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Expression of the *Saccharomyces cerevisiae RAD50* gene during meiosis: steady-state transcript levels rise and fall while steady-state protein levels remain constant

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Abstract. In *Saccharomyces cerevisiae*, the *RAD50* gene is required for repair of X-ray and MMS-induced DNA damage during vegetative growth, and for synaptonemal complex formation and genetic recombination during meiosis. We show below that the *RAD50* gene encodes major and minor transcripts of 4.2 and 4.6 kb in length which differ primarily at their 5′ ends. Steady-state levels of both *RAD50* transcripts increase coordinately during meiosis, reaching maximal levels midway through meiotic prophase, about 3 or 4 h after transfer of cells to sporulation medium. The 5′ ends of the major *RAD50* transcript in both meiotic and vegetative cells map to the same cluster of sites approximately 20 bp upstream of the amino-terminal ATG of the *RAD50* coding sequence. We conclude that the increased *RAD50* transcript level observed during meiosis does not reflect utilization of a new promoter. In contrast, steady-state levels of Rad50 protein do not increase during meiosis. Thus, changes in *RAD50* transcript levels are not neccessarily accompanied by commensurate changes in Rad50 protein levels. Possible explanations are considered.

Key words: *RAD50* – Meiosis – Recombinational repair – Transcription

Introduction

In *Saccharomyces cerevisiae RAD50* is one of several recombinational repair genes that are required for mitotic repair of X-ray- and MMS-induced DNA damage and for meiotic recombination (Game and Mortimer 1974; Game et al. 1980; Malone and Esposito 1981). *RAD50* is also required during meiosis for formation of the synaptonemal complex, possibly as a consequence of its role in recombination (Farnet et al. 1988; Alani et al. 1990). Mutations in each of two other genes, *XRS2* and *MRE11*, result in phenotypes indistinguishable from those of *rad50* mutants (Ivanov et al. 1992; Ajimura et al. 1993).

RAD50 is distinct from other well-characterized *RAD* genes in the recombinational repair group (*RAD51, 52, 54,* and *57*) with respect to its roles in meiosis and in DNA repair (Haynes and Kunz 1981; Resnick 1987). During meiosis, *RAD50* acts at a very early step in recombination: *rad50-1* and *rad50Δ* diploids are distinct from *rad51, rad52,* and *rad57* mutants in their failure to produce any intermediates or products of meiotic recombination (Morrison and Hastings 1979; Borts et al. 1986; Resnick et al. 1986; Cao et al. 1990; Shinohara et al. 1992), and *rad50-1* is genetically epistaticaly to *rad52-1* for meiotic viability under certain conditions (Malone 1983). Moreover, *rad50Δ* and *rad50-1* mutants fail to make synaptonemal complex, while *rad52-1* mutants make synaptonemal complex (Resnick et al. 1986; Alani et al. 1990). During mitotic growth, *rad50-1* and *rad50Δ* mutants are capable of homologous linear plasmid integration (Alani et al. 1990), show wild-type or elevated levels of spontaneous gene conversion and reciprocal recombination (Saeki et al. 1980; Malone 1983; Gottlieb et al. 1989), and are capable of, though not fully wild-type at, mating-type switching (Malone 1983; Sugawara and Haber 1992; A. McKee and N. Kleckner, unpublished data). In contrast, *rad51-1, rad51Δ, rad52-1,* and *rad54-1* mutants exhibit severe defects in these processes (Prakash et al. 1980; Saeki et al. 1980; Malone and Esposito 1981; Orr-Weaver et al. 1981; Weiffenbach and Haber 1981; Game 1983; Shinohara et al. 1992). The relationship between the mitotic and meiotic roles of *RAD50* is of particular interest in light of these phenotypic distinctions between *rad50* and other recombinational repair mutants. Intriguingly, both Rad50 and Rad51 proteins exhibit ATP-dependent DNA-binding activities in vitro (W. Raymond and N. Kleckner, submitted; Shinohara et al. 1992).

In this study, aspects of *RAD50* gene regulation have been examined in order to identify differences in *RAD50*

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expression between the mitotic cell cycle and meiosis. Gene regulation normally acts at transcriptional and translational levels to control gene function temporally or developmentally. As initial steps toward understanding the regulation of the *RAD50* gene, steady-state levels of *RAD50* transcripts and Rad50 protein were determined in cells growing either mitotically or meiotically. A ten-fold increase in steady-state levels of the major *RAD50* transcript during meiosis is not accompanied by a change in the sites of transcription initiation or by an increase in steady-state Rad50 protein levels.

Materials and methods

Yeast strains. All *S. cerevisiae* strains used were derivatives of the homothallic prototrophic diploid strain SK-1 (kindly provided by Robert Roth; Kane and Roth 1974). NKY147 (**a**/α *HO/HO lys2/lys2*) was selected on α-aminoadipate media (Chattoo et al. 1979) as a spontaneous *lys2* mutant of a spore of SK-1. NKY278 (**a**/α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3*) was constructed as follows. The *HO* gene of NKY147 was replaced by the *Hin*dIII *ho::LYS2* fragment of pNKY14 (described below), and tetrad dissection of the resultant **a**/α *ho::LYS2/HO lys2/lys2* strain (NKY272) produced the heterothallic haploid strains NKY210 (α *ho::LYS2 lys2*) and NKY212 (**a** *ho::LYS2 lys2*). NKY273 (α *ho::LYS2 lys2 ura3*) was obtained as a spontaneous mutant of NKY210 showing resistance to 5-fluoro-orotic acid (FOA) (L. Cao and N. Kleckner, unpublished data; Boeke et al. 1984). NKY273 and NKY212 were crossed, and sporulation of the resultant diploid produced NKY274 (α *ho::LYS2 lys2 ura3*) and NKY276 (**a** *ho::LYS2 lys2 ura3*). NKY278 is a diploid produced by mating NKY274 with NKY276. NKY551 (**a**/α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 rad50Δ::hisG/rad50Δ::hisG*) has been previously described (Alani et al. 1990).

Bacterial strains. E. coli strains JM101 (*lac thi supE/F traD36 proA proB lacI^{Q} lacZM15*) and MM294 (F^{-} *endA hsdR supE44 thiA*) were used as hosts for all plasmid manipulations. JM101 was also used as host for the preparation of single-stranded DNA from plasmids bearing M13 replication origins. NK5830 (*recA56 arg$_{am}$ Δlac-pro$_{XIII}$ NalA rif^{r}*/F′ lacI^{Q} *L8 pro*) was used as host for the production of Tn*10* transposase-Rad50 fusion proteins.

Media and genetic techniques. Yeast strains were constructed using standard methods for mating, sporulation, and tetrad analysis (Mortimer and Hawthorne 1969; Sherman et al. 1983). YPD (Sherman et al. 1983), YPA (YPD with 1% potassium acetate instead of 2% glucose) and sporulation (SPM; Kassir and Simchen 1976) liquid media were employed. *E. coli* strains were grown in L broth (LB; Miller 1972) supplemented when appropriate with ampicillin (Sigma) to 100 μg/ml.

