

Transcriptional control of *RAD51* expression in the ciliate *Tetrahymena thermophila*

Joshua J. Smith, Eric S. Cole¹ and Daniel P. Romero*

Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, MN 55455, USA and

¹Department of Biology, St Olaf College, Northfield, MN 55057, USA

Received June 28, 2004; Revised and Accepted July 27, 2004

ABSTRACT

The expression of Rad51p, a DNA repair protein that mediates homologous recombination, is induced by DNA damage and during both meiosis and exconjugant development in the ciliate *Tetrahymena thermophila*. To completely investigate the transcriptional regulation of *Tetrahymena RAD51* expression, reporter genes consisting of the *RAD51* 5' non-translated sequence (5' NTS) positioned upstream of either the firefly luciferase or green fluorescent protein coding sequences have been targeted for recombination at the macronuclear *btu1-1* (K350M) locus of *T. thermophila* strain CU522. Expression from *RAD51*-luciferase reporter constructs has been directly quantified from transformant whole cell lysates. Luciferase is induced to maximum levels in transformants harboring the full-length *RAD51*-luciferase reporter gene following exposure to DNA damaging UV irradiation. A series of truncations, deletions, insertions, substitutions and inversions of the *RAD51* 5' NTS have led to the identification of three distinct transcriptional promoter elements. The first of these sequence elements is required for basal levels of transcription. The second modulates expression in the absence of DNA damage, whereas the third ensures increased *RAD51* transcription in response to DNA damage and during meiosis. *Tetrahymena RAD51* is tightly regulated through these transcriptional elements to produce the appropriate expression during conjugation, and in response to DNA damage.

INTRODUCTION

Homologous recombination fulfills two seemingly conflicting roles. Recombination between sister chromatids during meiosis generates genetic diversity, while the same mechanism helps to maintain genetic stability when cells repair DNA damage incurred as a result of normal cellular processes and environmental agents. At the core of homologous recombination is the identification of homologous DNA by

a 'recombinase' protein, represented in eukaryotes by Rad51p and its various homologs (1). The Rad51p binds to single-stranded DNA and helps to scan double-stranded DNA until a homologous sequence is found, where it forms a nucleofilament on the single-stranded DNA and catalyzes homologous strand exchange [reviewed in (2,3)].

The expression of *RAD51* from the ciliate *Tetrahymena thermophila* varies in response to both environmental conditions and developmental signals. The Rad51 mRNA levels increase following exposure to DNA damaging agents (4), a property shared by homologs from other eukaryotes. There is a distinct pattern of *RAD51* expression through the cell cycle, with minimum levels coincident with micronuclear M phase and maximum expression corresponding to a period of macronuclear DNA replication that immediately follows cytokinesis (5). There is also a bimodal increase in the mRNA levels during conjugation, with periods of peak expression coinciding with meiotic prophase and exconjugant macronuclear development (5). *Tetrahymena* differs from most eukaryotes in its dependence on a single *RAD51* paralog for homologous recombination in both vegetatively dividing cells and cells undergoing meiosis (5). Other eukaryotes express multiple *RAD51* paralogs, one of which (*DMC1*) is only expressed during meiosis, where it is essential for sister chromatid exchange in yeast and humans (6,7).

Variations detected for *Tetrahymena* Rad51 mRNA levels are similar to those seen for *RAD51* in other eukaryotes. Changes in yeast and human Rad51 mRNA levels are largely mediated by either induction or repression of transcription (8–12), although post-translational inactivation of human Rad51p by caspase-mediated cleavage during apoptosis (13) or phosphorylation by c-Abl after ionizing radiation has also been documented (14,15).

It was unclear whether the increases in *Tetrahymena RAD51* expression in response to DNA damaging agents (4) and during conjugation (5) are due to transcriptional or post-transcriptional regulation. Although the major mechanism for regulating mRNA abundance in *Tetrahymena* is differential transcription (16), mRNA degradation can play a role in gene products such as the SerH3 surface antigen (17). In an effort to determine whether the *Tetrahymena RAD51* expression is under transcriptional control, a series of reporter genes under control of the putative *RAD51* transcriptional promoter have been constructed and introduced into *Tetrahymena*

*To whom correspondence should be addressed. Tel: +1 612 624 8997; Fax: +1 612 625 8408; Email: romero@med.umn.edu

Present address:

Joshua J. Smith, University of Virginia Health Systems, Biochemistry and Molecular Genetics, Charlottesville, VA 22908, USA

transformants. We have used this methodology to define the inducible *RAD51* transcriptional promoter and to identify *cis* elements critical to its differential expression in response to DNA damage and the developmental stages of conjugation.

MATERIALS AND METHODS

General methods

Tetrahymena genomic DNA was isolated by detergent lysis as described previously (18). RT-PCR protocols and molecular techniques were as described in (19). Total RNA was isolated from vegetatively dividing and conjugating cells using Qiagen RNeasy Total RNA kits (Valencia, CA). The specific ³²P-radiolabeled DNA probes were generated by a PCR strategy as described previously (20), and Southern-blot analysis was performed as described previously (5). Treatment of total cellular RNA from *Tetrahymena* with glyoxal prior to agarose gel electrophoresis and northern-blot analysis was as described previously (19).

Tetrahymena thermophila strains and growth conditions

The *T. thermophila* strains CU522, CU725 and CU727 (Jacek Gaertig, University of Georgia, Athens, GA) express the mutant β -tubulin *btu1-1* (K350M) allele that confers hypersensitivity to the microtubule-stabilizing drug paclitaxel (Table 1). These strains were transformed with various reporter constructs by the targeted disruption of the *btu1-1* allele throughout this study (21). The cells were grown in 2% PPYS (2% proteose peptone, 0.2% yeast extract and 0.1% sequestrene) at 30°C on a platform shaker (100 r.p.m.) as described previously (18). All *Tetrahymena* cultures were maintained in 1× PSF (Penicillin, Streptomycin and Fungizone; Life Technologies, Rockville, MD) to prevent bacterial and fungal growth.

