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Global Gene Expression Responses of Fission Yeast to Ionizing Radiation[D]

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A coordinated transcriptional response to DNA-damaging agents is required to maintain genome stability. We have examined the global gene expression responses of the fission yeast *Schizosaccharomyces pombe* to ionizing radiation (IR) by using DNA microarrays. We identified ~200 genes whose transcript levels were significantly altered at least twofold in response to 500 Gy of gamma IR in a temporally defined manner. The majority of induced genes were core environmental stress response genes, whereas the remaining genes define a transcriptional response to DNA damage in fission yeast. Surprisingly, few DNA repair and checkpoint genes were transcriptionally modulated in response to IR. We define a role for the stress-activated mitogen-activated protein kinase Sty1/Spc1 and the DNA damage checkpoint kinase Rad3 in regulating core environmental stress response genes and IR-specific response genes, both independently and in concert. These findings suggest a complex network of regulatory pathways coordinate gene expression responses to IR in eukaryotes.

INTRODUCTION

Exposure of cells to DNA-damaging agents such as ionizing radiation (IR) poses a significant threat to genome stability. Cells have therefore evolved complex response mechanisms to maintain genetic integrity after DNA damage. These include cell cycle delay, repair of DNA damage, transcriptional responses, and programmed cell death. Because disruption of any one of these DNA damage responses can lead to genomic instability and cancer, a comprehensive understanding of the individual components and their regulation is crucial.

It is well established that many physiological responses to DNA damage are regulated at the level of gene expression. In *Escherichia coli*, the SOS response induces the expression of a network of genes after DNA damage through the regulatory LexA and RecA proteins (Friedberg *et al*., 1995). In lower eukaryotes, studies with the budding yeast *Saccharomyces cerevisiae* have identified a large number of damage-induced genes, including many involved in DNA metabolism and DNA repair (Aboussekhra *et al*., 1996; Gasch *et al*., 2001). In the distantly related fission yeast *Schizosaccharomyces pombe*, *rhp51*$^+$, *uvi15*$^+$, *uvi31*$^+$, *UVDE*$^+$, and *rhp16*$^+$ have been shown to be DNA damage inducible (Bang *et al*., 1996; Davey *et al*., 1997; Shim *et al*., 2000).

DNA damage checkpoint pathways function to delay the eukaryotic cell cycle in response to DNA damage, thus providing an opportunity for DNA repair. Central to the DNA damage checkpoint pathway are two highly conserved phosphoinositol-related kinases, Ataxia telangiectasia mutated (ATM) and ATM and *Rad3*-related (ATR) and their yeast homologs *Sp*Tel1 and *Sp*Rad3 in *S. pombe*, and *Sc*Tel1 and *Sc*Mec1 in *S. cerevisiae*. Activation of ATM and ATR kinases by DNA damage leads to cell cycle arrest through a number of downstream effector molecules including the effector kinases CHK1/*Sp*Chk1/*Sc*Chk1 and CHK2/*Sp*Cds1/*Sc*Rad53 (for review, see Nyberg *et al*., 2002). In addition to regulating the cell cycle, DNA damage checkpoints also coordinate transcriptional responses that facilitate DNA synthesis and repair. In mammalian cells, genes encoding a number of key cell cycle regulators and proteins involved in DNA synthesis and DNA repair are regulated by the DNA damage checkpoint pathway. These include p21/Cip1, 14-3-3σ, GADD45, and p53R2 and several genes encoding classical DNA repair factors (for review, see Khanna and Jackson, 2001).

In mammalian cells, the JNK and p38 stress-kinase pathways play a significant role in regulating the transcriptional stress responses to a variety of DNA damaging agents, including IR, UV light (UV), and oxidative stress. After their activation, JNK and p38 MAP kinases regulate the transcriptional responses to stress through phosphorylation and transactivation of transcription factors c-Jun (Hibi *et al*., 1993; Kyriakis *et al*., 1994), ATF2 (Gupta *et al*., 1995; Livingstone *et al*., 1995) and Elk-1 (Cavigelli *et al*., 1995; Whitmarsh *et al*., 1995). The activation of both the c-Jun NH_2-terminal kinase (JNK) and p38 stress-kinases in response to IR is dependent on the ATM checkpoint kinase (Shafman *et al*., 1995), and a role for p38γ in the DNA damage checkpoint has been identified (Wang *et al*., 2000). Thus, there seem to be significant links between the stress-kinase and the DNA

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damage checkpoint pathways in the response to ionizing radiation.

In *S. cerevisiae*, *RNR2* and *RNR3*, which encode the small and large subunits of ribonucleotide reductase, respectively (Elledge and Davis, 1987; Hurd *et al*., 1987), are transcriptionally induced in a checkpoint-dependent manner: After activation of the Mec1-Rad53-Dun1 checkpoint pathway, Crt1 (a transcriptional repressor) is hyperphosphorylated. This leads to its promoter-dissociation and the subsequent derepession of the *RNR2* gene in response to DNA damage (Zhou and Elledge, 1993; Allen *et al*., 1994; Huang *et al*., 1998). Microarray analysis has recently identified a Mec1-dependent gene expression signature in response to DNA-damaging agents that includes a large set of genes responsive to diverse environmental stresses (Gasch *et al*., 2001). A role for the DNA damage checkpoint pathway in regulating transcriptional responses to DNA-damaging agents has also been identified in fission yeast (Harris *et al*., 1996; Shim *et al*., 2000), although it is not known to what extent the checkpoint pathway coordinates damage-induced transcription in this microorganism.