Sporulation. Flasks used in each sporulation protocol were rinsed thoroughly with distilled H_2O (dH_2O) prior to sterilization to remove traces of soap that could interfere with sporulation efficiency. An overnight YPD culture made from a fresh single colony of the specified strain was used to inoculate 50 ml of liquid medium (YPA or YPD) in a 500 ml Erlenmeyer flask to an initial OD_{600} of 0.05. The cultures were grown at 30° C with vigorous shaking (250 rpm) to an undiluted OD_{600} of 2; 1:10 dilutions of such cultures have OD_{600} readings of 0.6 to 0.8. Cells were then washed once with 50 ml dH_2O, resuspended to homogeneity in several ml of SPM, brought to a final volume of 250 ml in prewarmed (30° C) SPM in a 2-l flask, and shaken at 250 rpm at 30° C.

DNA techniques. Restriction enzymes, linkers, Klenow polymerase, T4 DNA ligase, T4 polynucleotide kinase, and *Hin*dIII-digested lambda DNA were obtained from New England Biolabs and were used according to the manufacturer's instructions. Plasmid DNA preparation and constructions were performed as previously described (Maniatis et al. 1982). The preparation of single-stranded DNA derived from plasmids bearing M13 origins and dideoxy DNA sequencing of such single-stranded DNA were as previously described (Messing 1983). Yeast strains were transformed with plasmids as previously described, using lithium acetate (Ito et al. 1983).

Plasmids. Plasmids pSP64, pSP65 (Melton et al. 1984), pNKY74, pNKY101, pNKY1070 (Alani et al. 1989), and YCp50 (Johnston and Davis 1984) have been described previously. pNKY2042 was constructed by ligating the 8.7 kb *Bam*HI *RAD50* fragment from pNKY74 into the *Bam*HI site of YEp24 (E. Alani and N. Kleckner, unpublished data; Botstein et al. 1979).

pNKY14 (pACYC::*ho::LYS2*) is a plasmid containing a disrupted *HO* gene that was derived from plasmids pNKY7 and pNKY12. pNKY7 was constructed by ligating the 2.5 kb *Hin*dIII fragment of YCp50::*HO* (pHO-c12; Russell et al. 1986), containing the *HO* gene, into the *Hin*dIII site of pACYC184 (Chang and Cohen 1978). pNKY12 is a derivative of pSL42-2 (a gift of S. Carl Falco, which consists of the 4.8 kb *Eco*RI–*Hin*dIII fragment of *LYS2* (Barnes and Thorner 1986) ligated into the *Eco*RI–*Hin*dIII backbone of YIp5 (Struhl et al. 1979)). pNKY12 contains *Pst*I linker(s) (5′-GCTGCAGC-3′) at the *Eco*RI and *Hin*dIII sites flanking the *LYS2* gene of pSL42-2. The small *Pst*I fragment internal to the *HO* gene of pNKY7 was replaced by the 4.8 kb *LYS2 Pst*I fragment of pNKY12 to produce pNKY14.

Plasmids pNKY75 and pNKY76 were derived from pNK852 (Morisato and Kleckner 1987), a plasmid used to overproduce Tn*10* transposase protein via the *tac* promoter (Amann et al. 1983), and pNKY1002, a plasmid made by ligating the 4 kb *Hin*dIII–*Sal*I *RAD50* fragment from pSG205 (a gift of S. Gottlieb and R.E. Esposito; Alani et al. 1989) into the *Hin*dIII–*Sal*I backbone of YCp50. The *Ava*I site of pNK852 was filled in with Klenow and ligated with *Sal*I linkers (5′-GGTCGACC-3′) to produce pNK1279. The 2 kb *Pvu*II–*Sal*I *RAD50*

fragment of pNKY1002 was ligated into the *Stu*I–*Sal*I backbone of pNK1279 to produce pNKY75. The *Stu*I–*Sal*I *RAD50* fragment of pNKY1002 was ligated into the *Stu*I–*Sal*I backbone of pNK1279 to produce pNKY76.

Ten *RAD50* and one *SPO13* SP6-probe template plasmids were constructed and used as follows. pNKY36 (probe 10): The 0.8 kb *Eco*RI–*Sal*I fragment of pSG205 was ligated into the *Eco*RI–*Sal*I backbone of pSP65 to produce pNKY36. After digestion with *Sal*I, pNKY36 was used as a template to produce an RNA probe extending from *Eco*RI (5′) to *Sal*I (3′) sites within the *RAD50* 3′ flanking sequences. pNKY66 (probe 9): pNKY36 was digested with *Eco*RI and *Hpa*I and filled in with Klenow. The 3.3 kb backbone was gel-purified and ligated to produce pNKY66. After digestion with *Sal*I, pNKY66 was used as a template to produce an RNA probe extending from *Hpa*I (5′) to *Sal*I (3′) sites within the *RAD50* 3′ flanking sequences. pNKY32 (probe 8): The 4 kb *Hin*dIII–*Sal*I *RAD50* fragment of pNKY1002 was ligated into the *Hin*dIII–*Sal*I backbone of pSP64 to produce pNKY32. After digestion with *Sal*I or *Pvu*II, pNKY32 was used as a template to produce an RNA probe extending from the *Hin*dIII (5′) to the *Sal*I (3′) or *Pvu*II (3′) site, respectively, within *RAD50*. pNKY68 (probe 7): pNKY36 was digested with *Sal*I and *Hpa*I and filled in with Klenow. The 3.45 kb backbone was gel-purified and ligated to yield pNKY68. After digestion with *Hin*dIII, pNKY68 was used as a template to produce an RNA probe extending from *Eco*RI (5′) to *Hpa*I (3′) sites within the *RAD50* 3′ flanking sequences. pNKY33 (probe 6): The 4 kb *Hin*dIII-*Sal*I *RAD50* fragment of pNKY1002 was ligated into the *Hin*dIII-*Sal*I backbone of pSP65 to produce pNKY33. After digestion with *Pvu*II, pNKY33 was used as a template to produce an RNA probe extending from *Sal*I (5′) of *RAD50* to *Pvu*II (3′). pNKY34 (probe 5): pNKY33 was digested with *Hin*cII and religated to produce pNKY34. After digestion with *Cla*I, pNKY34 was used as a template to produce an RNA probe extending from *Hin*cII (5′) of *RAD50* to *Cla*I (3′). pNKY37 (probe 4): pNKY37 is a derivative of pNKY16. pNKY16 was made by ligating the 4 kb *Hin*dIII–*Sal*I (filled-in) *RAD50* fragment from pNKY1002 into the *Hin*dIII–*Cla*I (filled-in) backbone of pBR333 (Way et al. 1984). The junction of the filled-in *Cla*I and the filled-in *Sal*I sites regenerates a *Sal*I site. The 2 kb *Pvu*II–*Hin*dIII fragment of pNKY16 was ligated into the *Sma*I–*Hin*dIII backbone of pSP65 to produce pNKY37. After digestion with *Xba*I, pNKY37 was used as a template to produce an RNA probe extending from *Pvu*II (5′) of *RAD50* to *Xba*I (3′). pNKY35 (probe 3): pNKY33 was digested with *Bgl*II and *Bam*HI and religated to produce pNKY35. After digestion with *Xba*I, pNKY35 was used as a template to produce an RNA probe extending from *Bgl*II (5′) of *RAD50* to *Xba*I (3′). pNKY63 (probe 2): pNKY33 was digested with *Bst*EII, filled in with Klenow, digested with *Sma*I, and religated to delete *RAD50* sequences from *Sma*I (originally a *Sal*I site) to *Bst*EII and produce pNKY63. After digestion with *Hin*dIII, pNKY63 was used as a template to produce an RNA probe extending from *Bst*EII (5′) of *RAD50* to *Hin*dIII (3′). pNKY179 (probe 1): The 2.5 kb *Hin*dIII–*Eco*RI fragment from pNKY101, containing sequences 5′ of the *RAD50* gene, was inserted into the *Hin*dIII–*Eco*RI backbone of pSP64 to yield pNKY179. After digestion with *Bgl*II, pNKY179 was used as a template to produce an RNA probe extending from *Hin*dIII (5′) to *Bgl*II (3′). pNKY44: The 0.8 kb *Xba*I–*Eco*RI fragment of p(SPO13)8 (Wang et al. 1987) was ligated into the *Xba*I–*Eco*RI backbone of pSP64 to produce pNKY44. After digestion with *Bst*EII, pNKY44 was used as a template to produce an RNA probe extending from *Xba*I (5′) to *Bst*EII (3′) within *SPO13*.