Radiolabeled probes

The PCR products were radiolabeled by the incorporation of [α -³²P]dATP (sp. act. 3000 Ci/mmol) as described in (20), to be used as probes in Southern- and northern-blot analyses. The PCR primer pairs P1, P2, P3, P4 and P5 were used to synthesize probes specific for the 3' non-translated sequence (3' NTS) of the *Tetrahymena* β -tubulin 1 gene (accession no. L01415), and coding regions of *Tetrahymena* Rad51 (accession no. AF064516) *Tetrahymena* α -tubulin (accession

no. M86723), luciferase and green fluorescent protein (GFP), respectively.

P1(+) TCGGTCAGCTAAACCAAC
 P1(−) ATGCGGGTGAGTGCAGAA
 P2(+) GACGAATTCGGTATTGC
 P2(−) TCACTCGTTGAAGTC
 P3(+) GTTATTTCAATTCACG
 P3(−) AAGAAGACAGCTCTGG
 P4(+) AAGAAAGGCCCGGCGC
 P4(−) GAGGCAGAGCGACACC
 P5(+) GCCAATTGGAGTATTT
 P5(−) GTTGTCCCAATTCTTG

Construction of reporter genes for expression in *Tetrahymena*

The Rad51-luciferase reporter construct was initiated by introducing a BamHI site at −1.3 kb of the *RAD51* 5' NTS, and the HindIII and EcoRV sites at the third codon and the stop codon of the Rad51p coding sequence, respectively (4). The luciferase coding sequence present in the plasmid pGL3-Basic (Promega, Madison, WI) was amplified by PCR, using primers that introduce HindIII and EcoRV sites at the third codon and the stop codon, respectively, to accommodate replacement of the Rad51p coding sequence with that of luciferase. The resultant BamHI–EcoRV fragment was subcloned into the Litmus 28 vector (New England Biolabs). The *BTU2* 3' NTS, includes a polyadenylation site, was amplified by PCR from pHAB2 (J. Gaertig, University of Georgia) and cloned into the pLit28::RdLuc construct at unique EcoRV and SpeI sites. Site-directed mutagenesis by the method of Kunkel (22) was used to introduce unique BamHI and NsiI sites flanking the H4-Neo cassette in pHAB1, a plasmid designed for the targeted disruption of the *Tetrahymena BTU1* locus (23). The introduction of BamHI and NsiI sites in pHAB1 facilitated the replacement of the H4-Neo cassette with the Rad51-luciferase-*BTU2* reporter construct described above. The resultant reporter construct, targeted for the disruption of the *btu1-1* locus in strain CU522 and consisting of the *RAD51* promoter, luciferase coding sequence and the *BTU2* polyadenylation site, is referred to throughout the text as RdLuc (Figure 1A). Similar methodologies were used to construct other reporter genes targeted for *btu1-1* disruption, including luciferase expressed from the *BTU1* promoter (BtLuc; Figure 1A), and GFP expressed from the *RAD51* promoter (RdGFP; Figure 8A).

Table 1. *Tetrahymena thermophila* strains

Strain	Micronuclear genotype	Macronuclear genotype	Macronuclear phenotype
CU522	<i>mpr1/mpr1, btu1-1/btu1-1</i>	<i>mpr1, btu1-1</i>	mp-R, ory-R, pac-S, VI
CU725	<i>chx1/chx1, btu1-1/btu1-1</i>	<i>chx1, btu1-1</i>	cy-R, ory-R, pac-S, VII
TC202	<i>mpr1/mpr1, btu1-1/btu1-1</i>	<i>mpr1, btu1-1::Luc</i>	mp-R, ory-S, pac-R, VI BtLuc
TC232	<i>mpr1/mpr1, btu1-1/btu1-1</i>	<i>mpr1, btu1-1::RdLuc</i>	mp-R, ory-S, pac-R, VI RdLuc
TC296	<i>chx1/chx1, btu1-1/btu1-1</i>	<i>chx1, btu1-1::RdLuc</i>	cy-R, ory-S, pac-R, VII RdLuc
TC368	<i>mpr1/mpr1, btu1-1/btu1-1</i>	<i>mpr1, btu1-1::RdGFP</i>	mp-R, ory-S, pac-R, VI RdGFP
TC370	<i>chx1/chx1, btu1-1/btu1-1</i>	<i>chx1, btu1-1::RdGFP</i>	cy-R, ory-S, pac-R, VII RdGFP

chx1, cycloheximide resistance (cy-R); *mpr1*, 6-methylpurine resistance (mp-R); *btu1-1*, paclitaxel sensitivity (pac-S) and oryzalin resistance (ory-R). BtLuc, luciferase expressed from the *BTU1* promoter; RdLuc, luciferase expressed from the *RAD51* promoter; RdGFP, GFP expressed from the *RAD51* promoter. The genotypes and phenotypes of representative transgenic strains are listed in this table. Transgenic strains not listed include those expressing reporter genes from variously mutated promoters described in the text.

