The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity

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The *RAD6* gene of the yeast *Saccharomyces cerevisiae* is required for post-replication repair of UV-damaged DNA, DNA damage-induced mutagenesis, and sporulation. Here we demonstrate that the protein encoded by the *RAD6* gene, previously shown to be a ubiquitin-conjugating (E2) enzyme, multiply ubiquitinates histones H2A and H2B efficiently to give products containing as many as seven or more molecules of ubiquitin. We also show that the highly acidic 23-residue *RAD6* carboxy-terminal tail domain, which contains a total of 20 acidic residues, is essential for the histone-polyubiquitinating activity. Because the *RAD6* polyacidic tail is required for the sporulation function but not for the DNA repair and induced mutagenesis functions of *RAD6*, the present observations suggest that the histone-polyubiquitinating activity of *RAD6* protein is essential for sporulation but not for DNA repair and induced mutagenesis. Attachment of multiple molecules of ubiquitin to histones by *RAD6* protein may serve to target the histones for degradation via the ubiquitin-dependent proteolytic system or to alter chromatin structure. The *in vitro* system for synthesizing polyubiquitinated histones described herein provides a means for investigating these possibilities.

[Key Words: Histone polyubiquitination; chromatin; *RAD6*; sporulation; DNA repair; yeast]

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The *RAD6* gene of *Saccharomyces cerevisiae* is a highly pleiotropic gene required for a variety of chromosomal processes. *rad6* mutants are extremely susceptible to killing by various DNA-damaging agents such as ultraviolet (UV) light, ionizing radiation, and alkylating agents (Cox and Parry 1968; Game and Mortimer 1974; Prakash 1974), are deficient in postreplication repair of UV-damaged DNA (Prakash 1981), and do not undergo mutagenesis in response to DNA-damaging treatments (Prakash 1974; Lawrence and Christensen 1976; McKee and Lawrence 1979). In addition, mutations at the *RAD6* locus render cells defective in meiotic recombination and sporulation (Game et al. 1980; Montelone et al. 1981).

The 20-kD protein encoded by the *RAD6* gene contains 172 amino acids, with 20 of its 23 carboxy-terminal residues being acidic, including an uninterrupted stretch of 13 aspartates (Table 1; Reynolds et al. 1985). Unlike the remainder of the *RAD6* protein, the 23-residue polyacidic sequence is hypersensitive to proteinase K, and it endows *RAD6* protein with an aberrantly large Stokes radius. From these and other observations, Morrison et

al. (1988) deduced that in *RAD6* protein the polyacidic sequence constitutes a freely extending tail, appended to a globular domain, which comprises approximately the first 149 residues. To investigate the function of the 23-residue *RAD6* polyacidic sequence in DNA repair, induced mutagenesis, and sporulation, Morrison et al. (1988) examined the phenotypes of the *rad6-149* and *rad6-153* mutant alleles, which code for *rad6* proteins lacking all, and all but four, residues of the polyacidic sequence, respectively (Table 1). These studies revealed that the sporulation function, but not the repair and mutagenesis function of *RAD6* is dependent on the polyacidic tail.

RAD6 protein is one of the five yeast ubiquitin-conjugating (E2) enzymes that can be isolated from cell extracts by affinity chromatography on ubiquitin-Sepharose (Jentsch et al. 1987), and it may be homologous to the mammalian 20-kD E2 enzyme (Pickart and Rose 1985; Jentsch et al. 1987). Ubiquitin is a 76-residue polypeptide found in eukaryotes, either in its free state or covalently joined to other cellular proteins (for review, see Finley and Varshavsky 1985). The sequence of biochemical events leading to the attachment of ubiquitin to target proteins has been studied using pertinent pro-

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Table 1. Carboxy-terminal residues of wild-type RAD6 and mutant rad6 proteins

Protein	Residues									
	149		153					172		
RAD6	↓ Ser Trp Glu	(Asp) ₂	Met	(Asp) ₂	Met	(Asp) ₁₃	Glu	Ala	Asp	
rad6-153	Ser Trp Glu	(Asp) ₂	Met							
rad6-149	Ser Trp									

The start of the polyacidic sequence in RAD6 protein is indicated by the arrow. The 23-carboxy-terminal amino acids in RAD6 protein contain 20 acidic residues. The last 19 residues of the polyacidic tail have been deleted in the rad6-153 protein, and all 23 residues of the acidic tail have been deleted in the rad6-149 protein.

tein factors isolated from rabbit reticulocytes (Pickart and Rose 1985; Hershko and Ciechanover 1986). Results from various investigations indicate that the carboxy-terminal glycine of ubiquitin is first joined in an ATP-dependent reaction to a cysteine residue of the ubiquitin-activating enzyme, E1, via a thiolester bond. The E1-linked ubiquitin is then transferred to a cysteine residue of a distinct set of ubiquitin-conjugating, or E2, enzymes. The E2 enzymes then catalyze the formation of an isopeptide bond between the carboxy-terminal glycine of ubiquitin and the ϵ -amino group of lysines in target proteins, a reaction that may or may not require an additional protein cofactor, E3. Protein substrates that are dependent on E3 for their ubiquitination seem to acquire a multiply ubiquitinated configuration and to be destined for degradation via an ATP-requiring proteolytic system (Hershko et al. 1984a,b; Hershko and Ciechanover 1986). It is believed that ubiquitin attachment may also serve to modulate the biological activities of some target proteins in a nonproteolytic fashion (Finley and Varshavsky 1985; Hershko and Ciechanover 1986).