RNA techniques. Total yeast RNA was prepared essentially as previously described (Carlson and Botstein 1982), using diethylpyrocarbonate (Sigma)-treated solutions. 10 ml of vegetative mid-log or of sporulating cultures was pelleted, washed with 1 ml dH_2O, and resuspended in 0.2 ml breaking buffer (0.5 M NaCl, 0.2 M TRIS-HCl, pH 7.5, 0.01 M EDTA, pH 8, 1% SDS) in an Eppendorf tube. 0.4 g acid-washed glass beads (Thomas, 0.45 mm diameter) and 0.2 ml of a phenol – chloroform – H_2O-saturated isoamyl alcohol solution (25:24:1, v:v:v) were added, and the mixture was vortexed at top speed for 2 min. An aliquot (0.3 ml) of breaking buffer without SDS and 0.3 ml of phenol solution were added, the mixture was vortexed briefly, and the phases were separated by centrifugation. The 0.5 ml aqueous layer was reextracted with phenol solution, and the final aqueous layer was precipitated with ethanol. The pellet was washed once with 70% ethanol, resuspended in 100 µl dH_2O, and stored at −70° C. The concentration of RNA in such preparations ranged from 1.5 to 3.0 mg/ml.

Poly(A)$^+$ RNA was prepared essentially as described previously (Winston et al. 1984). RNA from 1 l of vegetative mid-log or of sporulating cells was loaded onto an oligo-dT cellulose (0.25 g, Type 3, Collaborative Research) column equilibrated with loading buffer (0.5 M LiCl, 0.01 M TRIS-HCl, pH 7.4, 0.1% lithium dodecylsulfate) at 4° C. Unbound material was reapplied to the column 5 times, and the column was then washed with loading buffer until the effluent OD_{260} was less than 0.02. Poly(A)$^+$ RNA was eluted with 4 ml of 60° C elution buffer (0.1 M TRIS-HCl, pH 7.4, 0.05% SDS, 0.01 M EDTA) and precipitated with ethanol.

For RNA blot analysis, RNA was electrophoresed on 1% agarose gels as previously described (Melton et al. 1984), except that the gel and electrophoresis buffer contained 6% formaldehyde. ^{32}P-end-labeled *Hin*dIII-digested lambda DNA fragments were run as molecular weight standards. Gels were incubated in 0.05 M NaOH for 30 min and neutralized in 0.1 M TRIS-HCl, pH 7.5 for 30 min prior to carrying out a standard capillary blot procedure in 1X SSC (Maniatis et al. 1982) to transfer the RNA to GeneScreen membrane (New England Nuclear). RNA was crosslinked to the membrane by UV irradiation (Church and Gilbert 1984). Fresh ^{32}P-labeled SP6 probes were prepared using SP6 polymerase (Promega Biotech) according to the manufacturer's in-

structions and were hybridized to the filters at 55° C (Melton et al. 1984). For blot analysis of meiotic time course RNAs, 4 µl from each 100 µl RNA sample was used, regardless of the concentration of RNA in each sample. Since the RNA preparations were made quantitatively, equal volumes represent the same numbers of cells. Duplicate RNA preparations were made and analyzed at each time point in order to reveal any variations due to variations in technique. A 4 µl aliquot of a 100 µl sample typically contained 6–12 µg RNA.

Primer extension analysis was performed using the oligonucleotide 5′-GT TTT ACC TGA ACC ATT CAT-3′ ($oATG_2$), which is homologous to bp 103–122 and includes the second ATG of the predicted *RAD50* open reading frame. The primer, prepared by the Harvard Biological Laboratories Microchemistry Facility, was 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]dATP, and 1 pmol was hybridized with yeast RNA overnight at 37° C in 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, 0.4 M NaCl, 0.2% SDS. After ethanol precipitation, primer extension reactions were performed using reverse transcriptase (Boehringer-Mannheim; Treisman et al. 1982). The reaction mixtures were run on 8% acrylamide sequencing gels in parallel with dideoxy DNA sequencing reactions initated from $oATG_2$, using single-stranded DNA from pNKY74 as template. Primer extension products separated in the sequencing gels were visualized by autoradiography.

S1 nuclease mapping was performed essentially as described by Nasmyth (Nasmyth 1983). ^{32}P-end-labeled $oATG_2$ was extended with Klenow polymerase in the presence of [α-^{32}P]dATP using pNKY74 single-stranded DNA as template. The partially double-stranded DNA was digested with *Hin*dIII and the resultant 200 nucleotide single-stranded radiolabeled probe was purified by electroelution from a 6% polyacrylamide sequencing gel. The probe and each RNA sample were coprecipitated and then hybridized overnight in a humid chamber at 65° C in 0.78 M NaCl, 0.1 M PIPES, pH 6.9, and 0.01 M EDTA, pH 8. Following digestion with S1 nuclease (BRL), the samples were run on 6% polyacrylamide sequencing gels, and S1 nuclease-protected products were visualized by autoradiography.

Preparation of antibodies against a Tn10 transposase-Rad50 fusion protein. Tn*10* transposase-Rad50 fusion proteins were produced in *E. coli* strain NK5830 as previously described for Tn*10* transposase protein (Morisato and Kleckner 1987). Cells from a 200 ml culture of NK5830 transformed with either pNKY75 or pNKY76 were concentrated into 6.7 ml of 50 mM TRIS-HCl, pH 8.0, 25% (w:v) sucrose after a 40–60 min induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma). 300 µl aliquots of the concentrated cell suspensions were frozen at −70° C.