In fission yeast, the Sty1/Spc1 stress-kinase is closely related to the mammalian and *Drosophila* JNK and p38 stress-kinase pathways, and to the Hog1 pathway in *S. cerevisiae* (Toone and Jones, 1998). In contrast to the Hog1 pathway in budding yeast, Sty1/Spc1 is activated in response to a wide variety of environmental stresses, including UV, methylmethane sulfonate (MMS), oxidative stress, osmotic stress, and heatshock (Millar *et al*., 1995; Shiozaki and Russell, 1995; Degols *et al*., 1996; Degols and Russell, 1997; Shieh *et al*., 1997). Sty1 coordinates the transcriptional response to stress, in part, through regulating the bZip transcription factors Atf1/Gad7 (Shiozaki and Russell, 1995; Takeda *et al*., 1995; Kanoh *et al*., 1996; Wilkinson *et al*., 1996; Gaits *et al*., 1998) and Pap1 (Toone *et al*., 1998). Deletion of these transcription factors results in altered sensitivity to a subset of stresses (Toda *et al*., 1991; Shiozaki and Russell, 1995; Degols and Russell, 1997; Nguyen *et al*., 2000; Quinn *et al*., 2002), indicating a role for these factors for modulating specific transcriptional stress responses. A central role for the Sty1 mitogen-activated protein kinase (MAPK) pathway has recently been identified in coordinating a core environmental stress response (CESR). The CESR defines a group of genes which are transcriptionally up-regulated in response to all, or most, environmental stresses, including oxidative stress, heat shock, osmotic shock, heavy metal stress, and the DNA-damaging agent MMS (Chen *et al*., 2003).

Fission yeast provide an excellent model system for studying the eukaryotic responses to DNA-damaging agents, because the cell cycle, DNA integrity checkpoint, stress-response, and DNA repair pathways are all highly conserved and well defined. The *S. pombe* genome has also been sequenced (Wood *et al*., 2002), thus facilitating a systematic analysis of global gene expression responses. We have therefore examined the global gene expression responses to IR in fission yeast, by using microarray technology. From these studies, we have defined both a general DNA damage response and a specific gamma radiation response signature for *S. pombe*, and have identified important roles for components of the DNA damage checkpoint and the stress-response pathways in regulating expression of a number of functional classes of genes in response to IR. Gene expression responses to IR in the budding yeast *S. cerevisiae*, an alternative but distantly related model eukaryote, have recently been reported. A comparison of IR-induced gene expression responses of fission yeast to those of budding yeast identifies commonalities likely to be shared among all eukaryotes.

MATERIALS AND METHODS

Cell Collection and RNA Isolation

Approximately 200 ml of asynchronously grown wild-type (WT) (*ade6-704 leu1-32 ura4-D18 h−*) cells were cultured in YEA medium at 30°C, shaking at 200 rpm until reaching OD_{600} of 0.2 (~4×10^6 cells/ml). Five OD units (25 ml) of cells were harvested by centrifugation and snap frozen in liquid nitrogen (unirradiated control) and immediately after, the remainder of the culture was exposed to 500 Gy of gamma irradiation at 12.5 Gy/min (a total of 40 min). Irradiated cells were allowed to recover for 20 or 120 min at 30°C, shaking at 200 rpm, before 5 OD units were harvested by centrifugation and snap frozen in liquid nitrogen. Asynchronously grown checkpoint mutant strains, including *rad3*Δ (*ade6-704 leu1-32 rad3::ura4*$^+$ *ura4-D18 h−*), *cds1*Δ (*leu1-32 cds::ura4*$^+$ *ura4-D18 h−*), and *chk1*Δ (*chk1::ura4*$^+$ *ura4-D18 h−*); stress-activated MAP kinase mutant *sty1*Δ (*sty1::ura4*$^+$ *ura4-D18 h−*); and a double delete *rad3*Δ*sty*Δ (*rad3::ura4*$^+$ *sty1::ura4*$^+$ *h−*) were cultured and irradiated under the same conditions. Mid-log phase wild-type (WT) cells were synchronized by centrifugal elutriation as described previously (Christensen *et al*., 2000), and G2 cells harvested before as well as after exposure to 500 Gy of gamma irradiation with further shaking 30°C at 200 rpm for 20, 50, 80, and 120 min. Each time course was repeated independently three times, and total RNA was extracted using a hot phenol method (Lyne *et al*., 2003).

Microarray Hybridization, Data Acquisition, and Visualization

Approximately 20 μg of total RNA was labeled by directly incorporating Cy-3- and Cy-5-dCTP by using Superscript (Invitrogen, Carlsbad, CA) reverse transcriptase, and the resulting cDNA was hybridized onto glass DNA microarrays containing PCR probes for 99.3% of all known and predicted fission yeast genes (for full details, see Lyne *et al*., 2003; http://www.sanger.ac.uk/PostGenomics/S_pombe/). Microarrays were scanned using a GenePix 4000B laser scanner and analyzed with GenePix Prosoftware (Axon Instruments, Foster City, CA). Unreliable signals were removed and data normalized using a Perl script (Lyne *et al*., 2003) and evaluated using Genespring software (Silicon Genetics, Redwood City, CA). Hierarchical clustering was performed with preselected log-transformed gene sets by using Cluster and Tree-View software (Eisen *et al*., 1998), with uncentered Pearson correlation and average linkage clustering.