In the work of Jentsch et al. (1987), RAD6 protein was shown to catalyze the monoubiquitination of histone H2B. In vivo, singly ubiquitinated histones H2A and H2B are found concentrated in regions of chromatin that are being transcribed actively, and the modified histones may aid the relaxation of compact chromatin structures necessary for transcription and other chromosomal processes (Levinger and Varshavsky 1982; Barsoum and Varshavsky 1985; Jentsch et al. 1987). To determine the role of the RAD6 polyacidic tail in histone ubiquitination and to correlate the histone-ubiquitinating activity of RAD6 protein to the in vivo functions of the protein, we purified the RAD6, rad6-153, and rad6-149 proteins from yeast strains genetically tailored to overproduce the proteins and compared the ability of the wild-type RAD6 and mutant rad6 proteins to ubiquitinate histones H2A and H2B. Unexpectedly, we observed that RAD6 protein catalyzes the formation of multiply ubiquitinated H2A and H2B species containing as many as seven or more molecules of ubiquitin. To our knowledge, this is the first time that a histone polyubiquitinating activity has been reported for any of the E2 enzymes. Our studies also show that the acidic domain of RAD6 protein is required for its histone-polyubiquitinating activity, as the rad6-149 protein lacks this activity. The implications of these findings in respect to the in vivo functions of RAD6 are discussed.

Results

Purification of RAD6 protein and of mutant rad6 proteins lacking varying lengths of the carboxy-terminal acidic tail

The rad6-149 and rad6-153 proteins, which lack all, and all but 4, residues of the 23-residue RAD6 polyacidic tail (Table 1), were overproduced in yeast by placing the coding frames for the proteins under the control of the *ADC1* promoter (see Materials and methods), a strong yeast promoter previously used to facilitate the purification of the RAD3 (Sung et al. 1987a,b) and RAD6 proteins (Morrison et al. 1988). In the present study, the RAD6 and rad6 proteins were purified to apparent homogeneity (Fig. 1, lanes 2–4) from extracts of yeast cells overproducing the proteins via a combination of affinity chromatography on ubiquitin–Sepharose (Hershko et al. 1983) and anion-exchange chromatography on an FPLC Mono Q column. RAD6 protein was also purified by the procedure of Morrison et al. (1988), which does not entail affinity chromatography on ubiquitin–Sepharose (Fig. 1, lane 1).

Figure 1. SDS-PAGE of purified RAD6 and rad6 proteins. RAD6, rad6-153, and rad6-149 proteins (lanes 2, 3, and 4, respectively; 2 μ g each), purified as described in Materials and methods were electrophoresed on a 13% denaturing polyacrylamide gel and stained with Coomassie blue. (Lane 1) 2 μ g of RAD6 protein purified, using the procedure of Morrison et al. (1988).

RAD6 protein catalyzes the polyubiquitination of histones H2A and H2B

RAD6 protein is one of a small number of yeast proteins that can be linked covalently to Sepharose-bound ubiquitin through a thiolester bond (Jentsch et al. 1987) cleavable by dithiothreitol (DTT). Using RAD6 protein from yeast that was eluted from a denaturing polyacrylamide gel on which the DTT eluate of a ubiquitin-Sepharose column had been fractionated, or an extract of *Escherichia coli* cells harboring a *RAD6*-containing plasmid, Jentsch et al. (1987) demonstrated that RAD6 protein is a ubiquitin-conjugating enzyme that catalyzes the monoubiquitination of histone H2B in the presence of E1 enzyme and ATP. We have discovered that under the experimental conditions used by Jentsch et al. (1987), homogeneous RAD6 protein obtained from yeast without any denaturing step (see Materials and methods) multiply ubiquitinates histones H2A and H2B (Fig. 2). Ubiquitinated histone H2B species possessing apparent molecular masses of 24.2, 31, 33, 38, 39.7, 46.8, 53.5, 61.2, and 69 kD were observed on a 12.5% polyacrylamide gel (Fig. 2, lane 2). Assuming that H2B bands with a size difference smaller than 3 kD correspond to histone species with the same number of ubiquitin molecules

conjugated to alternate lysine residues, the 69-kD H2B band may represent a hepta-ubiquitinated species of the histone. In the case of H2A, ubiquitinated species of apparent molecular masses of 22.7, 24.2, 30.2, 33, 37, 38.5, 45.8, 52.5, 61, and 69 kD were seen (Fig. 2, lane 3), with the 69 kD band possibly representing hepta-ubiquitinated H2A. The whole spectrum of polyubiquitinated histones was still present under conditions where a portion of the added histones remained unmodified (not shown), suggesting that the addition of multiple ubiquitin molecules to histones may occur in a processive fashion.

No ubiquitination of histones was detected when either E1 enzyme, RAD6 protein, or ATP was omitted from the reaction mixture (Fig. 2, lanes 4–6). In contrast to the high degree of ubiquitin conjugation to histones, horse heart cytochrome *c*, also a highly basic protein, is evidently a poor substrate for RAD6 protein (Fig. 2, lane 7). Interestingly, RAD6-ubiquitin conjugates with apparent molecular masses of 30 and 31 kD and an E1-ubiquitin conjugate with molecular mass of >130 kD were also seen on the autoradiogram (Fig. 2, lane 1). Because thiolesters formed between ubiquitin and cysteines are unstable under the reducing conditions used in the electrophoretic analysis of reaction products (see Materials and methods and Fig. 5), the RAD6-ubiquitin and E1-ubiquitin conjugates most likely resulted from the attachment of ubiquitin to lysine residues in the RAD6 and E1 proteins.