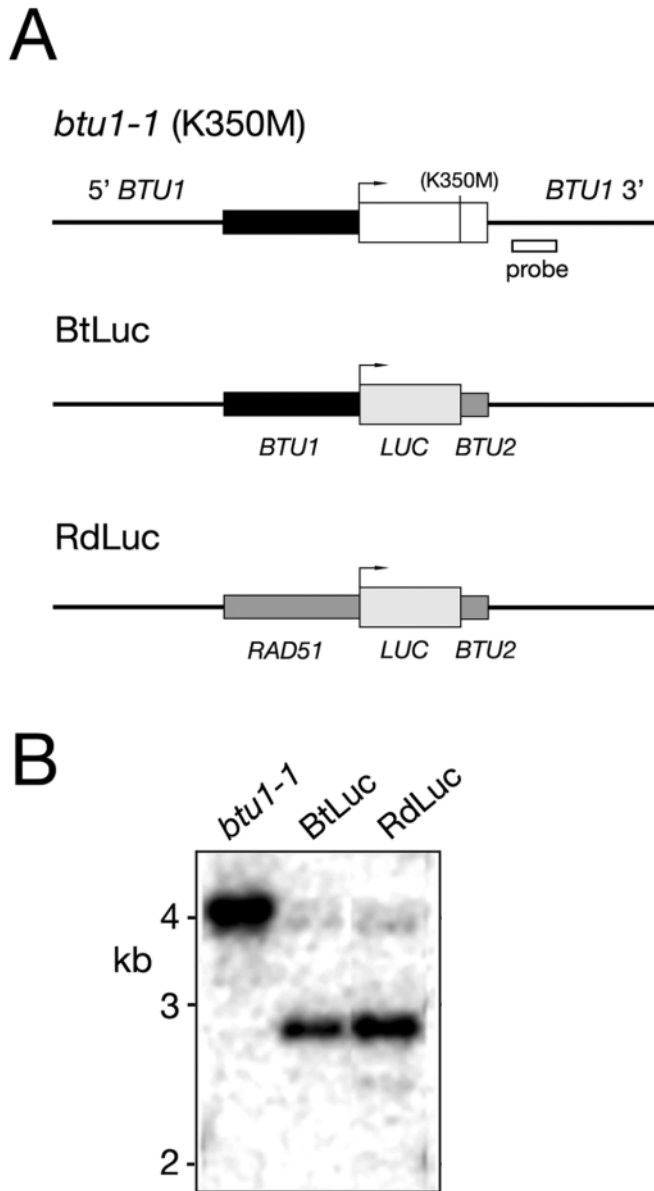


Figure 1. Reporter gene constructs. (A) Schematic representation of the *btu1-1* mutant allele that expresses β -tubulin (K350M) (white box), which is responsible for hypersensitivity of strain CU522 to the microtubule-stabilizing drug paclitaxel. Targeted disruption of the *btu1-1* allele with BtLuc [a reporter construct consisting of luciferase (light grey box) transcribed from the *BTU1* transcriptional promoter (black box), with a polyadenylation site from the *BTU2* gene (short dark grey box)] or RdLuc [luciferase expressed from the *RAD51* transcriptional promoter (long dark grey box)] is facilitated by the 5' and 3' *BTU1* flanking sequence (thin black lines). The location of the probe sequence used for Southern-blot analysis is also indicated. Not drawn to scale. (B) Southern-blot analysis of total DNA from paclitaxel resistant transformants. A restriction digest polymorphism (BglII and SphI digest) makes it possible to distinguish between intact and disrupted *btu1-1* alleles (4.0 and 2.8 kb, respectively).

Modification of the *RAD51*-luciferase reporter constructs

Manipulation of the *RAD51* promoter, including various mutations, truncations, deletions and inversions, were accomplished by introducing unique restriction sites by the site-directed mutagenesis method of Kunkel (22) and/or PCR amplification of selected regions of the promoter. For

example, the introduction of unique BglII sites within the promoter, followed by a double digest with BglII and BamHI and ligation of the compatible termini, resulted in a variety of reporter constructs with truncated promoters. Insertions and internal deletions were constructed using similar techniques. The sequence of constructs was confirmed by restriction digest analysis and DNA sequencing.

Biolistic transformation of *Tetrahymena*

Tetrahymena strain CU522 (also CU725 and CU727 for conjugation experiments) was grown in 100 ml cultures (2% PPYS) to a density between 1.0 and 3.0×10^5 cells/ml, and starved in 100 ml of 10 mM Tris-HCl (pH 7.5) for 14–21 h. The cells were pelleted by centrifugation and resuspended in 1–3 ml of 10 mM HEPES (pH 7.5) to a density of $\sim 1 \times 10^7$ cells/ml. The concentrated cells were then transferred onto a Petri dish (100 mm diameter) that contained a sterile Whatman 114 filter paper that was presoaked using 2 ml of 10 mM HEPES (pH 7.5). The Au particles (1.0 μ m diameter), coated with ~ 1.0 μ g of various reporter constructs digested previously with restriction enzymes KpnI and SacI, were introduced to the cell biolistically with the BioRad Gene GunTM, using 900 psi and a vacuum of 27 mm Hg as per the manufacturer's instructions. After biolistic transformation, the cells on the filter paper were incubated at 30°C for 2 h in 50 ml of pre-warmed 2% PPYS and then distributed to 96-well plates in 100 μ l aliquots. Selection of transformants resistant to 20 μ M paclitaxel at 30°C was monitored for 4–7 days. Southern-blot analysis of total DNA from resistant clonal cell lines confirmed the presence or absence of the reporter constructs in the *btu1-1* locus. Positive clonal lines were grown at low cell densities in 2% PPYS under continuous selective pressure (20–40 μ M paclitaxel) until 100% phenotypic assortment to the reporter construct at the targeted *btu1-1* locus had been achieved.

UV irradiation

Tetrahymena transformants and wild-type cells were irradiated with UV by the method described previously (4). Cells were allowed to recover at 30°C for 2 h before the preparation of cell extracts for luciferase activity assays.

Tetrahymena cell extract preparation for luciferase activity assays

The transformants were grown in 2% PPYS to densities of $1-3 \times 10^5$ cells/ml and pelleted by centrifugation (4 min at 1100 g). The cells were lysed in 300–800 μ l of 1 \times CCLR (Cell Culture Lysis Reagent; Promega), and lysates clarified by centrifugation at 16 250 g. The protein concentration of cell extracts was determined using BioRad Bradford Reagent (BioRad, Hercules, CA). The extracts were normalized for total protein (1.0 mg/ml), and the luciferase activity assay was performed using the procedure outlined in the Luciferase Assay Kit (Promega). A Turner Designs Luminometer (Model TD-20/20) was used to measure the luciferase activity. The sensitivity and Relative Light Units (RLUs) were normalized using luciferase levels present in BtLuc transformants, which constitutively express high levels of luciferase from the *BTU1* promoter (Figure 1A).

RESULTS

Reporter gene expression in *Tetrahymena*

The mutant β -tubulin allele *btu1-1* (K350M) confers hypersensitivity to the microtubule-stabilizing drug paclitaxel (23). Targeted disruption of this non-essential gene (a second β -tubulin gene, *BTU2*, is expressed in *Tetrahymena*) confers a selective advantage to transformants (paclitaxel resistance), making it possible to introduce and study foreign gene expression in *T. thermophila* (21). We have used this methodology to create stably transformed clonal lines that express foreign genes from either the *BTU1* or the *RAD51* transcriptional promoters. Chimeric reporter genes were constructed with either the *BTU1* or the *RAD51* 5' NTS positioned upstream of either the firefly *Photinus pyralis* luciferase coding sequence (Promega) or the GFP coding sequence (D. L. Chalker, Washington University, St Louis, MO). These reporter genes were subsequently cloned in a vector suitable for targeted disruption of the *btu1-1* (K350M) locus (Figure 1A). Following biolistic transformation of strain CU522, paclitaxel-resistant clonal lines were maintained under continuous selective pressure for 20–30 fissions to ensure 100% phenotypic assortment to the reporter allele (24,25), which was subsequently confirmed by Southern-blot analysis of the total DNA from transformants (Figure 1B; data not shown).