Frozen cells were rapidly thawed, mixed with an equal volume of 2× concentrated sample buffer (Laemmli 1970), and boiled for 5 min immediately prior to electrophoresis on preparative 5% polyacrylamide-SDS gels (Laemmli 1970). The abundant Tn*10* transposase-Rad50 fusion protein was visualized by staining the gel with Coomassie blue, and the stained, denatured protein was purified by electroelution from small gel slices. After extensive dialysis against 10 mM ammonium bicarbonate and subsequent lyophilization, the purified protein was resuspended in complete Freund's adjuvant and injected into the popliteal node of rabbits for antibody production (Sigel et al. 1983). For each injection, fusion protein purified from 30 ml of IPTG-induced *E. coli* cultures was used; this amounted to 0.5 mg of lyophilized solid (protein, SDS, and Coomassie blue) from the strain carrying pNKY75 and 1.3 mg of solid from the strain carrying pNKY76. Two weeks after the initial injection, subcutaneous injections of the same amount of protein were performed. Four weeks after the initial injection, blood was collected. Antiserum 891 was made against the fusion protein produced from pNKY76, and antisera 893 and 894 were made against the fusion protein produced from pNKY75. 894 preimmune serum was from blood withdrawn prior to inoculation from the rabbit that produced antiserum 894.

Yeast protein preparations. Total yeast protein was prepared according to Katz and Solomon (1988), with glass bead lysis of yeast cells in phosphate-buffered saline containing freshly added phenylmethylsulfonyl flouride (2 mM), leupeptin (1 µg/ml), pepstatin A (1 µg/ml), tosyl phenylalanine chloromethyl ketone (1 µg/ml), and aprotinin (0.1%, v:v). All protease inhibitors were from Sigma. Proteins were separated on 7% or 5% polyacrylamide gels containing 1% SDS (Laemmli 1970).

Protein blotting and immunostaining. Proteins were transferred electrophoretically from SDS polyacrylamide gels onto nitrocellulose (Towbin et al. 1979). Prestained molecular weight markers (BRL) run in the gel served as controls for complete transfer to the nitrocellulose. After protein transfer, the nitrocellulose was pretreated in TRIS-buffered saline (154 mM NaCl, 10 mM TRIS-HCl, pH 7.5; TBS) containing 10% (w:v) lowfat dry milk for 20 min at room temperature, washed in TBS containing 1% bovine serum albumin (Fraction V, US Biochemicals; TBS/BSA), and then incubated with shaking at room temperature for 2 h in a 1:500 dilution of 894 antiserum in TBS/BSA. The nitrocellulose was then washed twice for 1 min in TBS containing 0.1% (w:v) Tween 20 (Bio-Rad) (Batteiger et al. 1982), once in TBS/BSA, and then incubated for 1 h at room temperature with shaking in a 1:1000 dilution of affinity purified goat-anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad). The nitrocellulose was washed twice in TBS containing 0.1% Tween 20 and once in TBS, followed by development with hydrogen peroxide and HRP Color Development Reagent (Bio-Rad) according to the manufacturer's instructions.

Results

RNA blot analysis

The *RAD50* gene is present on a 4.1 kb *Hin*dIII–*Sal*I fragment (Kupiec and Simchen 1984; Huisman et al.

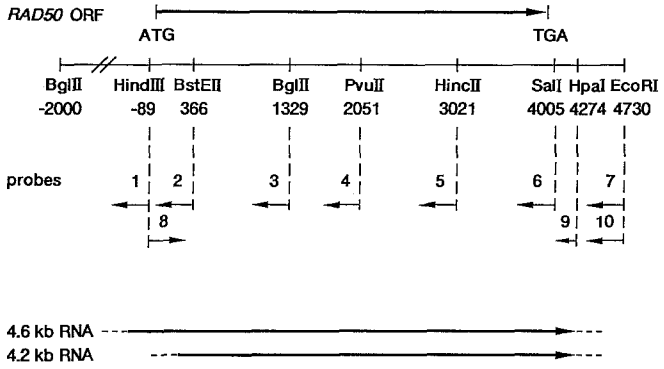

Fig. 1. Probes used in RNA blot analysis to identify *RAD50* transcripts. The 3936 nucleotide open reading frame (ORF) of the *RAD50* gene is contained on a 6.7 kb *Bgl*II–*Eco*RI fragment (Alani et al. 1989). Radioactive, single-stranded RNAs representing this region (probes 1–10) were used in RNA blot analysis to identify *RAD50* transcripts (Materials and methods). The 5′ end of each probe corresponds to the restriction site of the *RAD50* gene or flanking sequence nearest the SP6 promoter of the template plasmid; the 3′ ends are described in Materials and methods. Probes 2–6 and 9 hybridized to two transcripts, 4.6 and 4.2 kb in length. The orientation of these transcripts is that predicted by the *RAD50* ORF. Probe 1 hybridized to the 4.6 kb transcript but not the 4.2 kb transcript. Probe 8, of the opposite orientation, and probes 7 and 10, did not detectably hybridize to yeast RNA (data not shown)

1987) that contains an open reading frame of 3936 nucleotides (Alani et al. 1989). RNA blot analysis using six similarly oriented single-stranded probes covering the *RAD50* coding region identified two major *RAD50* transcripts in vegetative and meiotic cells, 4.2 and 4.6 kb in length (summarized in Fig. 1; Fig. 2A). Probes 2–6 hybridized to both *RAD50* transcripts, indicating that both transcripts span the entire *RAD50* open reading frame and are transcribed from the same template strand, in the orientation predicted by the open reading frame. Both *RAD50* transcripts are present in mitotically growing cells and throughout meiosis (Fig. 2A), and both transcripts are polyadenylated (Fig. 3A, lane 1 for the 4.2 kb *RAD50* RNA; data not shown for the 4.6 kb *RAD50* RNA). The two *RAD50* transcripts are the same length in strains of varying genetic background, including SK-1 (Fig. 2), AP-1 (Hopper et al. 1974), and RE821 (from R.E. Esposito; data not shown).

The 400 nucleotide length difference between the two *RAD50* transcripts can be accounted for primarily by distinct 5′ ends, with the 5′ end of the 4.6 kb transcript lying significantly upstream of the 5′ end of the 4.2 kb transcript (summarized in Fig. 1; data not shown). Probe 1, which extends 5′ of the *RAD50* open reading frame, hybridized to the 4.6 kb *RAD50* transcript but not to the 4.2 kb transcript. At the 3′ end of the *RAD50* gene, probe 9 hybridized to both transcripts, and probes 7 and 10 failed to detect either transcript. Because the 4.2 kb *RAD50* transcript is more abundant than the 4.6 kb *RAD50* transcript, and because it spans the entire *RAD50* open reading frame, it is considered here as the major *RAD50* transcript.