RAD6 protein purified by a procedure that does not entail affinity chromatography on ubiquitin-Sepharose (Morrison et al. 1988) gave identical results when used as the source of E2 enzyme in the experiments described above (not shown).

Figure 2. RAD6 protein multiply ubiquitinates histones. Conditions for the ubiquitin-conjugation assays are given in Materials and methods. Histones H2B and H2A (lanes 2 and 3) were efficiently modified to give multiple products containing one to seven molecules of ubiquitin, whereas an equivalent quantity of horse heart cytochrome *c* was only poorly ubiquitinated (uCytc, lane 7). No ubiquitination of H2A was observed when E1, RAD6 protein, or ATP was absent from the reaction mixture (lanes 4, 5, and 6, respectively). Omission of histone (lane 1) revealed ubiquitinated E1 (uE1) and ubiquitinated RAD6 protein (uRAD6). Unreacted ubiquitin (ub) is indicated.

RAD6 protein and E1 enzyme are the only protein components required for polyubiquitination of histones

The histone-polyubiquitinating activity was absent from the DTT eluate of ubiquitin-Sepharose when an extract of the *rad6* genomic deletion strain SX46A-6Δ harboring plasmid pSCW231, which contains the *ADC1* promoter but lacks any *RAD6* sequence, was used as starting material; whereas the activity was clearly present when an extract of SX46A-6Δ strain harboring the RAD6-overproducing plasmid pSCW242 was used (Fig. 3A). These observations indicate that when an extract of RAD6-overproducing cells is used as loading material for ubiquitin-Sepharose, RAD6 protein alone accounts for all, or almost all, of the histone-polyubiquitinating activity in the DTT eluate. The *S. cerevisiae* CDC34 gene, required for the transition from G1 to S phase in the cell cycle, also encodes a ubiquitin-conjugating enzyme. Extracts from *E. coli* expressing the *CDC34* gene monoubiquitinate histone H2B in an E1-dependent reaction (Goebel et al. 1988). In our studies, the CDC34 activity was not observed in *rad6*Δ extracts, most likely because of the relatively low abundance of the CDC34 protein (Goebel et al. 1988). It would be of interest to determine whether the CDC34 protein purified from yeast polyubiquitinates histones.

Figure 3. Histone polyubiquitinating activity is intrinsic to RAD6 protein. Extract from 25 g of the *RAD6* overproducing strain (SX46A-6Δ strain harboring pSCW242) was passed through a 1 ml column of ubiquitin–Sepharose and the bound E1 and various E2 enzymes, including RAD6 protein, were eluted with 20 mM DTT, as described for the purification of the *rad6* mutant proteins in Materials and methods. The DTT eluate was concentrated to 0.1 ml using a Centricon-10 concentrator (Amicon), diluted to 1 ml with buffer B (see Materials and methods), and reconcentrated to 0.1 ml. The DTT eluate that originated from SX46A-6Δ strain harboring pSCW231, which contains the *ADC1* promoter but lacks any *RAD6* sequence, was obtained and treated identically. (A) One microliter of the DTT eluate, which either lacks (lanes 1 and 2) or contains (lanes 3 and 4) *RAD6* protein, was added to ubiquitin–conjugation reaction mixtures, with (lanes 2 and 4) or without (lanes 1 and 3) histone H2A. It is clear that only the DTT eluate that originated from *RAD6*-overproducing cells possessed the histone-polyubiquitinating activity (lane 4). (B) Analysis of DTT eluates from the control cells lacking *RAD6* (lane 1) and from *RAD6*-overproducing cells (lane 2) on a 12.5% denaturing polyacrylamide gel stained with Coomassie blue revealed that the level of E1 and the other E2 enzymes was much lower in the eluate from the *RAD6*-overproducing cells. This accounts for the apparent absence in lane 3 (A) of the two E2–ubiquitin conjugates observed in lanes 1 and 2 (A).

The two radioactive bands present in lanes 1 and 2 of Figure 3A most probably correspond to isopeptide conjugates of ubiquitin and the two smallest E2 enzymes *E2_{14K}* and *E2_{16K}* (Jentsch et al. 1987). The lower intensity of these two radioactive bands seen when the DTT eluate that originated from *RAD6*-overproducing cells was used (see Fig. 3A, lane 3) is due to a general reduction of E1 and E2 enzymes in the eluate (Fig. 3B). It remains to be established whether this phenomenon stems from a reduced synthesis or enhanced degradation of E1 and the other E2 enzymes in *RAD6*-overproducing cells or is a result of the exclusion of the E1 and E2 enzymes from ubiquitin–Sepharose due to high amounts of *RAD6* protein.