Experimental Design

The IR time-course experiments with WT and mutant strains were performed as three independent biological repeats. For WT asynchronous, WT G2 synchronized and mutant strain time courses, labeled samples from each irradiated time point were hybridized with a labeled unirradiated sample. After data acquisition and normalization, the ratios represent the expression levels at each time point relative to the expression level of the untreated sample. Expression ratios for the biological repeats were averaged. Forty-eight microarrays in total were used in this study. The complete processed data are available from http://www.sanger.ac.uk/PostGenomics/S_pombe/

Identification of Checkpoint and Stress-dependent Genes

Gene expression was classified as dependent on checkpoint or stress-kinase genes when mean expression levels were statistically significantly different and changed by more than twofold between WT and mutant checkpoint or stress-kinase strains at the same time point. The Student's t-test was used to determine genes that were significantly different in mean expression level (p value cut-off of 0.05) and an F-test was run to determine whether the variances of the two populations were equal or unequal (p value cut-off of 0.05).

Northern Hybridization

Total RNA was extracted using the hot-phenol method described above. Northern blot analysis and hybridization of membranes to ^{32}P-labeled DNA probes were performed as described previously (Christensen *et al*., 2000). ^{32}P-Labeled probes were synthesized using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA) in accordance to the manufacturer's instructions.

Quantitative Polymerase Chain Reaction (PCR)

Approximately 20 μg of total RNA was treated with RQ1 RNAse free DNAse (Promega, Madison, WI) to remove contaminating genomic DNA, and reverse transcription was performed using Superscript (Invitrogen). Quantitative PCR (QPCR) was performed using Brilliant QPCR Core Reagent kit (Stratagene) and SYBR Green I (Molecular Probes, Eugene, OR) nucleic acid dye. Custom-made primers were designed using Primer 3 online software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi; Rozen and Skaletsky, 2000). Samples were run in duplicate on the MX4000 QPCR machine (Strat-

agene), and data were analyzed with the MX4000 software provided by the manufacturer. Only data from the log-linear portion of the amplification were chosen for analysis. Relative quantities of transcript were determined using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔCt is the difference in Ct of the gene of interest and Ct of the normalizer gene (*cdc2*); and ΔΔCt is the difference in ΔCt at time = t (irradiated) and ΔCt at time = 0 (unirradiated). Four fivefold serial dilutions of *S. pombe* genomic DNA were used to confirm that QPCR reaction efficiencies were equivalent.

RESULTS

To examine the gene expression responses to IR, the radiation dosage levels and response times were optimized for the detection of transcripts from known DNA damage-inducible genes in *S. pombe*. After exposure of asynchronously grown WT cells at doses between 100 and 500 Gy of gamma IR, the induction of *rad8*$^+$, *rhp51*$^+$, and the larger (1.9-kb) transcript of *suc22*$^+$ was observed by Northern blot hybridization (our unpublished data). From these induction profiles, an IR dosage of 500 Gy gamma-rays and sample times of 0 min (unirradiated control), 60 min (fast response), and 160 min (slow response) after radiation initiation were chosen (see MATERIALS AND METHODS).

IR-induced Gene Expression in Wild-Type Fission Yeast

Genome-wide gene expression profiles of WT (501) *S. pombe* cultures grown asynchronously were examined after exposure to 500 Gy of gamma IR by using whole-genome microarrays. Genes (204 in total) were identified that exhibited a twofold or greater change in expression levels after IR at either time points. One hundred sixty genes were induced greater than twofold, and 45 genes were repressed greater than twofold (a list of all 204 IR response genes together with gene annotations is available from our Website http://www.sanger.ac.uk/PostGenomics/S_pombe/). The stress response gene *rds1*$^+$ (Ludin *et al*., 1995) was induced greater than twofold after 60 min and repressed greater than twofold after 160 min. Only ~25% of the 160 induced genes have been characterized. The number of IR-response genes listed here should be regarded as a conservative estimate because some genes failed to make the twofold cut-off in all experiments (see MATERIALS AND METHODS).

Comparison of the IR-response genes with the *S. pombe* core environmental stress response (Chen *et al*., 2003) reveals that 108 of the 160 IR-induced genes are present in the CESR. Therefore, the majority of IR-induced genes encode proteins known or predicted to be involved in the environmental stress response, thus indicating an underlying stress response to IR (Figure 1A). IR-response genes common to the CESR include antioxidants such as *cta1*$^+$, *grx1*$^+$, *trx2*$^+$, and *gst2*$^+$ and genes involved in carbohydrate metabolism such as *tps1*$^+$, *zwf1*$^+$, and *ntp1*$^+$. These stress response genes largely exhibited similar expression profiles in response to IR: a severalfold induction was observed 60 min after initiating exposure to IR, which had returned to approximately unirradiated levels by 160 min (Figure 2A).

The remaining 52 induced IR-response genes not present in the CESR are involved in a variety of functions, including cell cycle control, signal transduction, transcriptional regulation, and cell metabolism (Table 1). Of the 52 non-CESR-induced IR genes, surprisingly few are known to be involved in DNA repair. These include genes encoding the homologous recombination proteins *rhp51*$^+$ and *rhp54*$^+$, the putative translesion DNA synthesis repair enzyme SPCC553.07c (*dinB*$^+$), and the meiotic recombination repair protein *rad50*$^+$. The transcriptional induction of *rhp51*$^+$ by a variety of DNA-damaging agents, including UV and MMS has been reported previously (Park *et al*., 1998). Notably, the mRNA levels of checkpoint genes *rad3*$^+$, *chk1*$^+$, and *cds1*$^+$ were found not to be significantly modulated in response to ionizing radiation, consistent with the finding that most checkpoint proteins are modulated posttranslationally in response to DNA damage in wild-type *S. pombe* (Humphrey, 2000).