Whole cell lysates from clonal lines transformed with the luciferase reporter constructs were routinely assayed for luciferase activity, making it possible to quantify its expression from a variety of different promoter constructs, and from cells subjected to a variety of environmental conditions. Luciferase expression from the *BTU1*-luciferase reporter (BtLuc) was ~ 100 times greater than that from the *RAD51*-luciferase construct (data not shown). All subsequent luciferase activity assays were normalized with respect to the constitutively high levels of luciferase detected in lysates from BtLuc transformants.

Induction of the *RAD51*-luciferase reporter by DNA damage

Transformants expressing the *RAD51*-luciferase reporter (RdLuc, Figure 1A) were irradiated with UV(c) (240 J/m^2) and assayed for luciferase activity over the next 6 h. An increase in luciferase was detected ~ 1 h after irradiation, with peak levels attained within 2 h (Figure 2). A similar pattern of induction was observed for transformants expressing the *RAD51*-GFP reporter (RdGFP, data not shown). The induction kinetics for the reporter genes reiterates those previously seen for Rad51 mRNA following UV irradiation (4).

In order to determine whether the *de novo* protein synthesis is required for the increased transcription of *RAD51* in response to DNA damage, transformants were UV-irradiated in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). The northern-blot analysis indicates that comparable levels of both Rad51 and luciferase mRNA from RdLuc transformants are attained with or without CHX, although maximal levels are delayed in the presence of the drug (Figure 3). In contrast, *ATU1* (α -tubulin) mRNA transcription decreases over time in the presence of CHX. The increase in Rad51 expression in response to UV irradiation is at the level of transcription and not due to changes in mRNA

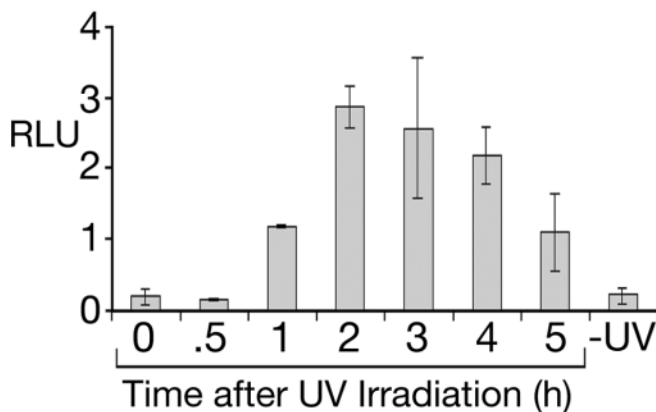


Figure 2. Luciferase expression from the *RAD51* promoter following UV irradiation of transformants. RdLuc transformants were irradiated with UV as described in the Materials and Methods, and allowed to recover for the time intervals indicated prior to the preparation of whole cell lysates. The cell extracts ($20 \mu\text{g}$ total protein) were assayed for luciferase activity in triplicate and normalized to the luciferase activity from BtLuc transformants, which constitutively express luciferase independent of UV irradiation (data not shown). The relative light units (RLUs) for non-irradiated RdLuc transformants are also shown. The bars represent the standard deviation for luciferase assays from three independently isolated RdLuc transformants.

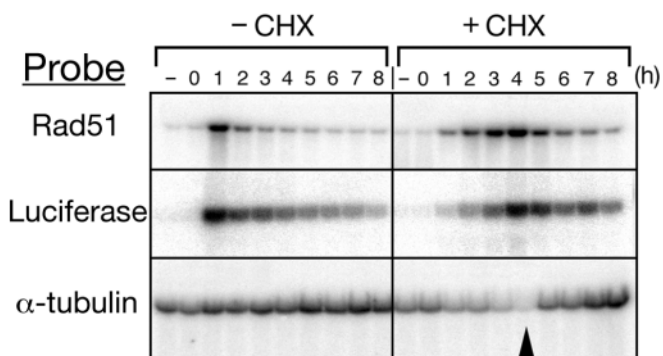


Figure 3. Transcription from the *RAD51* promoter in response to DNA damage is independent of *de novo* protein synthesis. RdLuc transformants were UV irradiated as described in the Materials and Methods in the presence (+CHX) or absence (–CHX) of the protein synthesis inhibitor CHX. Total RNA was prepared from the cells at the times indicated following irradiation, as well as from non-irradiated cells (–). CHX-treated cultures were washed extensively after 4 h and transferred to fresh media without CHX for an additional 4 h (arrow). Equal amounts ($10 \mu\text{g}$) of total RNA from each sample were separated in three identical electrophoretic gels. The RNA was transferred onto Nytran membranes, which were subsequently hybridized with probes specific for Rad51, luciferase and α -tubulin coding sequences.

stability, since both luciferase mRNA and the endogenous Rad51 mRNA follow the same kinetics in this experiment (Figure 3).