To ascertain that E1 enzyme is the only protein component required for the polyubiquitination of histones besides *RAD6* protein, an E1 preparation obtained after ubiquitin–Sepharose affinity chromatography and FPLC was size-fractionated on a column of Sephadex G-150 superfine (see Materials and methods) to yield apparently homogeneous E1 enzyme (Fig. 4A). As shown in Figure 4B, in reaction mixtures containing a fixed quantity of *RAD6* protein, histone H2B, and an aliquot of the fractions from the G-150 column, the histone–polyubiquitinating activity closely parallels the amount of E1 in the column fractions. An identical result was obtained

when H2A replaced H2B as substrate (not shown). These observations suggest that in terms of protein requirement, *RAD6* protein and the E1 enzyme together are sufficient for the polyubiquitination of histones.

rad6-149 and rad6-153 proteins retain the ability to form a thiolester bond and isopeptide conjugates with ubiquitin

Like *RAD6* protein, *rad6-149* and *rad6-153* mutant proteins missing all, and all but four, residues of the polyacidic tail sequence can be immobilized on ubiquitin–Sepharose and eluted from the affinity matrix with 20 mM DTT (see Materials and methods), suggesting that the *rad6* mutant proteins can form a thiolester bond with ubiquitin. To detect directly the formation of thiolesters between *RAD6* or mutant *rad6* proteins and ubiquitin, the proteins were incubated with ¹²⁵I-labeled ubiquitin and E1 enzyme in buffer R (see Materials and methods). The reaction was terminated with stop buffer that did not contain 2-mercaptoethanol, and samples were analyzed on a 12.5% polyacrylamide gel at 4°C without prior boiling (see Materials and methods). Figure 5A shows that there is approximately the same amount of thiolester formed between *RAD6* protein or the *rad6* mutant proteins and ubiquitin after a 30-min

Figure 4. E1 and RAD6 are sufficient for the polyubiquitination of histones. E1 enzyme purified by affinity chromatography and FPLC was further fractionated on a column of Sephadex G-150 (superfine), as described in Materials and methods. The fractions (0.5 ml) from the gel filtration column were examined for their content of E1 on a 9% denaturing polyacrylamide gel stained with Coomassie blue (A) and for their ability to support the polyubiquitination of histone H2B catalyzed by RAD6 protein (B). The level of histone polyubiquitination observed with fractions 29–33 clearly parallels the amount of E1 present in those fractions.

incubation. Electrophoretic analysis of reaction mixtures terminated at earlier times (2.5, 5, 9, and 15 min) revealed that the rate of thioester formation is unaffected by the RAD6 polyacidic tail (not shown). This implies that the polyacidic tail plays no role in the catalytic transfer of activated ubiquitin from E1 to the sole cysteine of RAD6 protein (Reynolds et al. 1985).

The inclusion of 5% (0.72 M) 2-mercaptoethanol in the stop buffer, plus a boiling step prior to electrophoretic analysis, greatly reduced the intensity of, but did not abolish, the RAD6–ubiquitin, rad6–ubiquitin, and E1–ubiquitin conjugates seen on the autoradiogram (Fig. 5B). The covalent conjugates of ubiquitin with RAD6, rad6, or E1 proteins are most likely isopeptide in nature, as they are stable to prolonged boiling in as much as 25% (3.6 M) 2-mercaptoethanol (not shown). As is discussed in greater detail below, attachment of ubiquitin to E1 and E2 enzymes through isopeptide linkages may represent a means of regulating the activity and/or stability of these enzymes, or it may serve an unknown function.

The acidic tail of RAD6 protein is required for recognition of histones in their polyubiquitination

RAD6 protein binds to the anion-exchange matrix Mono Q much more tightly than the other ubiquitin-conjugating enzymes in the DTT eluate of ubiquitin–Sepharose (not shown), indicating that RAD6 protein is the most acidic E2 enzyme present. Because histones are highly basic proteins, it is possible that the ability of RAD6 protein to multiply ubiquitinate histones is conferred by the RAD6 polyacidic tail. To test this hypothesis, histone H2A was incubated with either RAD6, rad6-153, or rad6-149 proteins, and the ability of the proteins to ubiquitinate the histone was compared. We found that rad6-149 protein, which lacks the entire polyacidic tail (Table 1), shows only a residual amount of histone H2A-monoubiquitinating activity and no detectable ability to polyubiquitinate H2A (Fig. 6, cf. lane 5 to lane 1). The addition of the first 4 residues of the 23-residue polyacidic tail, as in rad6-153 protein (Table 1), confers a considerable level of H2A-monoubiquitin-

Figure 5. Mutant rad6 proteins are ubiquitinated at their cysteine residue and elsewhere. (A) Thioesters of E1 (lanes 1–3), and of rad6–149 (lane 1), rad6–153 (lane 2), and RAD6 (lane 3) proteins were visualized by omitting 2-mercaptoethanol in the sample buffer and performing electrophoresis at 4°C. (B) Only those ubiquitin conjugates that were stable to 2-mercaptoethanol remained after treatment of the protein samples in A with 0.72 M 2-mercaptoethanol at 100°C for 3 min.