Examination of RNA prepared at various times after the initiation of meiosis revealed that steady-state levels

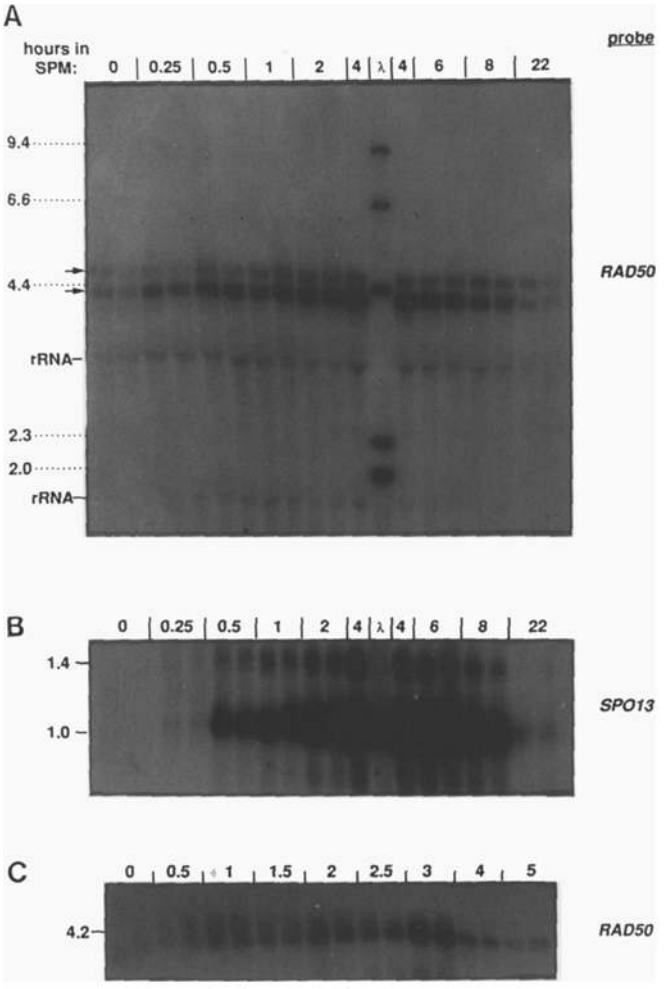

Fig. 2A–C. RNA blot analysis of *RAD50* and *SPO13* transcripts throughout meiosis. Total RNA from **a**/α diploid SK-1 strains was prepared at various times after transfer of a saturated culture into sporulation medium (SPM). Duplicate RNA samples were prepared quantitatively at each time point, and an equal volume of each RNA preparation, representing approximately 1×10^7 cells, was electrophoresed on 1% agarose gels and processed by RNA (Northern) blotting. **A** NKY147 was grown to saturation in YPD prior to transfer into SPM, and RNA was prepared at the indicated times thereafter. *RAD50* probe 3 was used in the RNA blot analysis. ^{32}P-end-labeled, *Hin*dIII-digested λ DNA fragments (λ) were included as size markers. *Arrows* point to the 4.6 and 4.2 kb *RAD50* transcripts. **B** The same blot shown in **A** was hybridized with a single-stranded, ^{32}P-labeled *SPO13* probe generated from pNKY44. The 1.4 and 1.0 kb *SPO13* transcripts are indicated to the left of the blot. **C** NKY278 was grown to saturation in YPA prior to transfer into SPM, and RNA was prepared at the indicated times thereafter. *RAD50* probe 3 was used in the RNA blot analysis. NKY278 and NKY147 (used in Figs. 2A and B) are closely related strains (see Materials and methods)

of the 4.2 and 4.6 kb *RAD50* transcripts rose and fell coordinately during meiosis (Fig. 2A), mimicking the pattern of a control RNA, *SPO13*, in the same RNA samples (Fig. 2B; Wang et al. 1987). Figure 2 shows RNA blot analysis of total yeast RNA harvested from diploid strains of the SK-1 genetic background during mitotic growth and at various times during meiosis. Total RNA was prepared from duplicate aliquots of cells

harvested at various times after transfer to SPM. The same volume of each RNA preparation, representing 1×10^7 cells, was analyzed so that any observed change in transcript level represents a change in the number of transcripts per cell. We did not attempt to normalize *RAD50* transcript levels to those of another gene because all meiotically expressed genes examined thus far exhibit significant changes in transcript levels during meiosis (Kaback and Feldberg 1985). During meiosis, the 4.2 and 4.6 kb *RAD50* steady-state transcript levels increased to levels approximately 10 times greater and three times greater, respectively, than the basal vegetative level of the *RAD50* transcripts. The peak of *RAD50* steady-state transcript levels occurred prior to the first meiotic division, at about the time of synaptonemal complex formation in our strains (Alani et al. 1990 and data not shown). This meiotic increase in *RAD50* steady-state transcript levels observed in **a**/α strains was not observed in **a**/**a** or α/α strains (NKY475 and NKY480; data not shown). Maximal *RAD50* transcript levels were quite modest compared to *SPO13* transcript levels at the same time in meiosis (Fig. 2B).

The growth regimen prior to incubation in sporulation medium affected the apparent timing and relative increase of meiotic *RAD50* RNA levels. Growth to saturation in YPD prior to sporulation resulted in a relatively broad peak of *RAD50* steady-state transcript levels centered at 4 h (Fig. 2A), while growth to saturation in YPA prior to sporulation resulted in a sharp peak of *RAD50* steady-state transcript levels at 3 h (Fig. 2C). The latter regimen results in a more synchronous meiosis in the SK-1 genetic background (Alani et al. 1990). Whether the growth regimen also affects resolution of the 4.2 and 4.6 kb *RAD50* transcripts is unclear (cf. Fig. 2A and 2C).

Analysis of plasmid subclones containing varying amounts of genomic DNA upstream of the *RAD50* coding region suggests that production of the 4.2 kb *RAD50* transcript requires more than 90 bp of sequence upstream of the first ATG and that 690 bp of 5′ flanking sequence are sufficient for the production of both the 4.2 and 4.6 kb transcripts. Specifically, both transcripts are produced by an *ARS1–CEN4* plasmid containing 990 bp of 5′ flanking sequence and 750 bp of 3′ flanking sequence and by a 2 μ plasmid carrying 690 bp of 5′ flanking sequence and 3.1 kb of 3′ flanking sequence (pNKY1070 and pNKY2042 respectively; data not shown). In contrast, a plasmid containing the *RAD50* gene and only 90 bp of 5′ flanking sequences and 3 kb of 3′ flanking sequence produced only an aberrant 6 kb transcript (pNKY1002; data not shown).

Mapping of the 5′ ends of the 4.2 kb RAD50 *transcript*

The 5′ ends of the 4.2 kb *RAD50* vegetative and meiotic transcripts map to the same cluster of sites located approximately 20 nucleotides upstream of the 5′-most ATG of the *RAD50* coding sequence (Fig. 3A). The ends of the 4.2 kb transcript were mapped by primer extension analysis using as primer a synthetic oligonucleotide, $oATG_2$, complementary to the region around the second in-frame ATG of *RAD50*. Three major 5′ ends, at −23, −19, and −15, were detected with wild-type vegetative and meiotic RNAs (Fig. 3A) and with RNA produced from the 2 μ plasmid pNKY2042. Many minor 5′ ends are also detected, and all but one (at +33) are upstream of the 5′-most ATG of the *RAD50* coding sequence. A transcript beginning at +33 would not encode the entire predicted Rad50 protein. The results of primer extension analysis were confirmed by S1 nuclease mapping of meiotic 4.2 kb *RAD50* transcripts, which revealed 5′ ends at −21, −20, −16, and −15 (Fig. 3B). The results of primer extension and S1 nuclease mapping