Identification of *RAD51* promoter elements

A series of truncations of the 1.3 kb 5' NTS in the RdLuc reporter construct have revealed three separable *cis*-sequence elements within the *RAD51* transcriptional promoter. The results from these experiments are summarized in Figures 4 and 5. Truncation of the Rad51 5' NTS to nucleotide positions

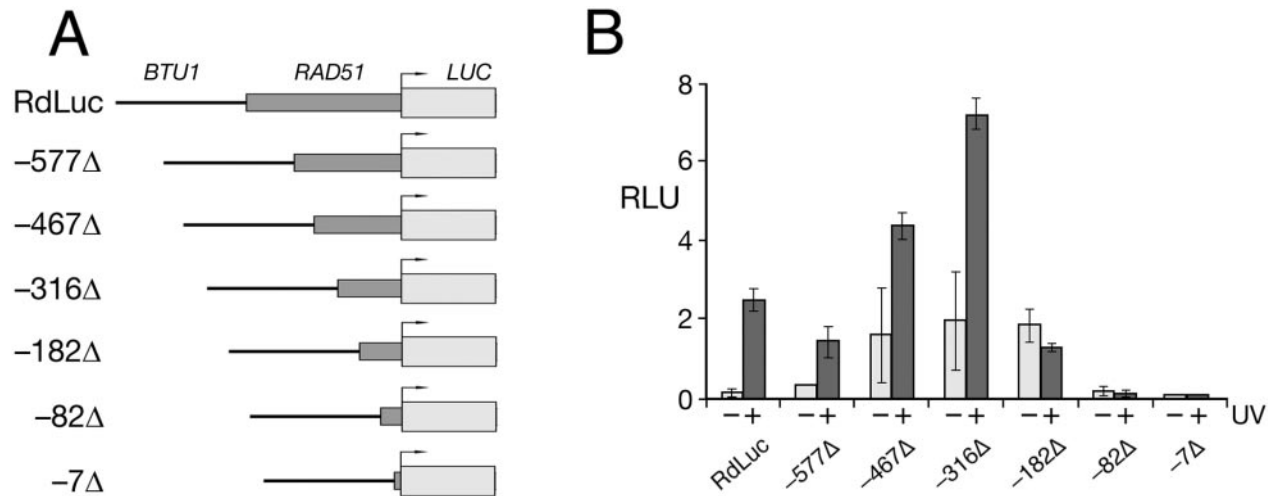


Figure 4. Luciferase expression from the truncated *RAD51* promoter. (A) The full-length *RAD51* promoter (1.3 kb) in the RdLuc construct as well as six successively larger truncations are shown schematically. The length of each truncated promoter (from its 5' terminus to the luciferase initiator codon) is indicated. (B) Luciferase activity from cells transformed with the various truncated RdLuc reporter constructs, with (+) and without (-) UV irradiation. Whole cell extracts were prepared 2 h following irradiation and assayed (20 μ g total protein) in triplicate for luciferase activity. Whole cell extracts from transformants that constitutively express luciferase (BtLuc) were used to establish the RLUs. Error bars represent the standard deviation.

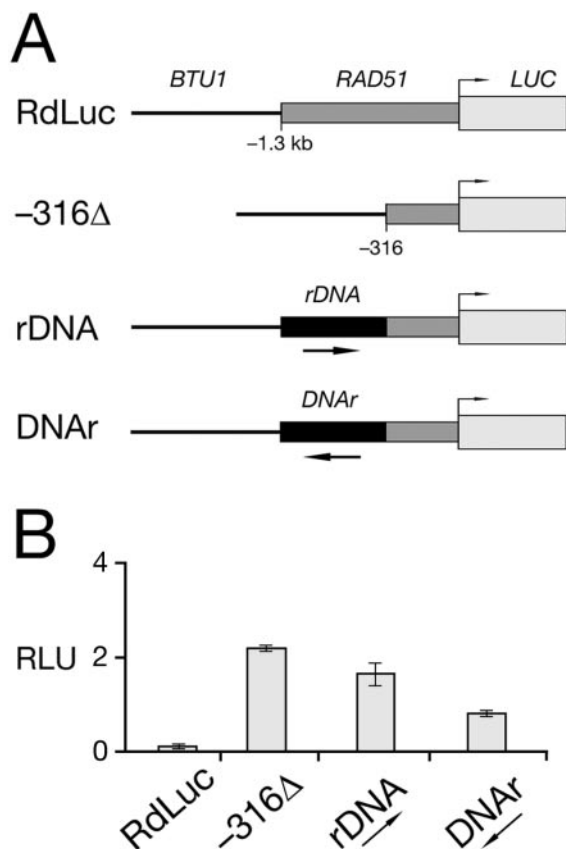


Figure 5. An upstream *cis* element contributes to low steady-state levels of expression from the *RAD51* promoter. (A) Schematic diagrams of the full-length RdLuc reporter construct, the -316 truncation [RdLuc(-316 Δ)], and the substitution of 1.0 kb of the Rad51 promoter (-1.3 kb to -0.3 kb) with 0.9 kb of *Tetrahymena* rDNA 3' NTS (accession no. X54512; 9363-10262 nt) in both sense [RdLuc(rDNA)] and anti-sense [RdLuc(DNAr)] orientations. Not drawn to scale. (B) Basal luciferase activities of lysates from transformants expressing the four reporter constructs (Figure 5A) was as described for non-irradiated cells in Figure 4. Error bars represent the standard deviation. See text for discussion.

-7 or -82 (relative to the translational start codon) eliminates all measurable luciferase from transformant lysates, with or without UV irradiation. In contrast, luciferase is still expressed in transformants with a reporter that includes nucleotides from -182 to -1 of the 5' NTS [RdLuc(-182 Δ)], although expression from this construct is unaffected by exposure to UV(c). Only when the proximal 316 bp (or more) of the Rad51 5' NTS is retained in reporter constructs do luciferase levels increase in response to UV(c) irradiation [RdLuc(-316 Δ); Figure 4B]. A similar response was observed during deletion analysis of the RdGFP reporter (data not shown). Taken together, these data indicate that at least two *cis*-sequence elements within the Rad51 5' NTS are required for (i) basal transcription (located between nucleotides -182 and -82) and (ii) inducible expression in response to DNA damage (located between nucleotides -316 and -182).

Steady-state luciferase activity was relatively low for transformants expressing the full-length RdLuc reporter without UV irradiation. This basal activity increased significantly for the various truncation constructs, with the increase somewhat proportional to the length of the 5' NTS truncation, being the most pronounced for the -467 Δ , -316 Δ and -186 Δ reporter constructs (Figure 4B). It was unclear whether the basal level increase was due to the loss of a putative control element between -1.3 kb and -316 of the Rad51 5' NTS or the proximity of the *BTU1* 5' targeting sequence in these constructs. In order to distinguish between these two possibilities, reporter constructs were tested that included 0.9 kb of a non-specific sequence derived from the rDNA 3' non-transcribed sequence (26), which was inserted between the *BTU1* 5' targeting sequence and -316 of the Rad51 5' NTS (Figure 5A). Insertion of the non-specific 'spacer' rDNA in either orientation reduced the basal luciferase levels, but not to those observed for full-length RdLuc transformants (Figure 5B). These data reveal the presence of a *cis*-element within the Rad51 5' NTS that contributes to the maintenance of low, steady-state levels of Rad51 observed in the absence of DNA damage.

