The EMBO Journal vol.15 no.23 pp.6641–6651, 1996

The *Schizosaccharomyces pombe rad3* checkpoint gene

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The *rad3* gene of *Schizosaccharomyces pombe* is required for checkpoint pathways that respond to DNA damage and replication blocks. We report the complete *rad3* gene sequence and show that *rad3* is the homologue of *Saccharomyces cerevisiae ESR1* (*MEC1/SAD3*) and *Drosophila melanogaster mei-41* checkpoint genes. This establishes Rad3/Mec1 as the only conserved protein which is required for all the DNA structure checkpoints in both yeast model systems. Rad3 is an inessential member of the 'lipid kinase' subclass of kinases which includes the ATM protein defective in ataxia telangiectasia patients. Mutational analysis indicates that the kinase domain is required for Rad3 function, and immunoprecipitation of overexpressed Rad3 demonstrates an associated protein kinase activity. The previous observation that *rad3* mutations can be rescued by a truncated clone lacking the kinase domain may be due to intragenic complementation. Consistent with this, biochemical data suggest that Rad3 exists in a complex containing multiple copies of Rad3. We have identified a novel human gene (*ATR*) whose product is closely related to Rad3/Esr1p/Mei-41. ATR can functionally complement *esr1-1* radiation sensitivity in *S.cerevisiae*. Together, the structural conservation and functional complementation suggest strongly that the mechanisms underlying the DNA structure checkpoints are conserved throughout evolution.

Keywords: ataxia telangiectasia/checkpoint/fission yeast/Mec1/Rad3

Introduction

In yeast model systems, and in mammalian cells, DNA structure-dependent checkpoint pathways act to prevent inappropriate progression through the cell cycle when DNA replication is incomplete or when the DNA is damaged (Weinert and Hartwell, 1988). In fission yeast, at least two distinct DNA structure-dependent checkpoint pathways have been identified, the S–M checkpoint which links mitosis to the prior completion of DNA replication, and the DNA damage checkpoint which arrests the cell cycle when DNA integrity is compromised (Sheldrick and Carr, 1993). Mutant cells which have lost the S–M checkpoint do not prevent mitosis when the DNA remains unreplicated, causing a lethal missegregation of unreplicated or partially replicated chromatin (Enoch and Nurse, 1990). Mutant cells which have lost the DNA damage checkpoint can attempt mitosis before DNA repair is complete, resulting in hypersensitivity to DNA-damaging agents (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992).

Initial studies on checkpoints in the two yeast model systems concentrated on the arrest of mitosis following S phase inhibition and DNA damage. A number of genes were identified, the products of which are required for DNA structure-dependent mitotic arrest (reviewed in Carr and Hoekstra, 1995). Several homologous gene pairs are evident between the two yeasts, which diverged ~330–600 million years ago (Berbee and Taylor, 1993), suggesting conservation throughout evolution. More recent work has demonstrated that the checkpoint proteins, which control cell cycle arrest responses at G_1, S phase and mitosis (Al-Khodairy *et al.*, 1994; Allen *et al.*, 1994; Siede *et al.*, 1994; Carr *et al.*, 1995; Paulovich and Hartwell., 1995), are required for multiple transcriptional responses to stress (Fernandez-Sarabia *et al.*, 1993; Allen *et al.*, 1994) and are probably involved directly in DNA repair events under specific circumstances (Griffiths *et al.*, 1995; Lydall and Weinert, 1995).

Biochemically, little is known about the checkpoint genes in *Schizosaccharomyces pombe*. While structural similarity to *Ustilago maydis* Rec1 suggests that Rad1 may be a nuclease (Carr, 1994; Long *et al.*, 1994) and conserved domains between Rad17 and RF-C (activator A) subunits hint that Rad17 may bind either DNA or replication proteins (Griffiths *et al.*, 1995), neither protein has been studied directly. Chk1 and Cds1 are potential protein kinases (Walworth *et al.*, 1993, Al-Khodairy *et al.*, 1994; Murakami and Okayama, 1995). Chk1 is not required for the S–M checkpoint, and has been shown to be modified by phosphorylation following DNA damage (Walworth and Bernards, 1996), but not S phase arrest. This modification is dependent on the integrity of the 'checkpoint Rad' proteins (Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1), suggesting that Chk1 acts downstream of the checkpoint Rad proteins in the DNA damage checkpoint. Unlike Chk1, the checkpoint Rad proteins are required for both the DNA damage and the S–M checkpoints. By extrapolation from work with *Saccharomyces cerevisiae* Rad53p, the *S.pombe* Cds1 kinase (which is a structural homologue) probably also acts downstream of the checkpoint Rad group of gene products (Sanchez *et al.*, 1996; Sun *et al.*, 1996), preventing at least some aspects of cell cycle progression in response to the inhibition of S phase.

The work to date, therefore, suggests that the checkpoint Rad proteins define a pathway or complex which acts at the beginning of the checkpoint pathway to recognize