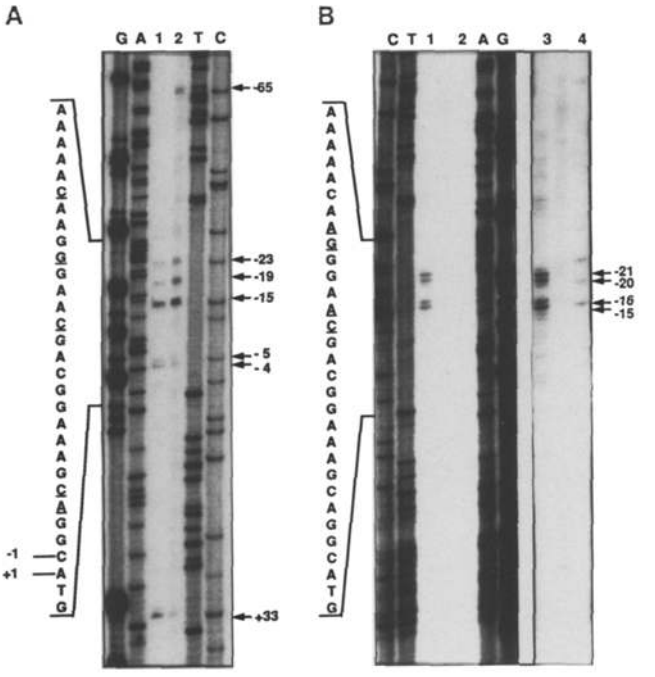

Fig. 3A, B. Primer extension and S1 nuclease mapping of the 5′ ends of the 4.2 kb *RAD50* transcript. **A** Primer extension analysis was used to map the 5′ ends of the 4.2 kb *RAD50* transcript from vegetative and meiotic RNA preparations. 5′ end-labeled primer $oATG_2$ was hybridized with yeast RNA and extended with reverse transcriptase as described in Materials and methods. The primer extension products were separated on 6% sequencing gels and visualized by autoradiography. *Arrows*, accompanied by *RAD50* nucleotide sequence numbers, point to primer extension products, and nucleotides to which primer extension products map are *underlined* in the accompanying sequence. Lane 1, primer extension products of poly(A)$^+$ vegetative RNA from NKY278; lane 2, primer extension products of total meiotic RNA from NKY278, 4 h after transfer of a saturated YPA culture into SPM; lanes G, A, T, C: dideoxy sequencing reactions obtained using the same ^{32}P-labeled primer and single-stranded DNA from pNKY74 to direct DNA synthesis in the presence of ddGTP, ddATP, ddTTP, or ddCTP, respectively. **B** S1 nuclease protection analysis of meiotic RNA was performed as described in Materials and methods. *Arrows*, accompanied by *RAD50* nucleotide sequence numbers, point to S1 nuclease-resistant products, and nucleotides to which these products map are *underlined* in the accompanying sequence. Lane 1, S1 nuclease-protected products of total meiotic RNA from NKY147, 30 min after transfer of a saturated YPA culture into SPM; lane 2, primer extension reactions of the same RNA as in lane 1; lanes G, A, T, C: as described for Fig. 3A. Lanes 3 and 4 are overexposures of lanes 1 and 2, respectively

```
-100
  *            HindIII                        +
  AAGCGGCTTT  CAAGCTTTGA  TCTTTAAATG  GAAATCTTTA  ATTCTAAGAA

 -50                                    +              -1
  *                              + >  >+   >>          ++  *
  GTGGTAGCAA  CCATTGAGAG  GCAAAAACAA  GGGAACGACG  GAAAGCAGGC

 +1
  *                                      +
  ATGAGCGCTA  TCTATAAATT  ATCTATTCAG  GGCATACGGT  CTTTTGACTC

+51
  *
  CAATGATAGG  GAAACTATTG  AATTTGGCAA  GCCTCTGACT  TTAATACTCG

+101
  *  _____oATG2_____
  GCATGAATGG  TTCAGGTAAA  ACGACTATCA  TCGAA
```

Fig. 4. Summary of primer extension and S1 nuclease mapping results. The *RAD50* sequence from −100 to +135 (Alani et al. 1989) is shown here. The 5′ ends of the 4.2 kb *RAD50* transcript determined by primer extension analysis are denoted by +, and those determined by S1 nuclease protection are denoted by >. The first two ATGs of the *RAD50* ORF are *underlined*, the *Hin*dIII site at −89 is *overlined*, and the sequence complementary to $oATG_2$, from +103 to +122, is *overlined*

of the 5′ ends of the 4.2 kb *RAD50* transcript are summarized in Fig. 4.

Rad50 protein analysis

Given the observed increase in *RAD50* transcript levels during meiosis, it was of interest to examine steady-state Rad50 protein levels during this process. To provide a tool for Rad50 protein detection, polyclonal antibodies that recognize Rad50 protein from *S. cerevisiae* were produced. Anti-Rad50 antibodies were raised against two different Tn*10* transposase-Rad50 fusion proteins produced in *E. coli*. pNKY75 (Fig. 5A) encodes a 112 kDa polypeptide that contains the C-terminal half of the predicted Rad50 protein and most of the Tn*10* transposase protein (Fig. 5B). A similar construct, pNKY76, containing a Tn*10* transposase-*RAD50* fusion gene with two-thirds of the *RAD50* coding sequence, produced a 149 kDa protein in *E. coli* (Fig. 5B). Antisera produced by rabbits injected with the pNKY75-derived fusion protein (antisera 893 and 894) and one antiserum produced by a rabbit injected with the NKY76-derived fusion protein (antiserum 891) recognized both Tn*10* transposase-Rad50 fusion proteins and endogenous yeast Rad50 protein on immunoblots (data not shown). Only antiserum 894, which had the strongest response to the *E. coli* fusion proteins, was further employed in this study.

Antiserum 894 recognizes the endogenous Rad50 protein of *S. cerevisiae*: it detects an endogenous *S. cerevisiae* polypeptide of approximately 150 kDa that is absent from NKY551 (a *rad50Δ/rad50Δ* diploid), is present in $Rad50^+$ strains, and is present in relatively greater amounts in strains bearing single- or multi-copy *RAD50* plasmids (Fig. 6A). The apparent molecular mass of this polypeptide is approximately that predicted by *RAD50* sequence analysis, 153 kDa (Alani et al. 1989). In addition to the 150 kDa protein specific to strains carrying the *RAD50* gene, the antiserum recognizes several other proteins that are present in all strains, including NKY551 (Fig. 6A), and thus are neither products of