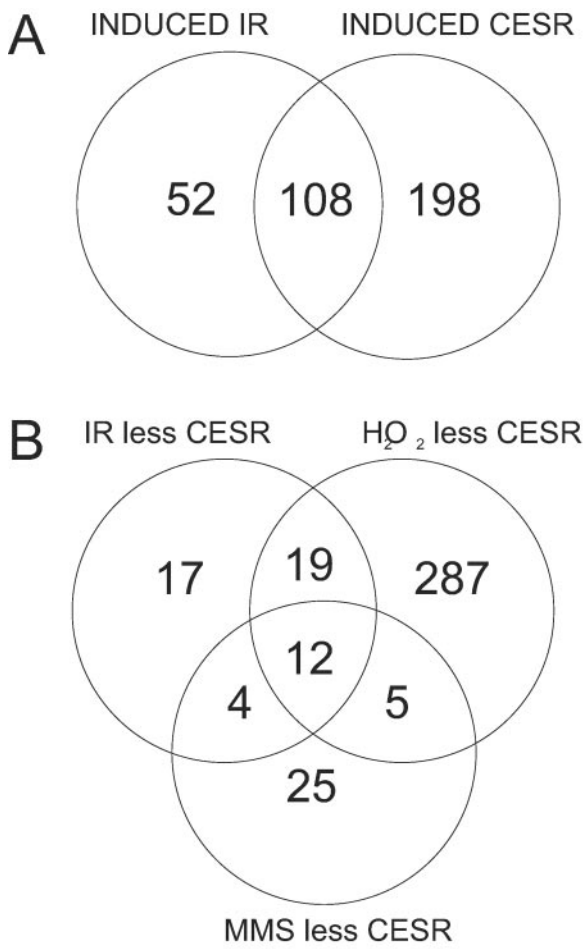

Figure 1. Induced IR genes are involved in a variety of cellular functions. (A) Venn diagram comparison of genes induced twofold or more in WT cells in response to 500 Gy of gamma IR (INDUCED IR) (this study) with genes induced in response to all, or most, environmental stresses (INDUCED CESR) (Chen *et al*., 2003). The number of genes common to these groups is shown within the overlapping circular regions. (B) Venn diagram comparison of non-CESR genes induced twofold or more in WT cells in response to 500 Gy of gamma IR (IR less CESR) (this study) with non-CESR genes induced twofold or more in WT cells in response to H_2O_2 (H_2O_2 less CESR) and non-CESR genes induced twofold or more in WT cells in response to MMS (MMS less CESR) (Chen *et al*., 2003). The number of common genes within these groups are shown within the overlapping circular regions.

Further analysis of the 52 IR non-CESR genes allows the characterization of a DNA damage-specific transcriptional response. The non-CESR transcriptional responses of *S. pombe* to DNA-damaging agents H_2O_2 and MMS (Chen *et al*., 2003) and IR (this study) were compared and 12 genes identified that were common to all three DNA-damaging agents (Figure 1B and Table 1). This list includes genes involved in the initiation of DNA replication (*cdc18*$^+$ and *cdt2*$^+$) and stress response (*trr1*$^+$).