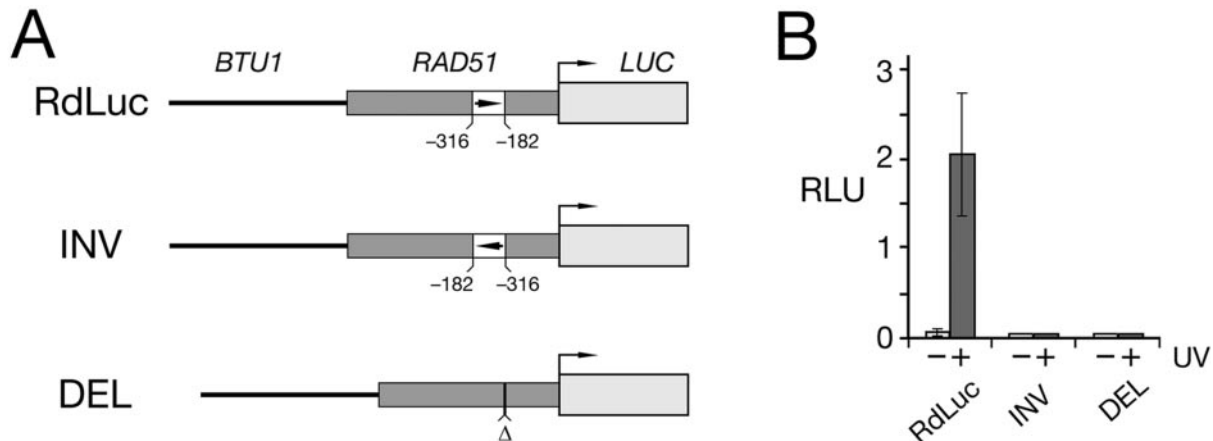


Figure 6. The induction region of the *RAD51* promoter. (A) A schematic diagram of luciferase reporter constructs expressed from the *RAD51* promoter with the 134 bp from positions -316 to -182 either inverted [RdLuc(INV)] or deleted [RdLuc(DEL)]. The reporter construct with the full length, wild-type *RAD51* promoter is also shown (RdLuc). Not drawn to scale. (B) Luciferase activity from transformants expressing the three reporter constructs in Figure 6A, with (+) and without (-) UV irradiation. Luciferase assays of whole cell lysates were as described for Figure 4.

Characterization of the *cis*-element required for *RAD51* induction

Luciferase induction by UV(c) from variously truncated reporter constructs is dependent on a relatively short *cis*-element within the Rad51 5' NTS (Figure 4). A more complete investigation of this putative DNA damage response element (DRE) is outlined below. Both inversion and deletion of the 134 bp segment between nt -316 and -182 completely eliminates any increase in the luciferase expression in response to UV(c) irradiation (Figure 6). Although luciferase is induced when full-length RdLuc transformants were treated with other DNA damaging agents, including UV(a), ionizing gamma irradiation (^{137}Cs source, 4 Gy) and methylmethane sulfonate, there is no induction in RdLuc(DEL) transformants (data not shown).

A search of the GenBank database failed to reveal any significant similarity between this sequence and any other known transcriptional promoter elements (data not shown). However, close examination of the 134 bp sequence revealed a 7 bp repeat (TTTCAAT) separated by a 14 bp. The direct repeat, identified as UV₁ and UV₂ (Figure 7A), is not found in either orientation elsewhere within the 1.3 kb Rad51 5' NTS.

The possibility that the UV₁ and/or UV₂ sequences are involved in Rad51 inducible expression was tested in a series of luciferase reporter constructs. Mutation of 5 of the 7 bp for each TTTCAAT repeat (UV₁ changed to TCCTAGG and UV₂ changed to GCTAGCT) had nearly identical effects, reducing luciferase induction in transformants by ~50% (Figure 7B). The effect of mutating both repeats in the same construct was additive, with the response to UV(c) reduced to ~10% that of the unaltered RdLuc construct. In contrast, the response to UV irradiation of reporter constructs with nucleotide substitutions at two randomly chosen sites relatively close to the UV₁/UV₂ repeats ('ATTGG' at positions -177 to -181 and 'AATATC' at positions -321 to -316) were indistinguishable from the full-length RdLuc reporter (data not shown).

The control of *RAD51* expression during meiosis

Maximal Rad51 expression in mating *Tetrahymena* is coincident with meiotic prophase, which normally occurs ~4 h

after conjugation has been initiated (5). In order to determine whether the Rad51 expression is under transcriptional control during meiosis, the kinetics of RdGFP expression during conjugation of transformants was investigated (Figure 8). Total RNA from cell lines expressing various RdGFP reporter constructs were monitored throughout the early stages of conjugation by northern-blot analysis. As an internal control, Rad51 mRNA levels (expressed from the endogenous *RAD51* locus) were also monitored in duplicate northern blots (data not shown). The results from these experiments are summarized below.

The GFP mRNA from transformants expressing the full-length RdGFP reporter, as well as from the -316 truncated version [RdGFP(-316Δ)], exhibited the same kinetics during conjugation as that of Rad51 mRNA expressed from the endogenous gene (5). In contrast, there was no marked increase in GFP mRNA during conjugation when expressed from RdGFP(-182Δ). Furthermore, mutation of the TTTCAAT direct repeat (UV₁ and UV₂; Figure 7) eliminated the dramatic increase in GFP mRNA levels during meiotic prophase. The expression pattern for these transformants closely resembled that from cells expressing RdGFP(-182Δ), as opposed to the pattern seen for RdGFP(-316Δ) transformants at the same stage of conjugation (Figure 8).