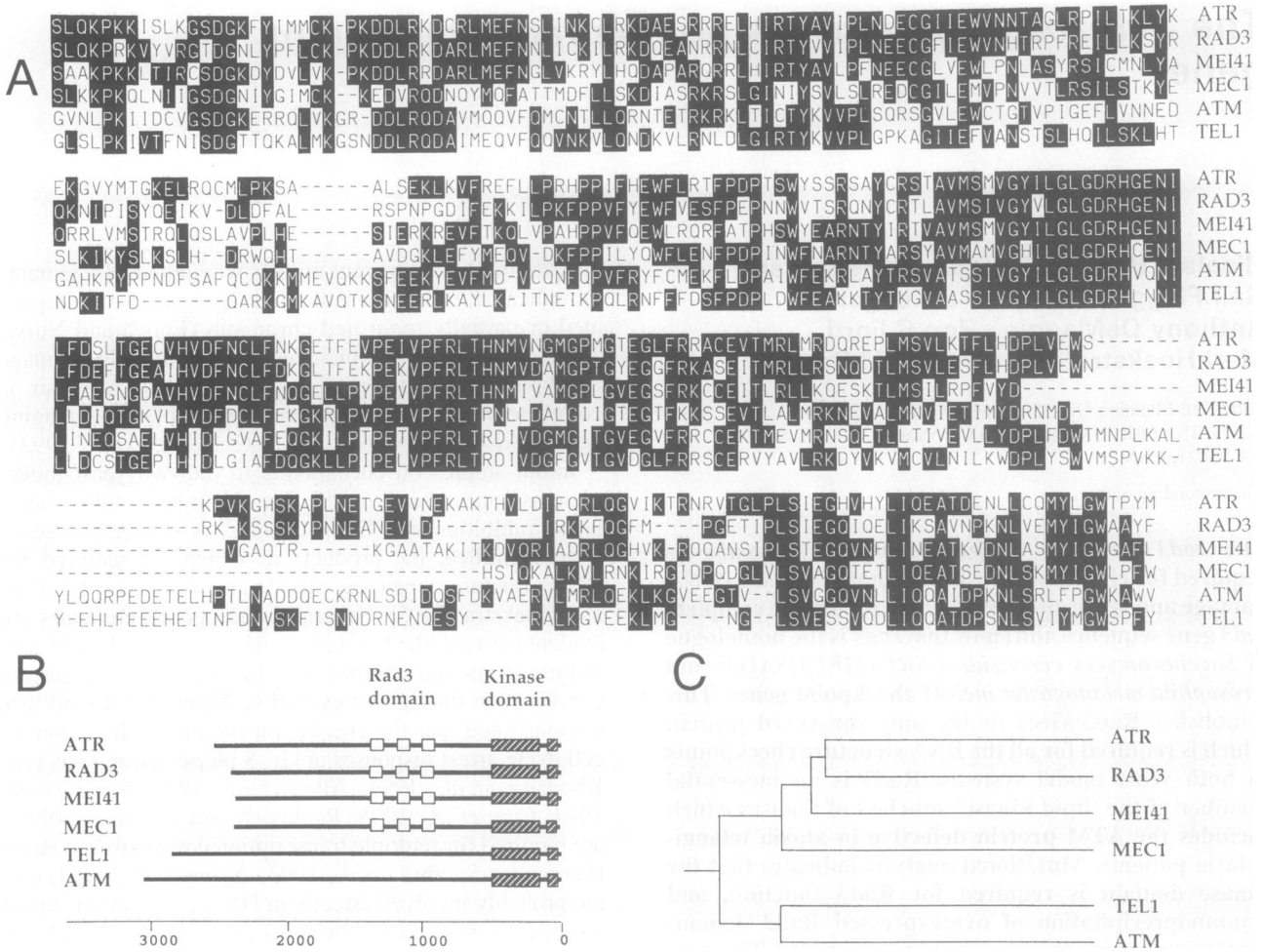

Fig. 1. The sequence and structure of *S.pombe* Rad3 and human ATR. (**A**) An alignment of kinase domains of ATR, Rad3, Mei-41, Mec1p, ATM and Tel1p. Boxed residues match the consensus, being present in three or more sequences. In the region shown, ATR is 53% identical to Rad3, 48% identical to Mei-41, 41% identical to Mec1p, and 31 and 32% identical to ATM and Tel1p respectively. ATM and Tel1p share 41% identity. (**B**) The overall structures of ATR, Rad3, Mei-41, Mec1p, ATM and Tel1p. The 'Rad3 domain' was defined by Savitsky *et al.* (1995b), boxes indicate highly conserved regions. (**C**) Dendrogram demonstrating the existence throughout evolution of two families of a large lipid kinase motif protein involved in checkpoint responses in eukaryotes. GenBank accession Nos: Rad3, U76307. ATR, U76308.

specific DNA or DNA–protein structures, and to translate this information into signals mediated by a series of protein kinases in order to affect cell cycle arrest in response to changes in DNA structure and integrity (Carr, 1996). Here we identify the complete sequence of the *rad3* gene from fission yeast. We provide evidence that Rad3 can self-associate, that it can act as a protein kinase and that the kinase domain is required for Rad3 function. We also show that 'kinase-dead' *rad3* constructs give a dominant-negative phenotype upon overexpression in wild-type cells, which suggests that Rad3 acts in a complex. In addition, we have identified a human homologue of Rad3 (ATR) which is closely related to the yeast checkpoint proteins, and demonstrate that *ATR* can functionally complement *S.cerevisiae esr1-1* mutants for radiation sensitivity. We discuss these results in the context of Rad3's similarity to the 'large lipid kinase motif' group of proteins which includes the *ATM* gene product defective in ataxia telangiectasia (A-T), a human multi-system genetic disease characterized by checkpoint defects, DNA damage sensitivity and cancer proneness.

Results

Cloning of full-length S.pombe rad3

Fission yeast *rad3* mutants have been characterized previously as checkpoint defective (Al-Khodairy and Carr, 1992; Jimenez *et al.*, 1992; Rowley *et al.*, 1992), and a partial genomic sequence has been reported (Seaton *et al.*, 1992) which is capable of encoding a protein of 1070 amino acids. We have cloned the complete *S.pombe rad3* gene. This work extends the published sequence in both the 5′ and 3′ directions and has identified a single open reading frame (ORF) of 2386 amino acids which contains at its C-terminus the consensus sequences that define the 'lipid kinase' subfamily of kinases (Figure 1). We have created two separate deletion mutants of the *rad3* gene (data not shown, see Figure 3 for schematic). In the first, the coding region corresponding to amino acids 1–1476 has been replaced by the *ura4* marker. In the second, the coding region corresponding to amino acids 1477–2271, including the majority of the C-terminal kinase domain, has been replaced with the *ura4* marker. The predicted

rad3 ORF, when cloned behind an inducible promoter in *S.pombe*, is able to fully complement both these *rad3* deletion mutants (data not shown), suggesting that it encodes the entire Rad3 protein.

Several large proteins recently have been reported which contain the lipid kinase domain (large lipid kinase motif proteins), including the checkpoint proteins Esr1p/Mec1p and Mei-41, the ATM protein and its potential *S.cerevisiae* homologue Tel1p, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the Tor1/2-related proteins (Helliwell *et al.*, 1994; Kato and Ogawa, 1994; Greenwell *et al.*, 1995; Hari *et al.*, 1995; Hartley *et al.*, 1995; Morrow *et al.*, 1995; Sabers *et al.*, 1995; Savitsky *et al.*, 1995a). Within the family of large lipid kinase motif proteins, Rad3 shows the most extensive homology with the *S.cerevisiae* Esr1p/Mec1p and the *Drosophila melanogaster* Mei-41 checkpoint proteins (Kato and Ogawa, 1994; Hari *et al.*, 1995) (Figure 1). This homology extends over the length of the protein, and is not restricted to the kinase domain. Thus, by function and structure, the Rad3/Esr1p/Mei-41 protein is clearly conserved throughout evolution.

The human rad3 homologue, ATR

The high degree of similarity between Rad3, Esr1/Mec1p and Mei-41 suggested that this protein may be conserved in higher eukaryotes. In order to identify a human form of *rad3*, a combination of degenerate PCR and expressed sequence tag (EST) library screening was applied. Degenerate primers were used to amplify a small region of human cDNA which translated to an amino acid sequence that aligned with the Rad3 and Esr1/Mec1 proteins. This region was used as a probe to identify a 5′-truncated 3.0 kb cDNA by hybridization. The same region was encompassed in a cDNA clone identified from an EST database. The 5′ end of the complete cDNA was obtained by RACE–PCR (see Materials and methods). Through these approaches, we have cloned the entire coding region of a human gene, which we have named *ATR* (ataxia and rad related). *ATR* is capable of encoding a 2644 amino acid protein which is much more closely related to the products of *S.pombe rad3*, *S.cerevisiae ESR1/MEC1* (Kato and Ogawa, 1994) and *D.melanogaster mei-41* genes (Hari *et al.*, 1995) than to the human *ATM* and *S.cerevisiae TEL1* genes (Greenwell *et al.*, 1995; Morrow *et al.*, 1995; Savitsky *et al.*, 1995a) (Figure 1). *mec1/sad3* checkpoint mutants (Allen *et al.*, 1994; Weinert *et al.*, 1994) and Mei-41 mutants (Hari *et al.*, 1995) have an equivalent phenotype to *rad3* and it is likely that *ATR* is therefore the true homologue of these genes.

ATR is less closely related to the human checkpoint gene *ATM*, although it does contain a C-terminal potential lipid kinase domain and has a similar overall structure (Figure 1B). Evolutionary relationships based on sequence alignments demonstrate clearly that the *rad3/ESR1(MEC1/SAD3)/mei-41/ATR* genes define a conserved protein in each organism, and suggest that *TEL1* and *ATM* may also represent a conserved gene, the fission yeast and *Drosophila* homologues of which have not yet been reported (Figure 1C). Using fluorescence *in situ* hybridization (FISH) and PCR analysis, we have mapped *ATR* to chromosome 3q22–3q23 (data not shown). This region is not associated with reported cancer-prone syndromes. These data are consistent with the report of Cimprich *et al.* (1996), which describes the cloning, sequencing and localization of the same sequence.