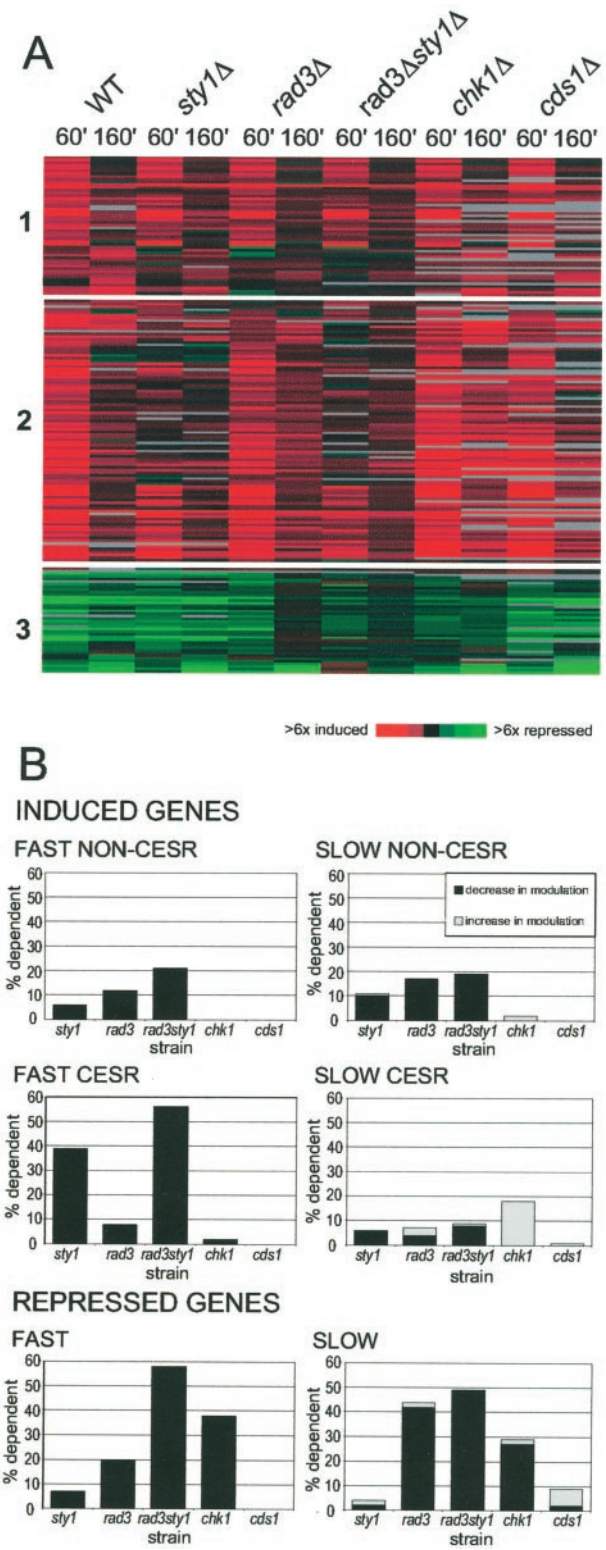

Figure 2. Regulation of gene expression during exposure to ionizing radiation. (A). The expression patterns of ~200 genes whose transcript levels changed significantly more than twofold after exposure to 500 Gy of gamma IR in WT cells are shown. The columns in the figure represent samples taken at 60 min (60′) and 160 min (160′) after IR treatment in WT, *sty1*Δ, *rad3*Δ, *rad3*Δ*sty1*Δ, *chk1*Δ, and *cds1*Δ cells. Analysis was subdivided into induced non-CESR genes (1; 51 genes), induced CESR genes (2; 96 genes), and repressed genes (3; 44 genes). Hierarchical clustering was performed as described in MATERIALS AND METHODS. The fold changes in transcript levels relative to the untreated levels are color coded with induced genes as red, repressed genes as green, no change as black, and missing data points as gray. The fold change in transcript abundance is indicated by the key at the bottom of the figure. Genes with missing data for >50% of the samples are not shown. (B) Analysis of the number of genes whose expression response to IR is dependent on the DNA checkpoint and/or stress response transduction kinases. Histograms depicting the percentage (% dependent) of induced non-CESR genes (top), CESR genes (middle), and repressed genes (bottom), which exhibited a twofold or more expression difference in *sty1*Δ, *rad3*Δ, *rad3*Δ*sty1*Δ, *chk1*Δ, and *cds1*Δ strains compared with WT after 60 min (FAST; left) and 160 min (SLOW; right) after initiating exposure to 500 Gy of gamma IR. Black bars indicate percentage where induction/repression level is one-half or less in the mutant cells compared with WT cells (decrease in modulation). Gray bars indicate the percentage where induction/repression level is twofold or more in the mutant cells compared with WT cells (increase in modulation).

The 17 induced IR-response genes whose expression was not modulated by MMS or H_2O_2 may represent an IR-specific response. However, these gene numbers can only be approximate and will change depending on the chosen cutoff. Moreover, both lists may be narrowed as more DNA-damaging agents and types of cellular stress are studied using microarray technology. For example, the DNA repair genes *rhp51*$^+$ and *dinB*$^+$, both present in the IR-specific gene list, are also induced by UV (Park *et al*., 1998) and MMS, respectively (Kai and Wang, 2003).

Of the 45 genes whose expression was repressed in response to IR, only five genes are involved the CESR. However, more than one-half show periodic cell cycle expression in G1/S (Rustici and Bähler, personal communication): Expression of the histone H4 gene (*hhf2*$^+$), found to be repressed after exposure to IR, is S-phase specific (Aves *et al*., 1985). Similarly *cdc15*$^+$, which is transcribed at the M-G1 transition (Anderson *et al*., 2002), was found to be repressed after exposure to IR, as was *eng1*$^+$, which is transcribed after septation (Martin-Cuadrado *et al*., 2003). It is likely that the repression is an indirect consequence of the IR-induced checkpoint-dependent G2-arrest in WT cells, which is expected to lead to reduced expression levels relative to unirradiated controls after DNA damage.

Confirmation and Extension of Array Data by Using QPCR

To verify the array data by a second method, and to extend the analysis in certain cases, QPCR was performed.

trr1$^+$ encodes thioredoxin reductase, which responds to redox changes, and is induced by H_2O_2 in *S. pombe* (Toone *et al*., 1998; Chen *et al*., 2003). QPCR confirmed the microarray observation that *trr1*$^+$ was induced severalfold after IR (see our Website).

dinB$^+$ is the *S. pombe* homolog of the *E. coli DinB* gene whose product is involved in transletion synthesis repair (Kai and Wang, 2003). The microarray data indicate that *dinB*$^+$ transcript levels were increased in asynchronous WT cells after exposure to IR and this up-regulation of *dinB*$^+$ in WT cells was confirmed using QPCR (see our Website).

rev1$^+$, like *dinB*$^+$, is a member of the Y-family of polymerases that are involved in error-prone translesion synthesis repair (Ohmori *et al*., 2001). Microarray data was not obtained for *rev1*$^+$ expression. QPCR, however, revealed a transcription profile for *rev1*$^+$ in which an approximately threefold increase was observed in WT cells after exposure to IR (see our Website).