DISCUSSION

Tetrahymena transcriptional promoters

Although nuclear run-on experiments have shown that the vast majority of *Tetrahymena* genes are under transcriptional control (16), the mechanism(s) that mediate(s) this control are poorly understood. It is generally assumed that the *cis*-sequence elements and *trans*-acting factors involved in this process for ciliates are similar to those described for other eukaryotes (27–30). One of the difficulties encountered in identifying *Tetrahymena cis*-sequence elements by homology to well-characterized promoters from other model organisms is the 75% A+T content for the *Tetrahymena* genome (31). As yet, no conclusive 'TATA' or 'GC' boxes have been identified for *Tetrahymena* genes, although a handful of *cis*-elements

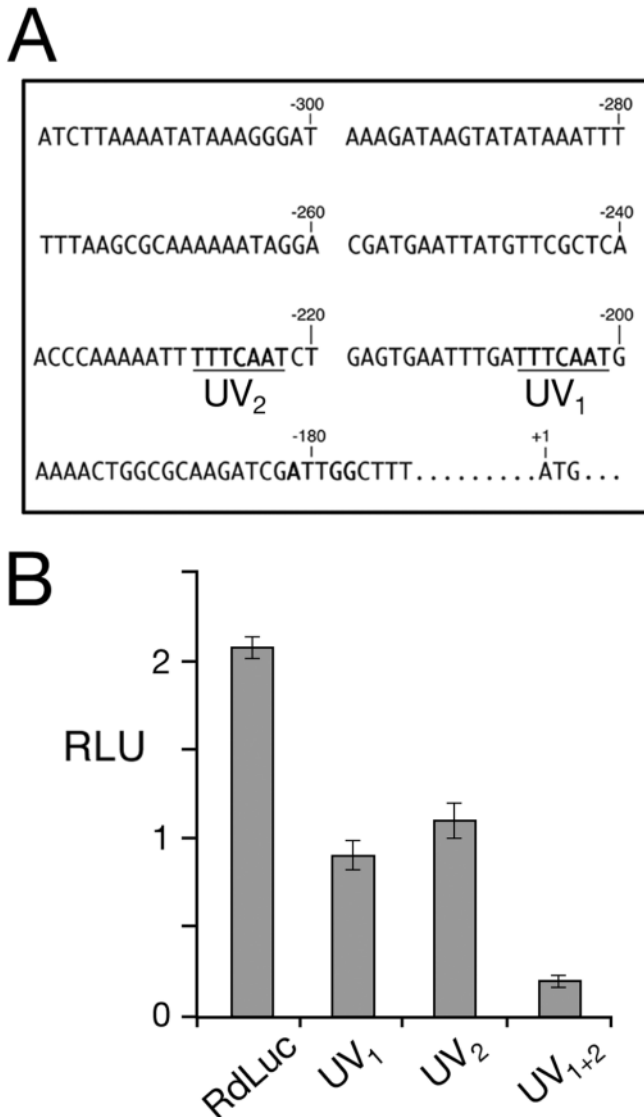


Figure 7. Analysis of a 7 bp tandem repeat within the *RAD51* promoter induction region. (A) Sequence of the *RAD51* promoter between nucleotide positions -320 and -180 is shown. Two 7 bp repeats (TTTCAAT) unique to the promoter region are underlined (UV₁ and UV₂). (B) Transformants expressing RdLuc reporter constructs that include UV₁ and UV₂ mutated singly [RdLuc(UV₁), RdLuc(UV₂)], and together [RdLuc(UV₁₊₂)] were assayed for luciferase activity 2 h after UV irradiation. Extracts from BtLuc transformants that constitutively express luciferase were used to establish the RLU. Error bars represent the standard deviation.

have been assigned that status. Characterization of the well-conserved histone genes has revealed a loose 'CCAAT' box consensus in the 5' non-transcribed sequence for *Tetrahymena* species (32–34). The *T. thermophila* telomerase RNA gene (*TER1*), a RNA polymerase III transcript (35), was found to contain a proximal sequence element at -55 (relative to the transcriptional start site) and an important A/T-rich element at -25 that are essential for the expression of a 'reporter' telomerase RNA (36).

In previous studies, the detection of foreign reporter gene expression in *Tetrahymena* has been limited to northern-blot analysis (36,37) and fluorescence microscopy (38). We have

exploited the powerful technique of stable transformation by targeted disruption of the non-essential *Tetrahymena btul-1* gene (23) to introduce a variety of luciferase reporter constructs. Our ability to quantify luciferase activity levels from transformant whole cell lysates has made it possible to characterize the *Tetrahymena RAD51* transcriptional promoter.

Once transformant cell lines under the selective pressure of 20–40 μ M paclitaxel have undergone 100% phenotypic assortment, transgenes are present at approximately the same copy number as endogenous macronuclear genes (Figure 1). The integrated reporters are positioned within macronuclear chromatin, placing it in the proper context for normal transcriptional control. This is borne out by the similar kinetics of endogenous Rad51 mRNA with those of luciferase or GFP mRNA from transformants in response to DNA damage (Figure 2) and conjugation (Figure 8). The *Tetrahymena RAD51* expression patterns (5) and transcriptional regulation appear to be similar to that from other eukaryotes. For example, the induction of *RAD51* expression in response to DNA damage, despite the presence of protein synthesis inhibitors (Figure 3), is similar to that seen for *RAD51* homologs in other eukaryotes (8,10,39).

It is unclear why there is a delay in peak levels of Rad51 mRNA when induced by UV in the presence of CHX. A possible explanation for this phenomenon is that the detection of DNA damage, and the subsequent induction of *RAD51* expression, may be coincident with a specific stage of the cell cycle. It is conceivable that a delay in the majority of cells reaching this particular stage in the cell cycle by treatment with CHX results in the observed delay (Figure 3). The UV induction of Rad51 in a culture treated with or without CHX that has been synchronized by starvation may show the same kinetics of Rad51 mRNA accumulation.

RAD51 transcriptional promoter *cis*-elements

Sequence analysis of transcriptional promoters for 10 DNA repair genes (including *RAD51*) from *Saccharomyces cerevisiae* revealed a high occurrence of MCB, HAP and UASH regulatory boxes, all of which are speculated to be involved in transcriptional regulation following irradiation (12). Increased expression of the human paralog *RAD51B* after exposure to DNA damaging agents is assumed to be mediated by 'consensus' promoter binding sites for both the AP2 and p53 proteins (40). However, due to the rapid divergence of sequences upstream of the relatively well-conserved Rad51p coding sequences, the identification of promoter elements by comparative analyses of *RAD51* homologs from yeast to ciliates to humans has not been possible.

