminal amino acids which are labilizing according to the N-end rule (α- and β-casein, Lys, Arg; β-lactoglobulin, Leu (Eigel et al., 1984)) and therefore could be targets of the UBRI gene product (Bachmair and Varshavsky, 1989; Bachmair et al., 1986). κ-Casein has a blocked N-terminal (pyroglutamic acid (Eigel et al., 1984)) and therefore is unlikely to be a UBRI substrate. In order to test if some or all of the E₆ activity could be attributed to the UBRI gene product, we examined the ubiquitination of substrates requiring E₆-R in extracts of a ubr1 null mutant (Fig. 5). Activity on all four substrates was found in this mutant indicating that E₆-R does not correspond with the UBRI protein. E₃-R has been prepared from the ubr1 null mutant, providing further support for this conclusion.

DISCUSSION

The above experiments show that the E₃ enzyme encoded by the RAD6 gene can act in two different modes: with or without an additional factor, E₅-R. Without E₅-R, it can transfer ubiquitin directly to histones, as was earlier reported (Jentsch et al., 1987; Sung et al., 1988). In the presence of E₅-R, it can transfer ubiquitin to a broader spectrum of target sequences.
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proteins than without it. E2-R resembles the ubiquitin-protein ligases (E2s) described previously in that it facilitates the transfer of ubiquitin from an E1 to specific target proteins (Reiss and Hershko, 1990; Heller and Hershko, 1990). Unlike the known E2s, it usually ligates 1, or sometimes 2, ubiquitin molecules to the target molecules tested rather than a multiubiquitin chain. It remains to be determined whether E2-R plays a similar role in target protein selection to the previously studied E2s. The presence of E2-R activity in a ubr1 null mutant shows that this activity is not due to the known E2 of yeast encoded by the UBR1 gene (Bartel et al., 1990). Since the E2-R preparation used in these experiments was not homogeneous, it is possible that it contained more than one type of E2 molecule with different specificities. This should become clear upon further purification.

The question arises whether E2-R, like the E2s described previously, is involved in protein degradation (Hershko, 1988). This is uncertain for the following reasons. E2-R apparently does not interact with the UBC1 and UBC4 gene products which have been clearly implicated in protein break-down (Jentsch et al., 1990; Seufert and Jentsch, 1990). Also, the attachment of multiubiquitin chains has been shown to be a prerequisite for the degradation of some proteins (Chau et al., 1989), whereas E2-R produces monoubiquitinated proteins (Figs. 1 and 3A). However, the existence of an unknown pathway degrading specific monoubiquitinated proteins cannot at present be excluded.

The most interesting feature of E2-R is that it seems to cooperate preferentially with the RAD6 protein. The specificity of the interaction is emphasized by the fact that even the product of the CDC34 gene, which is closely related to RAD6 (Goeb et al., 1988), collaborates only weakly with E2-R in the ubiquitination of the non-histone substrates. No activity of UBC1 and UBC4 gene products on the model substrates was detected in the presence of E2-R. Since so far only four of the seven cloned UBC gene products have been tested with E2-R in vitro, other E2s not tested so far may interact with it. However, the absence of activity on E2-R substrates in a rad6 null mutant (Fig. 4) suggests that none of the major E2 species present in growing cells cooperates with E2-R.

A reasonable assumption is that E2-R is involved in the function of the RAD6 gene which plays a key role in one of the DNA repair pathways of yeast (Friedberg, 1988). rad6 mutants, in addition to being extremely sensitive to UV light, x-rays, and chemical mutagens have low induced mutagenesis, increased frequencies of retrotransposition, and are defective in sporulation. It has been suggested that the multiple activities of the RAD6 gene product are related to its known in vitro activity, the direct transfer of ubiquitin to bovine histones (Jentsch et al., 1987, 1990; Sung et al., 1988). However, ubiquitinated histones have not been detected in S. cerevisiae (Swerdlow et al., 1990). Moreover, genetic modifications of yeast histones H2A and H2B in a manner which would prevent ubiquitination at the sites ubiquitinated in higher eukaryotes had no obvious effects on cellular function (Swerdlow et al., 1990; Schuster et al., 1986). Therefore, there is no good evidence to support the notion that histones are in vivo targets of RAD6. Our experiments show that RAD6 protein can function in vitro both with or without E2-R and that the involvement of E2-R can extend the spectrum of its target proteins. It is, thus, tempting to suggest that, although some RAD6 protein functions may involve a direct transfer of ubiquitin to target molecules, others may require the mediation of E2-R or similar factors. This assumption could explain the pleiotropic nature of rad6 mutations as well as the apparent division of RAD6 epistasis group genes into subtypes (Friedberg, 1988). It is, therefore, possible that known or unknown genes of the RAD6 epistasis group encode factors such as E2-R which modify RAD6 target specificity, while others encode corresponding target proteins.

Much attention has been given to the highly acidic C-terminal region of the RAD6 protein (Reynolds et al., 1985). This acidic tail has been found to be required for optimal ubiquitination activity on histones in vitro (Sung et al., 1988). Since S. cerevisiae mutants lacking all 23 amino acids of this C-terminal have impaired sporulation but are capable of normal DNA repair, it was suggested that sporulation requires histone ubiquitination whereas other functions involve the ubiquitination of target proteins not requiring the RAD6 acidic tail (Morrison et al., 1988). A possible explanation of these results, suggested by our findings, is that sporulation involves the direct ubiquitination of target proteins whereas other RAD6 activities are mediated by E2-R or similar factors. Another possibility is that all the RAD6 functions require factors of the E2-R type, with a different specificity for each function. The last hypothesis might explain why the product.
of the Schizosaccharomyces pombe rhp6+ gene, which corresponds with the S. cerevisiae RAD6 gene, lacks a polyacidic C-terminal but functions in both DNA repair and sporulation (Reynolds et al., 1990). Hopefully, further characterization of the E3s interacting with the RAD6 gene product will help to clarify some of the questions raised above.

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