The close structural similarity between ATR, Rad3 and Esr1/Mec1p suggests a common function. In order to investigate this, we have determined the ability of *ATR* to complement *S.pombe rad3* and *S.cerevisiae esr1-1* mutants. Expression of full-length human *ATR* from a thiamine-inducible promoter in fission yeast does not complement the *S.pombe rad3::ura4* C-terminal deletion mutant or the *rad3.D2249E* kinase-dead mutant (Figure 2A). Expression of full-length human *ATR* from a galactose-inducible promoter in *S.cerevisiae* complements the UV sensitivity of the *esr1-1* allele (Figure 2B), but not the hydroxyurea sensitivity (data not shown). The inability of *ATR* to complement in *S.pombe* is not due to expression problems since ATR protein can be detected with an affinity-purified ATR-specific antibody (Keegen *et al.*, 1996) in both yeasts (Figure 2C and D). Several DNA structure checkpoint and DNA repair proteins are conserved between *S.pombe* and *S.cerevisiae* and human cells, but only a very few human or budding yeast genes have been shown to be able to complement the equivalent mutants in *S.pombe* (McCready *et al.*, 1989; Carr *et al.*, 1994; Griffiths *et al.*, 1995; and our unpublished observations). Cross-species complementation is the exception rather than the rule, particularly when proteins are involved in multiple protein–protein interactions, and presumably reflects structural divergence. Thus, the complementation of the UV sensitivity of *mec1-1* by ATR (see Figure 2) strongly suggests a conserved function between these proteins.

Genetic analysis suggests the kinase activity is essential for rad3 function

Previous work on *S.pombe rad3* showed that a truncated clone, which did not contain the lipid kinase motif domain, could restore all the checkpoint phenotypes associated with loss of *rad3* function in both the *rad3.136* allele and in a mutant in which the *rad3* ORF had been interrupted by a copy of the *LEU2* gene (Jimenez *et al.*, 1992; Seaton *et al.*, 1992). This observation, and the large size of the Rad3 protein which creates potential for a structural role for Rad3 that may be independent of its kinase activity, suggested that the kinase activity may not be required for the DNA structure checkpoint functions lost in the *rad3* mutants.

In order to investigate the requirement for the kinase domain in establishing the DNA structure checkpoints, we created a deletion mutant of *rad3* in which a significant part of the protein, including the kinase domain, was deleted and replaced with the *ura4*$^+$ gene (Figure 3A). This mutant has an identical phenotype to *rad3.136* (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992) and the *rad3::LEU2* (Jimenez *et al.*, 1992; Seaton *et al.*, 1992) interruption mutant (Figure 3B–D). Next we created, by gene replacement of *rad3::ura4*, three separate mutant alleles of *rad3* in which single amino acids in the putative lipid kinase domain are changed to give 'kinase-dead' mutants (see Materials and methods). In all cases, these 'kinase-dead' alleles behaved identically to both the previously characterized *rad3* mutants and the new deletion mutant *rad3::ura4*. In each case, identical radiation sen-

sitivity (Figure 3B) and hydroxyurea sensitivity (Figure 3C) is seen. Furthermore, no significant checkpoint is evident following exposure of synchronous cultures to either radiation (Figure 3D) or hydroxyurea (Figure 3E). From this, we conclude that the kinase activity of Rad3 protein, and by extension of its homologues such as Mec1p and ATR, is likely to be essential for all its functions.

S.pombe Rad3 kinase-dead mutants cause dominant radiation sensitivity when overexpressed

The prediction that the kinase domain of Rad3 is required for its functions is not, at first sight, compatible with the report (Jimenez *et al.*, 1992) that a truncated *rad3* gene, which had lost the kinase domain, was able to fully complement both the *rad3.136* and *rad3::LEU2* alleles (see Figure 3A). One explanation for these data could be that they reflect specific intragenic complementation between the C-terminally truncated protein encoded by the plasmid and an altered form of Rad3 encoded from the genome. Such intragenic complementation can occur when a protein acts as a multimer. We have also found that moderate overexpression of a 'kinase-dead' *rad3* from an inducible vector (Maundrell, 1990; Basi *et al.*, 1993) causes a dominant radiation-sensitive phenotype in wild-type cells (Figure 4). This is likely to be a true dominant-negative effect since it can be partially reversed by co-expression of similar levels of wild-type *rad3* (data not shown). Dominant-negative effects are usually attributed to the defective protein forming complexes with other cellular components and rendering such complexes non-functional. This observation, therefore, supports the possibility that *rad3* acts in a complex with itself and/or other proteins. *ATR* expression does not cause a dominant-negative phenotype (data not shown).

In the above experiments, an attenuated *nmt1* promoter was used. Since we have thus far been unable to detect endogenous Rad3 with antibodies raised to Rad3-specific sequences, we are unable to state by how much Rad3 protein is overexpressed in these studies, although our best guess, based on the levels of other checkpoint proteins (Griffiths *et al.*, 1995; Walworth and Bernards, 1996; and our unpublished data), would be between 10- and 20-fold. pREP41 expression levels have been characterized in detail elsewhere (Basi *et al.*, 1993; Forsberg, 1993) and it is clear that the expected levels do not correspond to the massive induction seen with the unattenuated *nmt1* promoter (pREP1 plasmids). When we analyse the effect of high level overexpression of *rad3* and *rad3.KD* constructs in wild-type cells using the unmodified *nmt1* promoter in pREP1, we see that the *rad3.KD* constructs manifest an additional phenotype as the cells fail to form more than micro-colonies. The wild-type *rad3* construct and an ATR-expressing construct, under the same conditions, also slowed down the rate of colony formation when induced in wild-type cells, although this was less evident (data not shown).