Figure 6. Acidic tail of RAD6 protein is required for histone polyubiquitination. To compare the histone-ubiquitinating activity of the purified RAD6, rad6-153 and rad6-149 proteins, the RAD6 (lane 1), rad6-153 (lane 3), and rad6-149 (lane 5) proteins were added to ubiquitin-conjugation assay mixtures containing histone H2A as substrate. Equivalent reaction mixtures missing H2A (lanes 2, 4, and 6) were included to enable the identification of the bands corresponding to the ubiquitinated forms of RAD6 (lane 2), rad6-153 (lane 4), and rad6-149 (lane 6) proteins. As described in the text, deletion of the entire polyacidic tail, as in rad6-149 protein, inactivates the H2A-polyubiquitinating activity and greatly diminishes the ability to monoubiquitinate the histone (cf. lanes 5 and 1). The addition of only the first 4 residues of the 23-residue tail, as in rad6-153 protein, partially restores the ability to multiply ubiquitinate H2A (cf. lanes 3 and 1).

ating activity and, to a lesser degree, the ability to polyubiquitinate the histone (Fig. 6, cf. lane 3 to lane 1). Similar results were obtained when histone H2B replaced H2A as substrate (not shown).

The findings from these studies clearly demonstrate the importance of the RAD6 carboxy-terminal polyacidic sequence in the recognition of histones prior to their ubiquitination. However, it should be stressed that the RAD6 polyacidic tail is probably not the only structural determinant for substrate recognition, because (1) rad6-149 protein can still ubiquitinate histones to a limited extent and (2) the highly basic horse heart cytochrome *c* is only poorly ubiquitinated by RAD6 protein (Fig. 2, lane 7).

Discussion

Polyubiquitination of histones H2A and H2B by RAD6 protein

We find that among the various yeast ubiquitin-conjugating enzymes that can be isolated by affinity chromatography on ubiquitin-Sepharose, only RAD6 protein is capable of catalyzing the polyubiquitination of histone H2A and H2B in the presence of ubiquitin-activating enzyme E1 and ATP. Multiply ubiquitinated species of histones H2A and H2B with apparent molecular masses of 69 kD and below are observed, with the 69-kD species presumably representing hepta-ubiquitinated histones. In the study of Jentsch et al. (1987), RAD6 protein cata-

lyzed only the monoubiquitination of histone H2B. Whereas native RAD6 protein overproduced in and purified to apparent homogeneity from yeast was used in our study, Jentsch et al. (1987) used either RAD6 protein from yeast that was eluted from a denaturing polyacrylamide gel or an extract of *E. coli* cells harboring a RAD6-containing plasmid. Conceivably, the failure to detect the polyubiquitinated histone species by Jentsch et al. could have been due to the unfolding of RAD6 protein by SDS in the denaturing gel and the lack of post-translational modification of the RAD6 protein made in *E. coli*.

It remains to be seen whether the multiply ubiquitinated histones consist of single ubiquitin molecules being conjugated to different lysine residues on histones or ubiquitin-ubiquitin-branched structures (Hershko and Heller 1985) attached to perhaps only one or two lysine residues of histones.

Role of the RAD6 acidic tail in polyubiquitination of histones

Mutant rad6 proteins lacking all, and all but 4, residues of the 23-residue RAD6 polyacidic tail were purified to apparent homogeneity from extracts of yeast cells that overproduce the two proteins. The purified rad6-149 and rad6-153 proteins were used to ascertain the role of the RAD6 polyacidic tail in the histone ubiquitin-conjugation reaction. We observed that the polyacidic tail plays no role in the E1-dependent formation of thioester between RAD6 protein and ubiquitin. However, the polyacidic tail exerts a pivotal role in the recognition of histones in the transfer of ubiquitin from RAD6 protein to lysine residues of histones. The information for substrate specificity also resides in the globular domain of RAD6 protein, however, because rad6-149 protein, which lacks the entire polyacidic tail, still possesses residual histone-ubiquitinating activity. Overall, these results imply that the capture of histones by RAD6 protein requires both the globular domain and the carboxy-terminal polyacidic sequence of the protein, with the latter perhaps mediating the formation of a stable complex between RAD6 protein and histones, thereby increasing the efficiency and the processivity of the polyubiquitination reaction. Nevertheless, the fact that the highly basic horse heart cytochrome *c* is only minimally ubiquitinated by RAD6 protein indicates that high basicity alone is not sufficient to bring about ubiquitination by RAD6 protein.

Function of multiply ubiquitinated histones in sporulation

Typically, 10% of the total histone H2A and 1–1.5% of histone H2B in the nuclei of mammalian cells are conjugated to a single molecule of ubiquitin (West and Bonner 1980a,b). In monoubiquitinated H2A and H2B (uH2A and uH2B), the ubiquitin moiety is linked to lysine 119 of H2A (Goldnopf and Busch 1977) and lysine 120 of H2B (Thorne et al. 1987). Diubiquitinated H2A, H2B (u₂H2A,

u₂H2B), and triubiquitinated H2A (u₃H2A) have also been detected in the testis of developing rainbow trout with an anti-ubiquitin antibody (Nickel et al. 1987), indicating that multiply ubiquitinated histones are formed *in vivo*.

Existing evidence suggests that the distribution of uH2A on chromatin is not random, and uH2A may be enriched in regions of chromatin that are being transcribed actively (Levinger and Varshavsky 1982; Barsoum and Varshavsky 1985). It has been proposed that monoubiquitinated histones may aid the relaxation of compact chromatin and nucleosomal structures (Levinger and Varshavsky 1982; Barsoum and Varshavsky 1985; Jentsch et al. 1987) during processes such as transcription and replication (Varshavsky et al. 1979; Wu et al. 1979; Jakobovits et al. 1980; Saragosti et al. 1980; Levinger and Varshavsky 1982; Baer and Rhodes 1983; Sun et al. 1986). However, because monoubiquitinated histones possess biochemical and biophysical properties indistinguishable from those of unmodified histones (Goldknopf et al. 1977; Bonner and Stedman 1979; Martinson et al. 1979; Kleinschmidt and Martinson 1981; Levinger and Varshavsky 1982) and do not seem to be degraded rapidly (Wu et al. 1981; Grove and Zweidler 1984), the precise mechanism by which monoubiquitinated histones may help relax chromosomal packing is still obscure.