We have identified three separate *cis*-sequence elements within the *RAD51* transcriptional promoter, not by comparative analysis, but by expression of various reporter constructs *in vivo*. The first element, positioned between -182 and -82 bp (relative to the translational start site), ensures basal levels of expression, since truncation of the *RAD51* promoter to -82 in RdLuc(-82Δ) transformants eliminates significant expression of the luciferase reporter (Figure 4). As the *RAD51* 5' terminus has been mapped to position -100 (4), this basal transcriptional promoter element is most likely contained within the 62 bp between -182 and -120 .

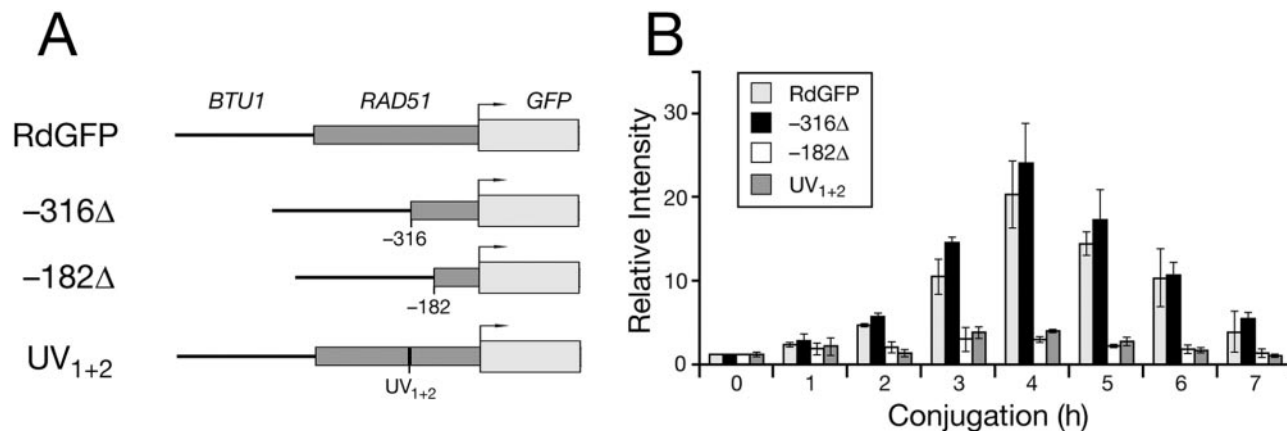


Figure 8. UV₁ and UV₂ repeats in the *RAD51* promoter are responsible for proper expression during conjugation. (A) Reporter constructs in transformants expressing GFP from the full-length, wild-type promoter (RdGFP), two promoter truncations [RdGFP(-316Δ) and RdGFP(-182Δ)], and the full-length promoter with mutated UV₁ and UV₂ repeats [RdGFP(UV₁₊₂)] are shown schematically as in Figures 1 and 4 for the RdLuc reporter. (B) GFP mRNA was monitored during the first 7 h of conjugation between transformant clonal lines. Each conjugation was repeated in triplicate. A northern blot of total RNA (10 μg per lane), isolated from each time point and hybridized with a GFP-specific radiolabeled probe, was quantified by PhosphorImager analysis. GFP mRNA levels are indicated by light grey bars for RdGFP, black bars for RdGFP(-316Δ), white bars for RdGFP(-182Δ) and dark grey bars for RdGFP(UV₁₊₂). Error bars represent the standard deviation.

A second sequence element, located within the 1000 bp between -1.3 kb and -316 bp, specifically limits Rad51 expression to relatively low levels in the absence of DNA damage or developmental signals. Progressively, larger truncations of this sequence from the 5' terminus of reporter constructs results in significantly higher basal luciferase activity, with the most pronounced increase detected at the transition between -577 and -467 (Figure 5B). These effects are not due simply to changes in the proximity of *BTUI* 5' targeting sequences in the truncation constructs, since insertion of a non-specific rDNA 3' NTS 'spacer' in either orientation does not restore luciferase activity to the low level seen in full-length RdLuc transformants (Figure 5). In lieu of a more extensive deletion analysis, this regulatory element can be broadly defined as contained within the 200 bp between -600 and -400 bp.

A third promoter element (located between -316 and -182) is required for the induction of *RAD51* expression in response to both DNA damage and meiosis (Figures 7 and 8). Within this inducible element are 28 bp (from -228 to -201) that include 7 bp direct repeats, TTCAAT, which are separated by 14 bp. Mutagenesis of the UV₁ + UV₂ repeats drastically reduces the induction of the luciferase reporter to ~10% of wild type (Figure 7). A very similar DRE has been mapped within the promoter for *RHP51*, the *Schizosaccharomyces pombe* *RAD51* homolog (9). Although DRE1 and DRE2 are not direct repeats, they are similar in length (11 bp), distance from each other (10 bp) and overall position (from -233 to -204) to those of UV₁ and UV₂ in *Tetrahymena*. The EMSA experiments revealed specific binding of two *S.pombe* proteins (59 and 45 kDa) to DRE1 and DRE2, although an increase in the presumed 'activator' proteins was not detected following DNA damage (9). A similar response is likely to exist for UV₁ and UV₂ in *Tetrahymena* cells that have incurred DNA damage or that have initiated meiosis.

In summary, *Tetrahymena RAD51* expression is controlled at the level of transcription, with levels varying in response to DNA damage and conjugation. The stable transformation of *Tetrahymena* with reporter constructs has made it possible to

define three *RAD51* promoter elements, one of which is critical to increased expression in response to these conditions. The identification of this DRE will make it possible to more completely characterize *trans*-acting factors involved in the transcriptional induction of *RAD51*, and possibly that of other genes, expressed during the early stages of conjugation in *Tetrahymena*.

ACKNOWLEDGEMENTS

This work was supported by NSF grant MCB 0220085 (D.P.R.) and NSF RU1 grant MCB 0131175 (E.S.C.).

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