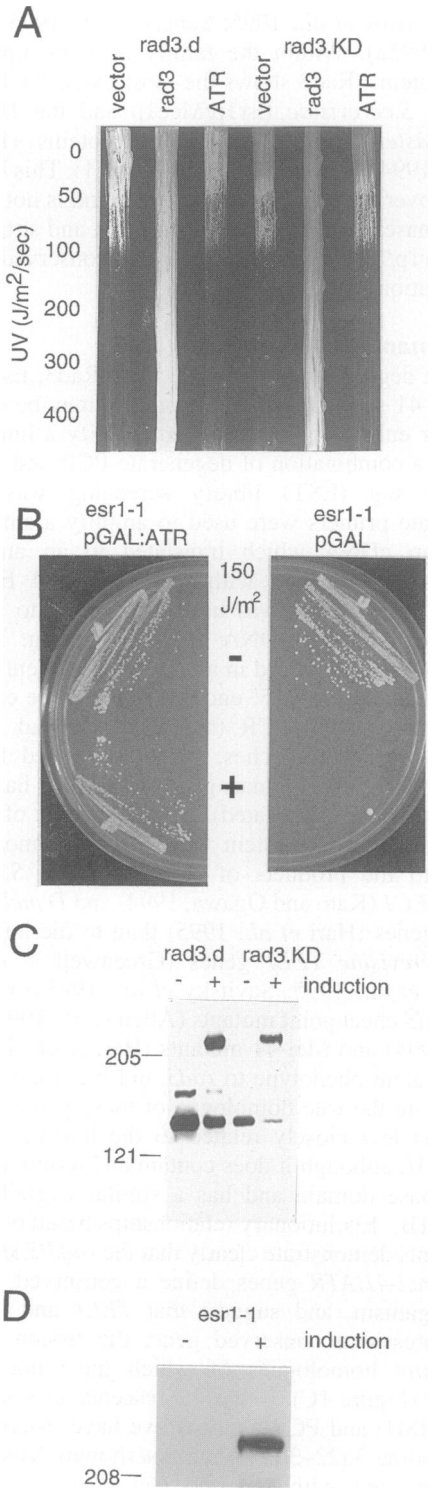

Fig. 2. Human *ATR* can complement *esr1-1* mutants, but not *rad3* mutants. (**A**) UV plate assay of full-length *rad3* and full-length *ATR* expressed in either a *rad3.d* C-terminal deletion mutant or *rad3.D2249E* (rad3.KD) kinase-dead mutant cells. Vector represents empty vector control. Rad3 complements as UV dosage increases down the plate, whereas ATR does not. (**B**) UV plate assay of ATR expressed in an *S.cerevisiae esr1-1* mutant. When induced for 8 h (pGAL:ATR), ATR allows survival of *esr1-1* mutant cells following exposure to 150 J/m^2 UV. The empty vector (pGAL) did not restore radiation resistance to *esr1-1* cells. (**C**) Western analysis using an antibody specific to the ATR protein (Keegan *et al.*, 1996) demonstrates that ATR is expressed in the *rad3.d* and *rad3.D2249E* mutants (mol. wt 301.5 kDa) when transcription is induced by removing thiamine from the media for 16 h prior to extract preparation. Cross-reacting bands below 205 kDa are non-specific, since they are seen in the uninduced as well as the induced lanes. (**D**) Similar analysis following 8 h induction indicates that ATR is also expressed in *esr1-1* cells.

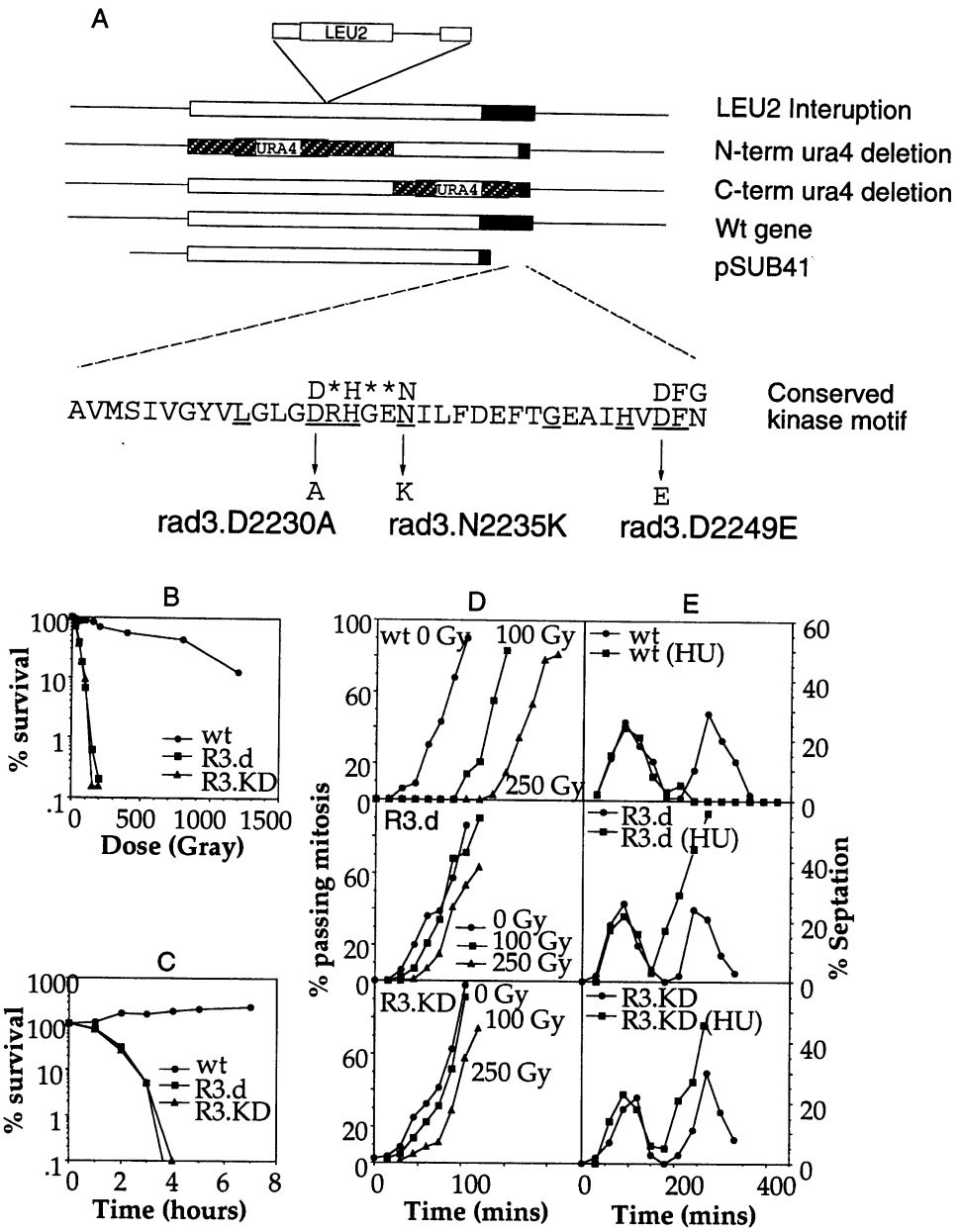

Fig. 3. Construction and characterization of *rad3* mutants in *S.pombe*. (**A**) The *rad3* locus is shown, along with the regions replaced by *ura4*$^+$ in the *rad3.d* deletion mutants, the structure of the *LEU2*$^+$ interruption mutant from Seaton *et al.* (1992) and the specific point mutants created in the kinase domain. Also shown is the extent of the pSUB41 clone (Seaton *et al.*, 1992). (**B**) Survival of wild-type (wt), *rad3* C-terminal deletion (R3.d) and *rad3.D2249E*, a representative *rad3* kinase-dead mutant (R3.KD) following increasing doses of ionizing radiation. (**C**) Survival over time of the same strains following exposure to 10 mM hydroxyurea. (**D**) Comparison of γ-ray checkpoint response in wild-type (wt) *rad3* C-terminal deletion (R3.d) and the representative *rad3* kinase-dead mutant (R3.KD) cells. Synchronous G_2 cells from lactose gradients were treated with either 0, 100 or 250 Gy of ionizing radiation and scored at 15 min intervals for passage through mitosis (Al-Khodairy *et al.*, 1994). (**E**) A similar analysis was performed for the S–M checkpoint by incubating G_2 cells with or without hydroxyurea and scoring the septation index at 30 min intervals (Al-Khodairy *et al.*, 1994). The data indicate that the kinase activity of Rad3 is required for its checkpoint functions.