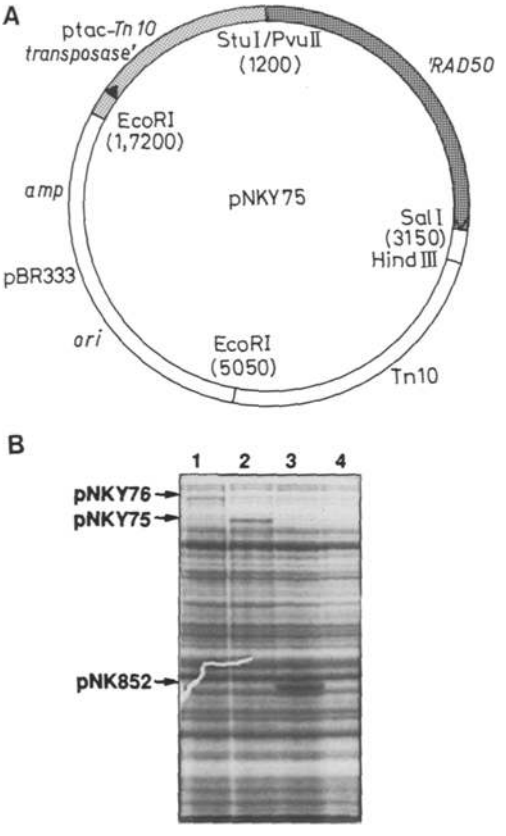

Fig. 5A, B. Production of Tn*10* transposase-Rad50 fusion proteins in *E. coli*. **A** pNKY75 is a pBR322-based plasmid containing a Tn*10* *transposase-RAD50* fusion gene whose expression is under the control of a *tac* promoter and optimized Shine-Delgarno sequences. The fusion gene consists of approximately 1 kb of Tn*10* transposase coding sequence at the 5′ end and approximately 2 kb of the *RAD50* 3′ coding sequence at the 3′ end. **B** Coomassie blue-stained SDS-polyacrylamide gel of total cellular protein from *E. coli* strain NK5830 bearing pNKY76 (lane 1), pNKY75 (lane 2), and pNK852 (lanes 3 and 4). The cultures from which protein in lanes 1, 2, and 3 was prepared were grown in logarithmic phase in the presence of 1 mM IPTG for 40 min, and the culture from which protein in lane 4 was prepared was not exposed to IPTG. Proteins produced from each plasmid in the presence of IPTG are indicated by *arrows*

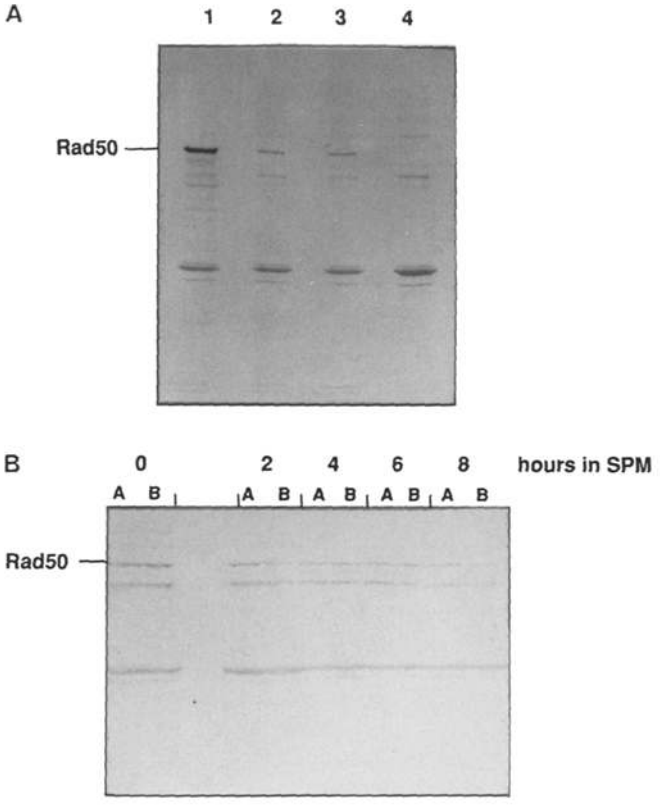

Fig. 6A, B. Immunoblots of total cellular protein from yeast using a polyclonal antiserum produced against a Tn*10* transposase-Rad50 fusion protein. Total cellular protein was produced by glass-bead lysis of yeast cultures and subsequent boiling of the lysates in buffer containing 1% SDS. Proteins were separated on 7% polyacrylamide-SDS gels, transferred to nitrocellulose, and probed with a 1:500 dilution of antiserum 894. The blots were developed as described in Materials and methods. **A** Protein from vegetative cultures of: lane 1, NKY278 (*RAD50/RAD50*) transformed with pNKY2042 (a multicopy 2μ-*RAD50* plasmid); lane 2, NKY278 transformed with pNKY1070 (a single-copy *ARS1-CEN4-RAD50* plasmid); lane 3, NKY278 transformed with YCp50 (a single-copy *ARS1-CEN4* plasmid); lane 4, NKY551 (*rad50Δ/rad50Δ*). **B** Protein from NKY278 at various times during meiosis. Duplicate aliquots of total yeast protein, labeled A and B, were prepared at the indicated times after transfer of a saturated culture of NKY278, grown in YPA, to SPM. This same culture was used for the preparation of RNA analyzed by RNA blot analysis in Fig. 2C

the *RAD50* gene nor proteins whose production depends on *RAD50*.

To examine steady-state levels of Rad50 protein throughout meiosis, total yeast protein was prepared from sporulating cultures at various times after transfer into SPM, and the presence and amount of Rad50 protein was determined by immunoblot analysis. At each time point, total protein was quantitatively prepared from duplicate aliquots of a sporulating culture. An equal volume of each protein preparation, representing protein from the same number of cells, was analyzed. The results of a representative experiment are shown in Fig. 6B. No apparent increase in the steady-state level of Rad50 protein occurred during meiosis, despite the observed 10-fold increase in steady-state *RAD50* transcript levels (Fig. 2A, C). Immunoblot development conditions were such that the level of Rad50 protein was well within the linear range of detection (Fig. 6A). Several different methods of protein extraction and analysis yielded the same result. These methods included lysis under acidic conditions to further minimize proteolysis, lysis in the presence of trichloroacetic acid to precipitate protein immediately, or loading protein samples onto gels immediately after they were prepared (rather than freezing early time point preparations and loading all samples simultaneously at the end of a meiotic time course; data not shown).

We have estimated that vegetative cells growing logarithmically contain on the order of 2000 Rad50 protein molecules per cell, using immunoblot analysis to measure the relative affinities of anti-Rad50 antibodies and anti-β-galactosidase antibodies to a Rad50-β-galactosidase fusion protein and then comparing signals produced by the fusion protein, known quantities of purified β-galactosidase, and cellular levels of Rad50 protein (W. Raymond and N. Kleckner, unpublished data).

Discussion

RAD50 is required for X-ray-induced DNA damage repair during mitotic growth and for meiotic chromosome synapsis and recombination. To determine whether *RAD50* expression varies between mitotic growth and meiosis, a study of *RAD50* transcription was undertaken. Two *RAD50* transcripts, 4.2 and 4.6 kb in length, were identified by RNA blot analysis using single-stranded probes covering the entire 3.9 kb *RAD50* coding sequence and flanking sequences. These transcripts differ in size from the 3.3 kb *RAD50* transcript previously reported (Kupiec 1986). Both *RAD50* transcripts are polyadenylated and are present during mitotic growth and meiosis. Steady-state levels of both transcripts rise and fall coordinately during meiosis, mimicking the pattern of *SPO13* expression in the same RNA samples, and peaking at levels approximately 10 times greater than vegetative levels for the more abundant 4.2 kb *RAD50* transcript. Maximum *RAD50* transcript levels occur prior to the first meiotic division, about midway through meiotic prophase (Alani et al. 1990 and data not shown). The observed meiotic increase in *RAD50* transcript levels could reflect increased stability of *RAD50* transcripts and/or an increased rate of *RAD50* transcription during meiosis. The increase in steady-state *RAD50* transcript levels is specific to sporulating **a**/α diploids: it is not observed in **a**/**a** or α/α diploids subjected to the same protocol.