The RAD6 polyacidic tail is required for sporulation, because α/α diploid strains homozygous for the *rad6-149* allele are highly defective in sporulation (Morrison et al. 1988). The results presented in this paper show that *rad6-149* protein, which lacks the entire polyacidic tail, does not polyubiquitinate histones. Interestingly, the addition of the first four residues of the polyacidic tail, as in *rad6-153* protein, partially restores both the histone-polyubiquitinating activity and the capability to effect sporulation (Morrison et al. 1988). These observations suggest that the sporulation defect accompanying the deletion of the RAD6 polyacidic tail may be due to the loss of the ability to multiply ubiquitinate histones. In higher eukaryotes, the process of spermatogenesis entails the replacement of nucleohistones by protamine. The polyubiquitinated histones synthesized during spermatogenesis in rainbow trout testis (Nickel et al. 1987) and the increase in the level of histone ubiquitin conjugates observed in rooster toward the end of spermiogenesis (Agell et al. 1983) may be important for nucleohistone replacement by protamine (Agell et al. 1983; Nickel et al. 1987). In yeast, polyubiquitination of histones may help bring forth the chromosomal modifications necessary to allow cells to proceed through sporulation. This notion is consistent with the recent observation that the yeast *UBI4* gene, which encodes a polymeric precursor of ubiquitin (Finley et al. 1987), is induced substantially during sporulation (Treger et al. 1988).

Multiply ubiquitinated histones may possess altered biochemical and biophysical properties that help realize perturbation of chromatin structure and perhaps dissolution of nucleosomal structure in various chromosomal

processes via either dissociation of the modified histones from the nucleosomal core, which may or may not require additional protein factors, or proteolytic degradation of the modified histones *in situ*, a strong possibility because of the existence of a multiply ubiquitinated configuration (Herskho et al. 1984b; Bachmair et al. 1986; Shanklin et al. 1987).

The *in vitro* system for synthesizing polyubiquitinated histones described here will be useful for studying the biological role of the modified histones in altering chromatin and nucleosomal structures. In particular, the biochemical and biophysical properties of the polyubiquitinated histones can be compared to those of unmodified histones by *in vitro* nucleosome assembly. The polyubiquitinated histones can also be used to identify the proteolytic system specific for the modified histones, if the modified histones are destined to be degraded by such a system *in vivo*.

Role of RAD6 protein in DNA repair

The *rad6-149* mutant shows an almost wild-type level of resistance to UV radiation and also displays unimpaired ability to convert lesions in DNA to mutations (Morrison et al. 1988). These observations indicate that the repair and mutagenesis functions of RAD6 protein are not as dependent on its histone-ubiquitinating activity as is sporulation. One may argue that the residual level of histone-ubiquitinating activity possessed by *rad6-149* protein can be utilized effectively in repair and mutagenesis via a hypothetical targeting mechanism that specifically directs the protein to DNA sites where the lesions are situated. However, because even a 20-fold underproduction of the *rad6-149* protein in a *rad6* genomic deletion strain does not result in a significant increase in UV sensitivity (Morrison et al. 1988), it seems reasonable to consider the possibility that RAD6 protein controls its DNA repair and mutagenesis functions via ubiquitination of nonhistone proteins that do not require the RAD6 polyacidic tail for their ubiquitination. The nonhistone substrates of RAD6 protein could include proteins encoded by genes in the *RAD6* epistasis group such as *RAD9*, *RAD15*, *RAD18*, *REV1*, *REV2*, and *REV3* (Haynes and Kunz 1981). Ubiquitin conjugation may serve to activate or modulate the biological activity of these proteins.

Possible significance of RAD6-ubiquitin isopeptide conjugate

E1, RAD6 protein, and various other E2 enzymes are themselves conjugated to ubiquitin through isopeptide linkages. The ubiquitination of these proteins may serve to regulate their biological activity and/or intracellular stability. The recent observation that ubiquitin may possess intrinsic proteolytic activity (Fried et al. 1987) raises the intriguing possibility that the E1 and E2 enzymes including RAD6 protein may be converted into *ad hoc* proteases upon their ubiquitination and that some of the biological functions of the E1 and E2 en-

zymes are fulfilled via their acquired proteolytic activity. In any event, the genetic significance of the ubiquitination of RAD6 protein can be examined by identifying and mutating the lysine residue in RAD6 protein to which ubiquitin is attached.