Table 1. Induced IR genes not present in the CESR

Gene name	Annotation
Cell cycle control	
pyp3	Protein-tyrosine phosphatase 3
*cdt2**	Target of CDC10 transcription factor
*cdc18**	Cell division control protein 18; replication initiation
wis2	Heat shock-inducible 40-kDa cyclophilin-like protein
DNA repair	
*rhp51:rad51^*	DNA repair protein RAD51 homolog; meiotic recombination protein
*dinB^*	Translesion DNA repair polymerase
*rhp54:rad54^*	DNA repair protein RHP54
rad50	DNA repair protein, yeast RAD50 homolog
Signal transduction and transcriptional regulation	
SPCC290.04	Putative transcriptional regulator
SPBPB2B2.06c^	Putative 5′ nucleotidase family protein
SPBC21B10.13c^	Homeodomain protein
SPAC869.02c*	Probable flavohemoprotein
Stress response	
SPBC3B9.01	Protein similar to yeast YBR101C involved in resistance to H_2O_2
SPBC216.04c*	Putative transcriptional regulator
*swo1:hsp90^*	Heat shock protein 90 Homolog
*trr1:caf4**	Thioredoxin reductase
*sti1^*	Activator of HSP70 and HSP90 chaperones
Transporters	
SPBC1683.03c	MFS transporter of unknown specificity
SPBC16A3.17c*	MFS efflux transporter of unknown specificity
SPAC17C9.16c	MFS multidrug efflux transporter
*nic1^*	Nicot heavy metal ion transporter
Sulphur metabolism	
SPCC1281.07c	Protein with glutathione *S*-transferase domain
SPCC191.09c*	Putative glutathione *S*-transferase
Nucleotide metabolism	
SPAC1002.17c^	Probable uracil phosphoribosyltransferase
SPCC965.14c^	Putative cytidine and deoxycytidylate deaminase zinc-binding protein
Other metabolism	
SPBC409.13*	6,7-Dimethyl-8-ribityllumazine synthase; DMRL synthase family
SPCC24B10.20	Hypothetical short-chain dehydrogenase
SPAC26H5.09c	Hypothetical oxidoreductase
SPCC576.02*	Putative hydantoin racemase
SPAC977.14c	Putative oxidoreductase
SPCC132.04c	NAD-specific glutamate dehydrogenase
SPCC4B3.06c*	Hypothetical protein
SPBC1539.07c	Probable formaldehyde dehydrogenase
SPCC1223.09	Uricase
Sequence orphans	
SPAC17H9.18c*	Very hypothetical protein
SPAPB1A10.05^	Hypothetical protein
SPBC1289.06c^	Hypothetical protein
SPCC338.08	Hypothetical protein
Unknown function	
SPCC18B5.02c*	
SPBC1271.05c	Zinc finger ZF-AN1 protein
SPAC1399.01c	Putative purine permease
SPCC285.04^	Putative transthyretin precursor
SPAC688.03c	Conserved hypothetical protein
SPBP4H10.12	Conserved hypothetical protein
SPAC5H10.05c	NADHDH_2 Domain protein
SPBC17D11.03c*	Hypothetical protein
SPCC663.07c	pseudogene
SPAC750.01	pseudogene
SPAC186.01^	Hypothetical serine/threonine-rich protein
SPBC1685.12c^	Very hypothetical protein
SPAC29B12.13	Hypothetical protein
SPAC630.04c^	Very hypothetical protein

Annotations for Table I are from GeneDB. http://www.genedb.org/genedb/pombe/index.jsp, From the comparison of IR, H_2O_2, and MMS less CESR (see Figure 1B), genes marked with * are common to all three lists and may represent a DNA damage response, and genes marked ^ are present only in the IR list and may represent an IR-specific response.

Ribonucleotide reductase (RNR) is the rate-limiting enzyme for DNA synthesis and catalyzes the conversion of ribonucleoside diphosphates to their corresponding deoxyribonucleotides. The small subunit of RNR in *S. pombe* is encoded by the constitutively expressed *suc22*$^+$ through a 1.5-kb transcript (*suc22S*). It has been demonstrated that a second larger transcript of 1.9 kb (*suc22L*) is induced after DNA damage and heat shock (Harris *et al*., 1996). The microarray analyses used in this study cannot distinguish between the smaller and larger *suc22* transcript. By using QPCR, an 11-fold induction was observed after 160 min in asynchronous cells for the larger transcript (see our Website). The more abundant *suc22S* transcript remains relatively unchanged after IR as determined from the array, confirming that it is the larger *suc22L* transcript that becomes more abundant in response to ionizing radiation.

Regulation of IR-induced Gene Expression Responses

We wished to examine the potential roles of the DNA checkpoint and stress response pathways in regulating IR-induced gene expression responses in fission yeast. To this end, microarray analysis was performed to determine transcript levels from cultures of three DNA checkpoint deletion mutants (*rad3*Δ, *chk1*Δ, and *cds1*Δ), an MAP kinase (*sty1*Δ) deletion mutant, and a strain in which both pathways were disrupted (*rad3*Δ *sty1*Δ) after exposure to IR. Using hierarchical clustering, the expression profiles of the ~200 genes whose expression was modulated greater than twofold in asynchronous WT cells were viewed in all genetic backgrounds at both time points. The gene list was subdivided into induced non-CESR, induced CESR, and repressed IR genes, and cluster analysis was performed (Figure 2A).

To determine whether expression of a particular gene was dependent on any of the above-mentioned kinases, mean expression levels in both WT and mutant cells after exposure to IR were compared. Genes whose expression levels exhibited a ≥2-fold change in expression in the mutant relative to WT after exposure to IR were classed as "dependent" on that gene (see MATERIALS AND METHODS). The analysis was again subdivided into induced IR genes (divided into non-CESR and CESR genes) and repressed IR genes, and a complete list of all checkpoint and MAP kinase-dependent IR genes is available from our Website. The percentage of genes whose expression was found to be dependent on each particular kinase after exposure to IR was determined (Figure 2B).