We have characterized this slow growth phenotype further by following cell number during *rad3.KD* induction in exponential cultures. The doubling time of the culture was significantly increased (from ~3.5 h to >12 h) as *rad3.KD* was induced. Furthermore, the cellular morphology changed subtly following induction. While the septation index did not change significantly, remaining at ~10%, the cell size at mitosis (estimated by averaging 25 measurements of randomly chosen septated cells) was reduced from 15 to 11.2 μm. This 'semi wee' and slow growth phenotype is not observed in the *rad3* deletion mutants and appears to be distinct from the dominant-negative radiation sensitivity seen with more modest *rad3.KD* overexpression from the attenuated *nmt1* promoter in pREP41. We do not know the reason why high levels of *rad3.KD* and, to a lesser extent, *rad3* and *ATR* expression cause this effect. It is possible that these proteins interfere in a second pathway whose function overlaps with that of Rad3 and acts to inhibit mitosis. A candidate for such a pathway is the *ATM/TEL1* pathway

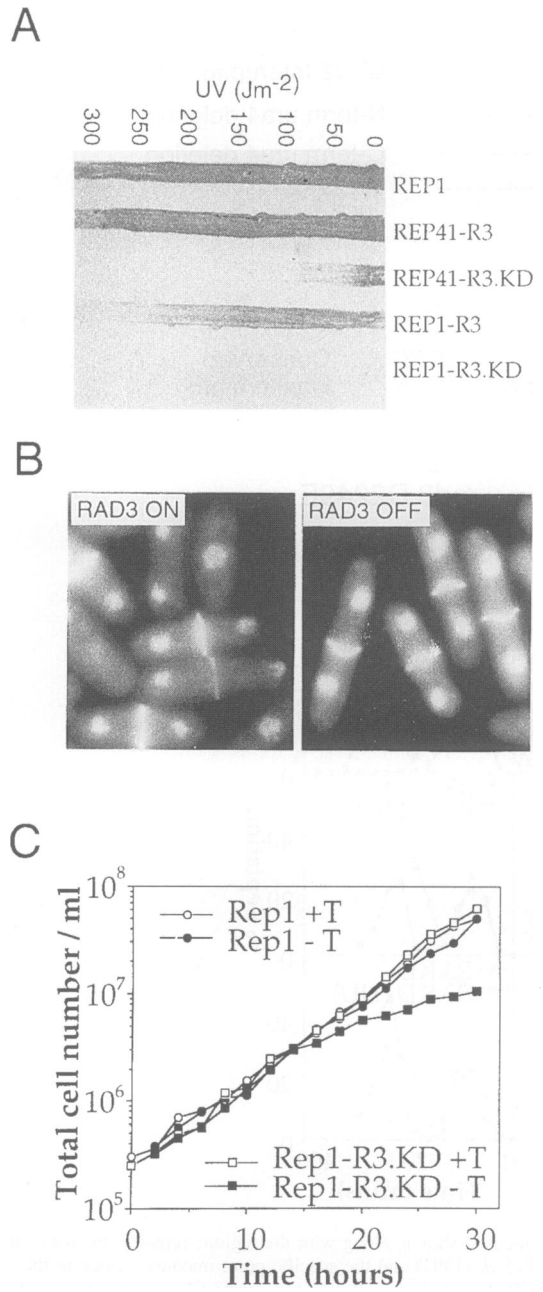

Fig. 4. The dominant-negative effect of overexpressing a *rad3* kinase-dead mutants. (**A**) Survival was measured after exposing freshly plated cells to increasing doses of UV radiation in 50 J/m^2 increments, from 0 to 300 J/m^2, right to left. Expression (REP41 = moderate, REP1 = high level) is induced by growing for 16 h without thiamine prior to plating. Wild-type cells expressing wild-type *rad3* (REP41-R3 and REP1-R3); expressing 'kinase-dead' *rad3.D2249E* (REP41-R3.KD and REP1-R3.KD); and empty vector control (REP1). (**B**) High level overexpression (REP1-KD) of kinase-dead *rad3* severely slows proliferation. However, cells (from A) show no evidence of cell cycle stage-specific arrest, maintaining a similar mitotic index and dividing at a smaller cell size than control cells (average of 25 measurements: 11.2 and 15 μm respectively). Examples of mitotic and septated cells are shown. Rad3 ON, induced, Rad3 OFF, uninduced. (**C**) Cell number increase following induction (–T) of either empty vector (REP1) or a *rad3* dominant-negative kinase-dead mutant (REP1-R3.KD) was measured by counting cells every 2 h using a haemocytometer during exponential growth. The promoter is induced at 16 h after removal of thiamine from the media (Maundrell, 1990), at which time overexpression of *rad3.D2249E* significantly slows down cell growth.

which has been shown to have some overlapping functions with the *ESR1(MEC1/SAD3)* pathway (Morrow *et al.*, 1995; Sanchez *et al.*, 1996).

S.pombe Rad3 can immunoprecipitate both Rad3 and ATR, suggesting multiple Rad3 proteins are present in a common complex

In order to investigate further the possibility that Rad3 acts as a multimer, we have created two separate tagged constructs of full-length *rad3* in pREP-based inducible vectors under the control of the attenuated *nmt1* promoter. In one, Rad3 is translated with two myc epitope tags at the N-terminus, while in the other these are substituted for a triple HA epitope tag. When both constructs are expressed together in wild-type cells, it is possible to co-precipitate the HA-tagged Rad3 with the myc-specific antibody, and the myc-tagged Rad3 with the HA-specific antibody (Figure 5). These data suggest that Rad3 may bind directly to Rad3 to form homomers, or that multiple Rad3 molecules may be present in complexes. We can eliminate the possibility that Rad3 immune complexes trap proteins non-specifically since, in control experiments, neither myc-tagged Rad4, Rad17 or Rad1 proteins will co-precipitate with HA-tagged Rad3 using HA-specific monoclonals. The observation that Rad3 immunoprecipitates with Rad3 is fully consistent with the complementation data of Jimenez *et al.* (1992) and the dominant-negative phenotype associated with *rad3.KD* induction.

Although the *ATR* gene could not complement the phenotype of the *rad3* mutants, we have investigated the ability of ATR to form a protein complex with *S.pombe* Rad3 by expressing both ATR and myc-tagged *S.pombe* Rad3 in the same yeast cells. Using an anti-ATR antibody (which does not precipitate *S.pombe* Rad3, see Materials and methods), we are able to co-precipitate the yeast protein. We were also able to precipitate the human ATR protein with myc-specific antibodies that recognize the *S.pombe* Rad3 (Figure 5). These data suggest that the human and yeast proteins can form a heteromeric complex, which supports the contention, based on the sequence similarity and the complementation of *mec1-1* by ATR, of a close functional relationship between these homologues.

Rad3 proteins have associated kinase activity

Since mutagenesis experiments suggest that the kinase activity of the Rad3 proteins *in vivo* appears to be essential for their function, we have investigated this activity further. Using *S.pombe rad3::ura4* cells expressing HA-tagged *S.pombe* Rad3, we have been able to detect a significant protein kinase activity which precipitates with HA-specific antibodies only when Rad3 is induced and which is not changed following irradiation (Figure 6) This activity, which is specific to Rad3 or co-precipitating kinases, appears to reflect phosphorylation of Rad3 itself, since the major band above 200 kDa that is phosphorylated can be detected by Western analysis with anti-HA antibody (Figure 6).