While the magnitude and exact timing of the increase in steady-state *RAD50* transcript levels during meiosis vary according to the growth regimen employed prior to transferring cells to SPM, a 5 to 10-fold increase is observed whether cells are grown to saturation in YPD (Fig. 2A), to saturation in YPA (Fig. 2C), or to mid-log phase in YPA (data not shown). The differences in magnitude and timing of the increase in steady-state *RAD50* transcript levels are probably related to differences in meiotic synchrony observed between one growth regimen and another.

By comparing the signals on RNA blots obtained using *RAD50*, *SPO13*, and *URA3* probes, the mid-meiotic *RAD50* transcript level is roughly estimated at one transcript per 10 cells (*SPO13* comparison) or one transcript per 40 cells (*URA3* comparison) (data not shown). These calculations are based on the reported quantitation of approximately two *SPO13* 1.0 kb transcripts per cell at the maximum level observed in meiosis (Wang et al. 1987) and of 3.3 *URA3* transcripts per vegetative cell (LaCroute et al. 1981). While these approximations of *RAD50* transcript levels may be underestimates, it is clear that *RAD50* transcript levels are low relative to levels of meiotic *SPO13* and mitotic *URA3* transcripts.

Primer extension and S1 nuclease analyses reveal that the 5′ ends of the major, 4.2 kb, *RAD50* transcript are distributed among several nucleotides located approximately 20 nucleotides upstream of the 5′-most AUG. The identical 5′ ends of the *RAD50* 4.2 kb transcript in mitotic growth and in meiosis suggest that the increase in *RAD50* steady-state transcript levels observed during meiosis is not a result of differential transcription initiation.

In some RNA samples, primer extension analysis revealed a minor 5′ end of the 4.2 kb *RAD50* transcript that maps to +33 of the *RAD50* coding sequence (Fig. 3A, B) and thus could not encode a full-length Rad50 protein. Translation initiating at the first AUG of this shorter transcript would produce a polypeptide lacking the consensus nucleotide-binding domain of the predicted Rad50 polypeptide (Alani et al. 1989); this consensus nucleotide-binding domain is required for *RAD50* function (Alani et al. 1990). Thus, the biological relevance of a minor *RAD50* transcript incapable of producing a functional Rad50 polypeptide is unclear, and no polypeptide of the predicted size has been detected (data not shown).

Rad50 protein levels throughout meiosis were monitored by immunoblot analysis of total yeast protein using a polyclonal antibody prepared against a Tn*10* transposase-Rad50 fusion protein (Fig. 6). Interestingly, steady-state Rad50 protein levels remained at or below their low mitotic levels throughout meiosis despite the observed increase in meiotic *RAD50* transcript levels. The level of Rad50 protein in mitotic cells has been estimated to be on the order of 2000 molecules per cell (W. Raymond and N. Kleckner, unpublished data). That this level does not increase during meiosis suggests that Rad50 protein is not a major structural component of the synaptonemal complex.

The observed increase in steady-state *RAD50* transcript levels during meiosis without a corresponding increase in Rad50 protein levels might reflect a real, in vivo situation, or it might be due to a technical limitation of the approach taken here. One interpretation of the apparent paradox is the possibility that new *RAD50* meiotic transcripts are not translated. *RAD50* translational repression might be effected directly by Rad50 protein; during mitotic growth, however, differing levels of Rad50 protein do not affect expression of a *RAD50-lacZ* fusion (L. Szubin, W. Raymond, and N. Kleckner, unpublished data). Alternatively, the constant steady-state level of Rad50 protein throughout meiosis despite increased *RAD50* transcript levels might be explained by a balance between increased Rad50 protein synthesis resulting from elevated *RAD50* transcript levels and in vivo meiotic proteolysis. Observations of high levels of meiotic proteolysis have been well-documented (Hopper et al. 1974; Zubenko and Jones 1981), although it is disputed whether such proteolysis is specific to meiosis or is a general consequence of nitrogen starvation.

Incomplete recovery of Rad50 protein from mid-meiotic cells could result in an artifactual observation of constant Rad50 protein levels throughout meiosis. That yeast lysates are boiled directly in sample buffer containing 1% SDS and that Rad50 protein overproduced during mitotic growth is soluble (W. Raymond and N. Kleckner, submitted) suggest that this is not the case. Furthermore, treatment with 8 M urea of the insoluble pellets produced after lysis did not extract detectable amounts of Rad50 protein (data not shown). Exogenous proteolysis during protein preparation is also unlikely to account for the reported results. Five protease inhibitors were included during cell lysis, and identical results were obtained when lysis was carried out under a variety of acidic conditions designed to minimize proteolysis further (data not shown). Finally, purified β-galactosidase added to meiotic cells prior to cell lysis is not degraded during the lysis procedure (data not shown).

The observation that steady-state *RAD50* transcript levels rise and fall during meiosis while steady-state Rad50 protein levels remain constant provides an example in which a moderate (10-fold) increase in meiotic transcript levels is not reflected by an overall increase in polypeptide levels. Moderate increases in meiotic transcript levels of other essential meiotic genes, such as *RAD51, RAD52,* and *RAD54*, are accompanied by increases in meiotic polypeptide levels (Cole et al. 1989; Shinohara et al. 1992). Whether the observed moderate increases in *RAD50, RAD51, RAD52,* and *RAD54* meiotic transcript levels are required for the meiotic functions of these genes is not known. Vegetative transcript levels of *RAD51* and *RAD54* increase in response to DNA damaging agents (Cole et al. 1987; Shinohara et al. 1992), but interestingly, the moderate increase in *RAD54* transcript levels observed as a result of DNA-damaging agents is not required for the mitotic DNA repair function of *RAD54* (Cole and Mortimer 1989). The effect of DNA damaging agents on *RAD50* expression during vegetative growth has not yet been examined.

Other essential meiotic genes, including *SPO11, SPO13, MER1*, and *RED1*, produce maximal transcript levels at the same time in meiosis as *RAD50* (Atcheson et al. 1987; Wang et al. 1987; Thompson and Roeder 1989; Engebrecht and Roeder 1990); *HOP1* and *MEI4* produce maximal transcript levels at approximately this same time (Hollingsworth et al. 1990; Menees et al. 1992). Unlike *RAD50, RAD51, RAD52,* and *RAD54*, however, these genes produce no detectable mitotic transcripts and therefore exhibit dramatic, rather than mod-

erate, meiotic transcript level increases. The increased meiotic transcript levels of *SPO11, SPO13, MEI4, MER1,* and *RED1* are accompanied by parallel increases in β-galactosidase activities of the corresponding *lac*Z fusions (Strich et al. 1989; Engebrecht and Roeder 1990; Rockmill and Roeder 1991; Menees et al. 1992). The dramatic transcriptional activation during meiosis of this set of essential meiotic genes thus directly reflects the restriction of their functions to meiosis. The role of comparatively moderate changes in steady-state meiotic transcript levels of *RAD50, RAD51, RAD52,* and *RAD54* remains to be elucidated.

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