Regulation of non-CESR Genes

Analysis of gene expression in strains in which the DNA checkpoint or stress-response pathways were disrupted indicated that deletion of *rad3*$^+$ or *sty1*$^+$ had only a minor effect on the induction of non-CESR genes, where 12% of induced fast response non-CESR genes were found to be modulated in a Rad3-dependent manner, and only 6% to be modulated in a Sty1-dependent manner. Similar percentages of Rad3- and Sty1-dependent non-CESR genes (17 and 10, respectively) were observed for the slow response (Figure 2A, 1; and B). From the cluster analysis, it is clear that the effect on gene expression in the *rad3*Δ *sty1*Δ double deletion strain was greater than the one seen for the single mutants (Figure 2A). The percentage of IR-induced fast-response genes whose expression was significantly reduced in the double mutant (21%) was greater than the sum of single deletion strains (Figure 2B, top), suggesting that although the DNA checkpoint and stress-response pathways seem to function largely independently, they may both contribute to the expression of particular genes in response to IR.

Table 2. Characterized checkpoint and MAP kinase-dependent induced IR genes (*cds1*Δ is omitted)

FAST				SLOW			
*sty1*Δ	*rad3*Δ	*rad3*Δ *sty1*Δ	*chk1*Δ	*sty1*Δ	*rad3*Δ	*rad3*Δ *sty1*Δ	*chk1*Δ
non-CESR							
	cdc18			*cdc18*	*cdc18*	*cdc18*	
	cdt2	*cdt2*			*cdt2*	*cdt2*	
	dinB	*dinB*			*dinB*	*dinB*	
					rhp51	*rhp51*	
	rhp54	*rhp54*					
sti1		*sti1*					
	swo1	*swo1*					*(swo1)*
CESR							
bfr1		*bfr1*					
cgs1		*cgs1*			*(cgs1)*		*(cgs1)*
	cta1	*cta1*				*cta1*	*(cta1)*
gpd1		*gpd1*					
		gpx1		*gpx1*			
gst2		*gst2*				*gst2*	
hsp16	*hsp16*	*hsp16*					*(hsp16)*
		meu8					
ntp1		*ntp1*					
		obr1					
pka1		*pka1*					*(pka1)*
		plr					
psi		*psi*					*(psi)*
ptc1		*ptc1*					
rds1		*rds1*			*(rds1)*	*(rds1)*	*(rds1)*
slt1		*slt1*					*(slt1)*
srk1		*srk1*			*(srk1)*		*(srk1)*
		tms1		*tms1*		*tms1*	
tps1		*tps1*					*(tps1)*

Genes in parentheses are those whose level of modulation after IR is increased in the mutant relative to WT.

Non-CESR genes whose expression in response to IR was determined to be Rad3-dependent included homologous recombination genes *rhp51*$^+$ and *rhp54*$^+$ and the translesion DNA synthesis repair gene *dinB*$^+$ (Table 2). These same genes show down-regulation in *rad3*Δ *sty1*Δ double mutants but were unchanged in *sty1*Δ cells compared with WT cells. These genes were however not Cds1 nor Chk1 dependent. The expression of these genes in response to IR is thus likely to be specifically regulated by a subbranch of the DNA checkpoint pathway.

A number of repressed IR genes showed significantly reduced levels of repression in response to IR in either *rad3*Δ or *chk1*Δ strains, especially at the later time point. This group again contains a number of cell cycle genes, including histone H4 gene (*hhf2*$^+$) and *eng1*$^+$, suggesting that these genes are inappropriately expressed as a result of disruption of the DNA checkpoint in these mutant strains (Figure 2A, 3; and B, bottom). Deletion of *cds1*$^+$, in contrast to deletion of *rad3*$^+$ and *chk1*$^+$, had little effect on the number of genes induced or repressed in response to IR, consistent with checkpoint dependent G2 arrest in this mutant (Murakami and Okayama, 1995).

Regulation of CESR Genes

Deletion of the stress-activated MAP kinase gene *sty1*$^+$ has a pronounced effect on the expression of IR-induced CESR genes, where the expression of 39% of the IR-induced fast

response CESR genes was reduced by more than twofold in a *sty1*Δ strain compared with wild-type (Figure 2A, 2; and B, middle). The Sty1 dependency of these CESR genes is mostly restricted to the 60-min time point after exposure to IR, consistent with a role for Sty1 in the transient expression of these genes in response to IR (Figure 2A, 2; and B, middle). The expression of a number of Sty1-dependent IR-response genes identified here had previously been shown to be *Sty*1-MAP kinase dependent after exposure to environmental stress, including *rds1*$^+$, *cgs1*$^+$, *tps1*$^+$, and *gpd1*$^+$(Chen *et al*., 2003). These same genes show down-regulation in *rad3*Δ *sty1*Δ double mutants but were unchanged in *rad3*Δ cells compared with WT cells.

Surprisingly, deletion of *rad3*$^+$ was also shown to significantly reduce expression of 8% of IR-induced fast response CESR genes (Figure 2A, 2; and B, middle), and many CESR genes showed a less than twofold reduction in expression in a *rad3*Δ strain. As observed with non-CESR genes, the percentage of CESR fast-response genes whose expression was modulated in response to IR in the double *rad3*Δ *sty1*Δ mutant (56%) was greater than the sum of the individual *sty1*Δ (39%) and *rad3*Δ (8%) mutants, again suggesting the DNA checkpoint and stress-response pathways functioned in concert to modulate particular genes in response to IR. Several uncharacterized induced CESR genes, including SPAC27D7.09c, SPAC4H3.08, and SPBC1271.08c show both Rad3 and *Sty*1 dependency (list available from our Website).