Materials and methods

Plasmids and strains

The *rad6-149* and *rad6-153* deletion mutant alleles were obtained using oligonucleotide-directed mutagenesis as described (Morrison et al. 1988). To facilitate the purification of RAD6, *rad6-149*, and *rad6-153* proteins from yeast, the open reading frames of the *RAD6* and *rad6* alleles were placed downstream of the promoter of the highly expressed and constitutive yeast dehydrogenase [*ADC1*] gene. The *ADC1* promoter is present in the vector pSCW231, which also carries the replication origin of the yeast 2 μ m plasmid and the yeast *TRP1* gene (Sung et al. 1987a). The 0.6-kb *EcoRI* fragments containing the *RAD6*, *rad6-149*, and *rad6-153* alleles were each introduced (Morrison et al. 1988) into pSCW231 to yield the plasmids pSCW242, pR619, and pR628, respectively. All the plasmids were maintained in the haploid strain SX46A-6 Δ (*MATa rad6 Δ :: URA3 ade2 his3-532 trp1-289*), whose genomic *RAD6* gene has been replaced by the *URA3* gene (Morrison et al. 1988). The overproduction of RAD6, *rad6-149*, and *rad6-153* proteins in SX46A-6 Δ was verified by the immunoblotting of cell extracts with affinity-purified anti-RAD6 antibodies. The RAD6 and *rad6* proteins each constitute ~0.5% of total protein in cell extracts from the various plasmid-bearing yeast strains (not shown). The haploid strain CMY135 (*MATa ade2-1 his3- Δ 200 lys2-801 trp1 Δ ura3-52 RAD⁺*) was used for the purification of E1 enzyme.

Purification of the ubiquitin-activating enzyme E1

The yeast E1 enzyme was purified to apparent homogeneity (Fig. 4A) via a procedure refined from that of others (Hershko et al. 1983; Jentsch et al. 1987). Yeast strain CMY135 was cultured in YPD (1% yeast extract, 2% bacto-peptone, and 2% dextrose) medium and harvested by centrifugation at a titer of 1.2×10^8 cells/ml. The cell paste (100 g) was washed once with distilled water, centrifuged, and resuspended in 60 ml of 20 mM Na-HEPES (pH 7.2). Following the addition of 2,4-dinitrophenol and 2-deoxyglucose to 0.2 and 20 mM, the cell suspension was agitated vigorously for 2 hr at 30°C. This treatment was designed to deplete the intracellular ATP that would otherwise lower the level of free ubiquitin-activating E1 and ubiquitin-conjugating (E2) enzymes by enabling the conjugation of these enzymes to endogenous ubiquitin (Hershko et al. 1983). After being washed with a large volume of distilled water, the treated cells were collected by centrifugation and stored at -70°C .

Unless stated otherwise, all the purification steps were carried out at 0–4°C. To prepare cell extract, the frozen yeast cake (100 g) was broken into small pieces with a sharp instrument to aid thawing. The cells were suspended in 150 ml of 10 mM Tris-HCl (pH 7.5), containing the protease inhibitors chymostatin, pepstatin A, leupeptin, and aprotinin at 10 $\mu\text{g}/\text{ml}$, and disrupted by the passage of the cell suspension through a French press at 20,000 psi. The cell lysate was clarified by centrifugation at 100,000g for 75 min and layered onto a column of DEAE-cellulose (2.6 $\times$ 18 cm; DE52 from Whatman) equilibrated in 3 mM KH_2PO_4 (pH 7.0) containing 1 mM DTT. The column was washed with 250 ml of 3 mM KH_2PO_4 (pH 7.0), containing 20 mM KCl and 1 mM DTT, and eluted with 0.5 M

KCl and 1 mM DTT in 20 mM Tris-HCl (pH 7.2). Fractions (3 ml) with $\text{OD}_{280} > 0.15$ were pooled, and the proteins precipitated by the addition of solid ammonium sulfate to 90% saturation (0.66 g/ml). The protein precipitate was collected by centrifugation (20,000g for 20 min), dissolved in 10 ml of buffer A (50 mM Tris-HCl containing 5% glycerol, 0.2 mM DTT, 1 mM EDTA and 1 mM ATP), and dialyzed overnight against 1 liter of the same buffer. The dialysate was clarified by centrifugation (100,000g for 30 min), and ATP and MgCl_2 were added to 4 mM and 10 mM, respectively. The protein solution was diluted with 10 ml of buffer A, warmed to 25°C, and applied onto a column of ubiquitin-Sepharose (0.6 $\times$ 3.5 cm; 22 mg ubiquitin/ml Sepharose) prepared according to Ciechanover et al. (1982). The E1 and E2 enzymes, being linked covalently to the Sepharose-bound ubiquitin via a thiolester bond (Hershko et al. 1983), were eluted with 20 mM DTT in 50 mM Tris-HCl (pH 7.5) at 25°C after the column had been washed with 20 ml each of 1 M KCl in 50 mM Tris-HCl (pH 7.5) and the Tris buffer without KCl. Fractions (0.4 ml) were collected on ice and screened for their content of E1 and E2 enzymes by SDS-PAGE on a 12% gel. The peak fractions were pooled (2 ml) and dialyzed overnight against 1 liter of 100 mM KCl in buffer B [20 mM Tris-HCl (pH 7.5) containing 5% glycerol, 0.5 mM DTT, and 0.2 mM EDTA]. The dialysate was applied onto an FPLC Mono Q column (HR 5/5; Pharmacia) equilibrated in buffer B. After being washed with 5 ml of 100 mM KCl in buffer B, the column was developed with a 35-ml gradient of 100–550 mM KCl in buffer B. Fractions (0.5 ml) containing the E1 enzyme, which elutes between 215 and 225 mM KCl, were pooled (1 ml) and concentrated to 0.3 ml (400 μg E1/ml) using a Centricon-30 concentrator (Amicon).