Attempts to identify convenient *in vitro* substrates such as myelin basic protein, RP-A and several purified *S.pombe* checkpoint proteins have so far proved unsuccessful and thus we have analysed the phosphorylation of Rad3 as the only currently available assay. When the immunoprecipitation (IP) *in vitro* kinase assay is performed with

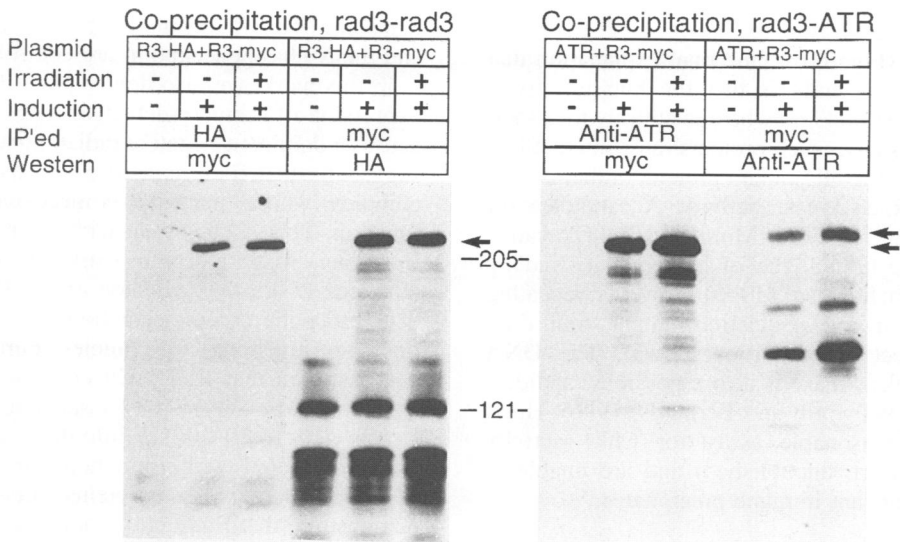

Fig. 5. Rad3 proteins can form homomeric complexes and can complex with ATR. Self-association of Rad3 was investigated by expressing two separate tagged constructs in wild-type cells from the attenuated *nmt1* promoter of pREP41 (left panel). Extracts from cells expressing both triple HA- and double myc-tagged Rad3 proteins were prepared 18 h after removing thiamine and immunoprecipitated with either anti-HA or anti-myc monoclonals, and then the immune complexes electrophoresed and Western blotted with the complementary antibody. Anti-HA antibodies can precipitate myc-tagged Rad3 and vice versa. The potential interaction between Rad3 and the human homologue ATR was investigated by expressing both myc-tagged Rad3 and full-length ATR in the same cells and immunoprecipitating from extracts with either anti-myc or anti-ATR antibodies (right panel). Co-precipitation of the human protein is seen with anti-myc antibodies and co-precipitation of the myc–Rad3 protein is seen with anti-ATR antibodies. Bands corresponding to tagged Rad3 (277 kDa) and ATR (301.5 kDa) are indicated with arrows. Bands at and below 121 kDa (left panel) represent cross-reaction with antibodies since they are seen in the non-induced controls. Bands between 121 and 205 (right panel) are probably degradation products of Rad3 or ATR respectively, since they are not seen in the uninduced controls. In no case was a significant reproducible difference in association seen upon irradiation. Extensive controls have established that each antibody is specific to its respective construct and there is no cross-reaction or non-specific precipitation.

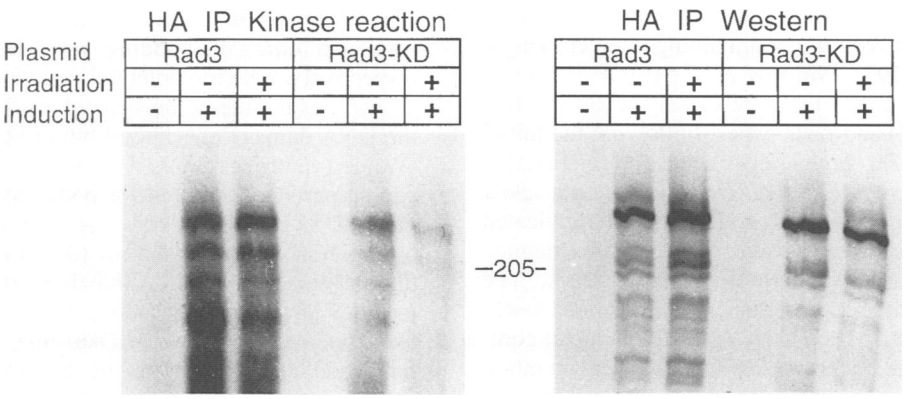

Fig. 6. Rad3-associated protein kinase activity. Using anti-HA antibodies, HA-tagged Rad3 was immunoprecipitated from *rad3.d* C-terminal deletion mutant extracts either uninduced (–) or induced (+) for Rad3 (Rad3) or Rad3.D2249E (Rad3-KD) expression from the attenuated *nmt1* promoter of pREP41. Tagged Rad3 has a mol. wt of ~277 kDa. The experiment was also performed on cells irradiated with 100 Gy 30 min prior to extract preparation (++). IP pellets were assayed directly for kinase activity. The products were separated by SDS–gel electrophoresis and transferred to a nylon membrane. In the left panel, ^{32}P incorporation was detected by autoradiography. In the right panel, the Rad3 protein was detected by Western analysis using anti-HA monoclonal antibody. Bands below the major Rad3 band are presumably degradation products still containing the N-terminus, since they are absent in the uninduced control lanes. The kinase activity associated with Rad3 appears to phosphorylate Rad3 and possibly some Rad3 degradation products, and this activity was significantly but not completely reduced when IPs were prepared from extracts expressing kinase-dead Rad3.

cells overexpressing a 'kinase-dead' D2249E version of Rad3, the associated kinase activity precipitated by HA-specific antibody is reduced significantly (Figure 6). There are several possible explanations for this, the first being that the measured kinase activity solely reflects Rad3 activity. In this case, the residual activity seen with the kinase-dead Rad3 could reflect the fact that it is not unknown for the equivalent D to E mutation in other protein kinases to produce a biologically inert protein with residual *in vitro* biochemical activity. Since attempts to perform kinase assays following renaturation of Rad3 after electrophoresis and transfer to membranes have proved unsuccessful, we have addressed this by repeating the IP kinase analysis with a second construct carrying a more severe D2249A mutation. This gave similar results (not shown), suggesting that the residual activity may not be due to Rad3 itself.

It is likely, therefore, that the kinase activity we measure in HA immunoprecipitates is due to a combination of Rad3 and other associated or contaminating kinases, the

ratio of these being the difference between the Rad3 and Rad3.KD activities (Figure 6). One final explanation that we cannot exclude currently is that the kinase activity which phosphorylates Rad3 may be due entirely to associated proteins. In this case, such proteins must interact less effectively with the D2249E mutant protein, or themselves be stimulated by Rad3 kinase activity. A candidate for such an activity would be Cds1 (Murakami and Okayama, 1995), the *S.pombe* homologue of *S.cerevisiae* Rad53p (Sanchez *et al.*, 1996; Sun *et al.*, 1996). However, repeating the IP kinase assay in a *cds1* deletion mutant strain does not appear to affect activity (not shown). The DNA damage-specific Chk1 kinase is also a possible candidate (Ford *et al.*, 1994; Walworth and Bernards, 1996). However, we have a reasonable assay for Chk1 activity (H.Lindsay and A.Carr, unpublished) and are unable to detect this in the relevant immune precipitates.

Discussion

Much of the work in mammalian cells on DNA structure-dependent checkpoints has focused on the checkpoint controlling progression from G_1 phase into the cell cycle and the relationship between DNA damage and apoptosis (see Hartwell and Kastan, 1994). Only a single genetic disorder defective in a G_2 checkpoint control, namely A-T, has yet been identified (Beamish and Levin, 1994). The gene defective in A-T patients has been cloned and is structurally related to a number of proteins identified by yeast genetics. The *ATM* gene is most closely related to *TEL1*, which is involved in maintaining telomere length (Greenwell *et al.*, 1995; Morrow *et al.*, 1995). However, *ATM* function appears to be more closely related to that of the *ESR1(MEC1)* and *rad3* genes. Following the initial discovery of the *ATM* gene and its sequence relationship to *TEL1* and to *rad3/ESR1(MEC1)*, it was not clear whether, as in many cases in yeast, the gene had duplicated and diverged, or whether the two yeast proteins defined conserved sub-families of closely related genes. The identification of a human gene, *ATR*, which is more closely related to *rad3/ESR1(MEC1)/mei-41* and which can complement some of the phenotypes associated with mutation in the *ESR1/MEC1* gene, helps to define two structurally distinct checkpoint-related sub-families of protein/lipid kinases that are conserved throughout eukaryotic evolution. An ORF identical to *ATR* has been reported recently (Cimprich *et al.*, 1996) as a sequence structurally related to FRAP proteins.

Although the proteins in these two sub-families may have some overlapping functions, they probably control different processes. For example, the *rad3* sub-family in yeast control all the G_1 and G_2 DNA damage checkpoints in response to both UV and ionizing radiation, and the S phase checkpoint which prevents mitosis following inhibition of replication (Al-Khodairy and Carr, 1992; Allen *et al.*, 1994; Weinert *et al.*, 1994). In contrast, A-T cells have abnormal responses to a narrow range of DNA-damaging agents, including ionizing radiation, bleomycin and neocarzinostatin, which produce strand breaks in DNA as a consequence of radical attack. The response to UV and most chemical carcinogens is normal, as is the response to the inhibition of DNA synthesis. It is possible that some or all of the remaining DNA damage checkpoints and the S phase checkpoint are controlled by *ATR*.

In this work, we also demonstrate that moderate overexpression of a kinase-defective *rad3* mutant in *S.pombe* causes a dominant-negative radiation-sensitive phenotype, suggesting that Rad3 is acting as a member of a protein complex whose integrity is necessary for checkpoint function. This is consistent with the genetic data derived from our work and the work of Subramani's group (Jimenez *et al.*, 1992; Seaton *et al.*, 1992) that indicates that intragenic complementation can occur between distinct non-functional *rad3* alleles. Furthermore, we have demonstrated that Rad3 will co-immunoprecipitate with Rad3 (by co-expressing two separate tagged *rad3* constructs ~10- to 20-fold). While there are significant problems associated with immunoprecipitation experiments involving overexpressed proteins, our control experiments demonstrate that Rad3 co-immunoprecipitation is at least partially specific. Together, these unrelated approaches (on the one hand genetic and on the other hand biochemical) provide good evidence that Rad3 exists in a complex containing two or more Rad3 proteins *in vivo*. Deletion mutants of *rad1*, *rad9*, *rad17*, *rad26* and *hus1* all have phenotypes that are essentially indistinguishable from *rad3.d* (reviewed in Carr and Hoekstra, 1995), and it is therefore possible that, together with Rad3, these proteins form a 'guardian complex' which underlies an essential step in the checkpoint pathways.

There are significant differences between the organization of the checkpoints in *S.pombe* and *S.cerevisiae* which can be informative. For example, the homologues of *rad1* and *rad17* in budding yeast (*RAD17* and *RAD24* respectively, note the potential confusion in nomenclature as *rad17* is not the homologue of *RAD17*) are required for the DNA damage checkpoint but not the S–M checkpoint (Weinert and Hartwell, 1993), whereas their *S.pombe* counterparts are required for both checkpoints. Similarly, the *RAD53* gene in *S.cerevisiae* is required for both these checkpoints in this organism (Allen *et al.*, 1994), while the *S.pombe* homologue, *cds1*, is involved primarily only in the S phase arrest response and not the DNA damage checkpoint (Murakami and Okayama, 1995). Uniquely so far, Rad3 and its homologue Esr1(Mec1)p is the only checkpoint protein required for all the DNA structure-dependent checkpoints in both organisms, placing it central to these pathways.

Esr1(Mec1)p has been implicated in the phosphorylation of Rad53p in *S.cerevisiae* (Sanchez *et al.*, 1996; Sun *et al.*, 1996) and Rad3 has been shown to be required for the phosphorylation of Chk1 following DNA damage in *S.pombe* (Walworth and Bernards, 1996). Thus it seems that the central function of Rad3/Esr1(Mec1)p is at an early step, possibly monitoring the structure of the DNA by interacting directly with particular DNA or DNA–protein structures common to replication and repair pathways. Using the paradigm of DNA-dependent protein kinase, where the Ku70–Ku80 dimer recognizes DNA ends and recruits the DNA-PKcs catalytic subunit and stimulates its activity, it is tempting to speculate that Rad3 may interact with its own recognition subunits, possibly some of the checkpoint Rad proteins, in order to generate a signal for cell cycle arrest. In this model, different checkpoint Rad proteins may mediate specific interactions

with repair and replication structures and direct the activity of Rad3 appropriately.

Models such as these can be refined once we know which proteins interact with Rad3. The identification of the Rad3–Rad3 complex reported here is the first step in identifying the protein components of any 'guardian complex'. Similarly, the substrates of Rad3 will be important in understanding the mechanisms by which Rad3 and its human homologues establish the checkpoint signal. The identification of a potential protein kinase activity for Rad3 suggests that, like the DNA-PKcs protein, it can act as a protein kinase. Delineation of the interacting proteins and the potential kinase substrates of Rad3 by genetic and biochemical analysis is therefore the aim of current work in our laboratories.

Materials and methods

Strains, plasmids and media

Standard genetic techniques, growth conditions and media for *S.pombe* are described in Gutz *et al.* (1974). The *S.pombe* strain sp011 (*ura4.D18, leu1.32 ade6.704 h*$^-$) has been described previously (Murray *et al.*, 1992). Plasmid pSUB41 was a gift from S.Subramani (Seaton *et al.*, 1992). The *S.cerevisiae* strain NR110ABU is the diploid: *Mat***a** *leu2-1 his4-290 can1 ura3 cyh2 ade6 ade2 esr1-1. Mat***a** *leu2-27 his4 trp1 met2 ade2 esr1-1.*

Radiation and S–M checkpoint analysis

Cultures of synchronous cells were prepared on a 7.5–30% lactose gradient as described in Barbet and Carr (1993). G_2 cells were recovered from the top of the gradient and inoculated into fresh YES media. For radiation checkpoint analysis, samples were subjected to either 0, 100 or 250 Gy ionizing radiation using a Gammacell 1000 ^{137}Cs source (12 Gy/min) and incubated at 29°C. Aliquots were removed at 15 min intervals and fixed in methanol for estimation of passage through mitosis by 4′,6′-diamidino-2-phenylindole (DAPI) and calcofluor staining (Al-Khodairy *et al.*, 1994). For S–M checkpoint analysis, samples were incubated either with or without hydroxyurea, and samples removed at 30 min intervals for DAPI and calcofluor staining.