To purify further E1 enzyme to apparent homogeneity, 0.15 ml of the FPLC-purified E1 pool was diluted with an equal volume of buffer A and size-fractionated on a column of Sephadex G-150 superfine (1 $\times$ 45 cm; Pharmacia), equilibrated in and eluted with buffer A according to Jentsch et al. (1987; see Fig. 4A). The E1 enzyme pool (1.2 ml) was concentrated to 0.07 ml (800 μg E1/ml), which was stored in small aliquots at -70°C .

Purification of RAD6, *rad6-149*, and *rad6-153* proteins

The RAD6 and *rad6* proteins were purified to apparent homogeneity (Fig. 1), using a combination of affinity chromatography on ubiquitin-Sepharose and anion exchange on an FPLC Mono Q column as in the purification of the E1 enzyme, but with some modifications. SX46A-6 Δ strains harboring the plasmids pSCW242, pR619, and pR628, which overproduce RAD6, *rad6-149*, and *rad6-153* proteins, respectively, were cultured in complete synthetic medium lacking tryptophan and harvested at a titer of 1.2×10^7 cells/ml. The cells were treated with 2,4-dinitrophenol and 2-deoxyglucose (see the preceding section) before being stored at -70°C .

For the purification of RAD6 protein, cell extract from 60 g of SX46A-6 Δ [pSCW242] was fractionated on columns of DEAE-cellulose (2.6 $\times$ 11 cm) and ubiquitin-Sepharose (0.6 $\times$ 3.5 cm), as described for the purification of the E1 enzyme. The protein pool from the ubiquitin affinity step was dialyzed overnight against 1 liter of 100 mM KCl in buffer B, and the dialysate was applied onto the Mono Q column. Subsequent to being washed with 5 ml of 100 mM KCl in buffer B, the column was eluted with a 25-ml gradient of 300–550 mM KCl in buffer B. Fractions (0.5 ml) containing RAD6 protein, which elutes between 425–435 mM KCl, were pooled and concentrated to 0.1 ml (1 mg RAD6 protein/ml) using a Centricon-10 concentrator (Amicon). RAD6 protein was also purified by the method of

Morrison et al. (1988), which does not entail the use of affinity chromatography on ubiquitin–Sephacrose and anion exchange on Mono Q.

In the purification of rad6-149 and rad6-153 proteins, cells (60 g) of the strains SX46A-6Δ[pr619] and SX46A-6Δ[pr628] were lysed in 50 mM Tris-HCl (pH 7.5) containing 5% glycerol, 0.2 mM DTT, 1 mM EDTA, and protease inhibitors (see the preceding section). After the addition of ATP and MgCl₂ to 3 and 5 mM, the cell lysates were applied onto the ubiquitin–Sephacrose column without prior fractionation on DEAE–cellulose. The rad6 protein pools were dialyzed against buffer B and further fractionation on the Mono Q column. A 35-ml gradient of either 0–135 mM or 135–225 mM KCl in buffer B was used, depending on whether rad6–149 protein or rad6–153 protein was being purified. Individual pools (1 ml each) of rad6–149 and rad6–153 proteins, which elute between 65–75 and 170–180 mM KCl, respectively, were concentrated to 0.1 ml (1 mg rad6 protein/ml). The concentrated RAD6 and rad6 preparations were stored in small aliquots at –70°C.

Ubiquitin-conjugation assays

Reaction mixtures (20 μl) were assembled in buffer R [50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM ATP, 50 mM KCl, 0.2 mM DTT, and 100 μg/ml bovine serum albumin] and contained 100–200 ng of RAD6 protein, 400 ng of E1 enzyme, 1 μg of bovine histone H2A or H2B, and 550 ng of ¹²⁵I-labeled ubiquitin (3 × 10⁵ cpm). After 30 min at 30°C, the ubiquitin-conjugating reaction was terminated by the addition of 10 μl of stop buffer [200 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, and 0.06% bromophenol blue]. The samples were boiled for 3 min before being electrophoresed at 25°C on 12.5% polyacrylamide gels at 1 mA/cm for 4 hr, as described by Laemmli (1970). The gels were dried, and the radioactive bands revealed by autoradiography using Kodak XRP1 film at –70°C in cassettes fitted with DuPont Cronex Lightning Plus intensifying screens. To detect the formation of thioesters between ubiquitin and the E1 and E2 enzymes, 2-mercaptoethanol was omitted from the stop buffer and samples were electrophoresed at 4°C without prior boiling.

Other procedures

Yeast cells were transformed by the method of Ito et al. (1983). Bovine ubiquitin (370 μg) was iodinated for 40 sec at 25°C using 500 μCi of Na¹²⁵I (Amersham) and chloramine T (330 μg/ml) in 30 μl of 166 mM KH₂PO₄ (pH 7.5), to a specific radioactivity of 0.45 μCi/μg or 5.6 × 10⁵ cpm/μg (Ciechanover et al. 1980). The ¹²⁵I-labeled ubiquitin was separated from the unreacted iodide and other components of the labeling mixture by filtration through a column of Sephadex G-25 fine (0.6 × 18 cm; Pharmacia), equilibrated in and eluted with 20 mM KH₂PO₄ (pH 7.5). SDS–PAGE was carried out according to Laemmli (1970).

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The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity.

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