For *S.cerevisiae* complementation studies, NR110ABU cells growing on SC-URA plates containing 2% glucose were streaked onto SC-URA plates containing 0.005% casamino acids and 2% galactose. Plates were grown at room temperature for 8–12 h prior to exposure to 150 J/m^2 of UV light in a UV Stratalinker. Plates were incubated at room temperature and scored for growth after several days.

Cloning of S.pombe rad3

A 4.0 kb *Kpn*I fragment was excised from pSUB41 (Seaton *et al.*, 1992) and sequenced in both directions to obtain the 5′ *rad3* sequence. The 3′ clone was identified from a genomic library (Barbet *et al.*, 1992) by colony hybridization using a 1 kb 3′ probe derived from the published *rad3* sequence (Seaton *et al.*, 1992), and sequenced in both directions. In this way, the sequence of the entire *rad3* gene was assembled.

'Kinase-dead' and deletion mutants of rad3

Two deletion mutant constructs of *rad3* were created. The first, an N-terminal deletion mutant, replaced the first 1476 amino acids (from an *Nde*I site introduced at the ATG to a *Bam*HI site corresponding to amino acid position 1476) with the *ura4*$^+$ marker gene. The second, a C-terminal deletion mutant, replaced 794 amino acids between amino acids 1477 and 2271 (including the kinase motif domain) with the *ura4*$^+$ gene. These mutant constructs were created using the methodology described in Barbet *et al.* (1992). Linear fragments of these were used separately to transform sp011 to uracil prototropy, and single copy integration at the *rad3* locus was checked by Southern blotting. To create the site-specific kinase-dead mutations, a C-terminal 3.01 kb *Bam*HI–*Sal*I fragment of *rad3* was mutated with either A (GTTTTCGCCATGGCGCGCTCCCAAACCCAA), B (TTCATCAAACAATATCTTTTCGCCATGGCG) or C (CAAAAAGACAGTTGAATTCGACATGGATAG) in order to introduce either the D2230A, N2235K or D2249E mutations into the kinase domain. Analogous changes have been used previously in the analysis of PI3 kinase *VPS34* of *S.cerevisiae* (Schu *et al.*, 1993). These fragments were then used to transform the *rad3.d* deletion mutant corresponding to the C-terminal deletion, and gene replacements were selected by their ability to grow on fluoro-orotic acid (FOA)-containing media (Grimm *et al.*, 1988). All strains were checked by Southern blotting. Full-length expression constructs of *rad3.D2249E* were created in pREP1 and pREP41 (Maundrell, 1990; Basi *et al.*, 1993) by standard subcloning following introduction of an *Nde*I site at the ATG and mutation of three internal *Nde*I sites.

Cloning of ATR

To isolate an appropriate probe for identifying cDNAs corresponding to a human *rad3* homologue, degenerate oligonucleotides were designed against the amino acids LGLGDRH (5′ oligo; oDH18) and HVDF[D/N]C (3′ oligo; oDH16) of Rad3/Esr1p. Inosine was incorporated at positions of 4-fold degeneracy, and primers were tailed with *Bam*HI (oDH18) and *Eco*RI (oDH16) to facilitate cloning. DNA sequence analysis of the ~100 bp PCR product obtained from amplification of peripheral blood leukocyte cDNA demonstrated significant similarity to *MEC1/rad3*. This sequence was used to synthesize a non-degenerate primer (oDH23; GACGCAGAATTCACCAGTCAAAGAATCAAAGAG) for PCR with an additional degenerate primer (oDH17) designed against the amino acid sequence KFPP[I/V][L/F]Y[Q/E]WF of Rad3/Esr1p. The 174 bp product of this reaction was used directly to screen a macrophage cDNA library. Four positive clones were isolated (the largest was ~3 kb).

In parallel, database searches with full-length *S.pombe rad3* identified a human cDNA clone, HSAAADPDG, as a potential homologue of *rad3*, if a single frameshift was allowed for in the 233 bp sequence. The entire clone (1.6 kb) was sequenced and lies within the cDNA clones identified by degenerate PCR and library screens. To identify the whole gene, RACE–PCR experiments were performed on cDNA derived from placental and thymus mRNA using the instructions provided with a Clontech Marathon Kit. Gene-specific primers were derived from the cDNA clones. From these experiments, a 8202 bp cDNA sequence was assembled with an internal ORF of 2644 amino acids, a 79 bp 5′ non-coding region, a 188 bp 3′ non-coding region and a poly(A)$^+$ tail. Parts of the sequence were determined solely by PCR. To avoid errors, clones from a minimum of three independent PCR reactions were sequenced in both directions.

To detect ATR protein following expression in the yeasts, ATR polyclonal antibody #5018 (Keegan *et al.*, 1996) at a dilution of 1:500 was added to Western blots for 2 h at room temperature. A goat anti-rabbit secondary antibody at 1:10 000 was added for 1 h and visualized by chemiluminiscence.

Mapping ATR

We mapped the *ATR* gene to chromosome 3 by a combination of FISH- and PCR-based assays. FISH analysis using a cDNA clone identified the *ATR* gene on chromosome 3, at approximately position q22–23. PCR analysis also identified *ATR* on chromosome 3. Two primers (oATR23: GACGCAGAATTCACCAGTCAAAGAATCAAAGAG and oATR26: TGGTTTCTGAGAACATTCCCTGA) which amplify a 257 bp fragment of the *ATR* gene were used on DNA derived from human–rodent somatic cell hybrids containing various human chromosome panels available from the NIGMS Human Genetic Mutant Cell Repository (Drwinga *et al.*, 1993). PCR with the same primers was used to sub-localize *ATR* to a specific region on chromosome 3. The templates for these amplifications consisted of DNA samples from patients with truncations along chromosome 3 (Leach *et al.*, 1994).

IP and kinase assays with Rad3

The *S.pombe rad3* and human *ATR* genes were cloned into pREP41 expression vector for complementation studies. To tag the proteins, versions of these vectors containing in-frame N-terminal tag sequences, either a double myc or a triple HA tag, were used (Griffiths *et al.*, 1995). Tagged proteins were expressed by growing in media without thiamine (Maundrell, 1990). Yeast cells were lysed in lysis buffer [25 mM Tris–Cl pH 7.5, 60 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1% Triton X-100, 50 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol (DTT)] by the addition of glass beads followed by treatment in a dismembrinator for 2 min. For IPs, 300 μg of total protein extract were incubated on ice with the appropriate antibody for 30 min and the immune complexes precipitated by mixing with protein G beads for a further 30 min at 4°C. For kinase assays, the immune complexes were washed four times with lysis buffer, once with kinase buffer (25 mM HEPES pH 7.7; 50 mM KCl; 10 mM $MgCl_2$; 0.1% NP-40; 2% glycerol;

1 mM DTT), and incubated in kinase buffer with 10 μM ATP (50 Ci/mmol) for 15 min at 30°C. We determined that the incorporation of ^{32}P into Rad3 reached a maximum between 5 and 10 min at this temperature. The reactions were stopped with 20 μl of 2× SDS sample buffer prior to separation on 6% polyacrylamide gels. Rad3 IPs contained several phosphorylated products, including one which co-migrated with the Rad3 protein itself, based on Western analysis.

Acknowledgements

We thank Sue Naylor for DNA samples of chromosome 3 derivatives used to map *ATR* and Chris Allen and Christi Woods for help with DNA sequence analysis.

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Received on July 15, 1996; revised on September 11, 1996