Surprisingly, some genes that are regulated in a *Sty*1-dependent manner under environmental stress conditions were found to be modulated in a Rad3-dependent manner in response to IR: Catalase, which is encoded by the *cta1*$^+$ gene in *S. pombe* and converts H_2O_2 to $H_2O + O_2$, has been previously shown to be transcriptionally regulated by the *Sty*1-MAP kinase signal pathway after oxidative stress (Toone *et al*., 1998; Nguyen *et al*., 2000). Studies here indicated *cta1*$^+$ to be rapidly and significantly (80-fold) induced in response to IR. Surprisingly, IR induction of *cta1*$^+$ was Rad3 dependent, whereas *cta1*$^+$ levels in *sty1*Δ cells were not significantly different from those of WT cells after IR.

The *hsp16*$^+$ gene encodes a polypeptide of predicted molecular mass 16 kDa that belongs to the HSP20/alpha-crystallin family. Expression of *hsp16*$^+$ in response to heat shock and nucleotide depletion is regulated via the *Sty*1-MAPK pathway (Taricani *et al*., 2001). After exposure to IR, *hsp16*$^+$ transcript levels were found to be increased approximately fourfold in WT cells, and although our data suggest that the increase is Sty1-dependent, consistent with what has been described previously, transcriptional induction was also Rad3 dependent.

Deletion of *chk1*$^+$ was also observed to significantly increase the IR-induced expression of several CESR genes at the later time point relative to WT cells (Figure 2A, 2; and B, middle). These genes include stress response genes *cgs1*$^+$, *cta1*$^+$, *hsp16*$^+$, *rds1*$^+$, *slt1*$^+$, and *tps1*$^+$ (Table 2). The IR-induced expression of these genes seems to be extended in *chk1*Δ cells compared with wild type. For example, *cgs1*$^+$ is induced approximately threefold in both WT and *chk1*Δ cells after 60 min, and in WT cells the expression level has returned to unirradiated levels after 160 min. In contrast, expression of *cgs1*$^+$ *in chk1*Δ cells is still elevated more than twofold after 160 min after exposure to IR. Unlike *rad3*Δ and *cds1*Δ mutants, *chk1*Δ cells can accumulate in S phase after IR through activation of the intra-S checkpoint. One possible explanation for these findings is that genes whose expression is elevated in *chk1*Δ at the later time point may be S-phase specific. Alternatively, these findings may point to an independent role for Chk1 in attenuating expression of these genes in response to IR.

IR-induced Gene Expression in G2 Synchronized Cells

To eliminate genes whose transcriptional "induction" after IR was an indirect result of cell cycle accumulation, we next examined the effects of irradiating cells that had been synchronized in G2. G2-synchronized cells were irradiated with 500 Gy of gamma IR and sampled at times 60, 90, 120, and 160 min and analyzed using microarrays (see MATERIALS AND METHODS). Genes (118 in total) were identified that exhibited a twofold or greater change in expression levels in G2-synchronized cells after IR. One hundred fourteen genes were induced greater than twofold, and only four genes were repressed greater than twofold in G2-synchronized cells after IR. However, there is relatively little overlap with those genes modulated in asynchronous cells after IR (Figure 3A). This was surprising because the majority (~70%) of asynchronous *S. pombe* cells are within the G2 period of the cell cycle. Fewer CESR genes are modulated in response to IR in G2 cells compared with those of asynchronous cells (Figure 3B). A complete list of genes whose expression was modulated more than twofold after irradiation of G2-synchronized cells is available from our Website.

A striking difference in the G2 and asynchronous expression profiles after IR exposure was observed for several Cdc10 targets, including *cdc22*$^+$, *cdt1*$^+$, *cdt2*$^+$, and *cdc18*$^+$ (Figure 3, C and D). These Cdc10 targets are transiently expressed during S phase (Lowndes *et al*., 1992; Kelly *et al*., 1993; Hofmann and Beach, 1994) and are under control of the DSC1 (DNA synthesis control) transcription factor (Lowndes *et al*., 1992; Hofmann and Beach, 1994; Baum *et al*., 1997). In asynchronous cells, IR induction levels of between approximately twofold (*cdc22*$^+$) and approximately fourfold (*cdt2*$^+$) after 160 min were observed (Figure 3C) and contrasted markedly with IR-induction levels of between approximately sixfold (*cdt2*$^+$) and ~30-fold (*cdc22*$^+$) in irradiated G2-synchronized cells (Figure 3D). Further analysis of the expression levels of these genes by QPCR revealed their expression levels to be significantly reduced, by between fivefold (*cdt1*$^+$ and *cdt2*$^+$) and 16-fold (*cdc22*$^+$ and *cdc18*$^+$) in the unirradiated G2 synchronized cells compared with those of unirradiated asynchronous cells (our unpublished data). Such differences in expression profiles are likely for genes that are expressed at lower levels in G2 compared with other phases of the cell cycle and are likely to account for the differences in IR expression profiles recorded between asynchronous and G2-synchronized cultures. These data raise the possibility that, in a G2 cell subjected to IR-induced damage, the checkpoint pathway activates the DSC1/Cdc10-dependent transcriptional machinery, possibly to ensure an adequate supply of factors for the DNA synthesis associated with repair.

Further examination of genes induced in G2 cells after IR revealed several genes involved in cell cycle control not identified after the IR treatment of asynchronous cells, including *smc3*$^+$ (2.5-fold induced), *pol1*$^+$ (twofold induced), *rep2*$^+$ (threefold induced), and *cig2*$^+$ (fivefold induced). The cohesin and DNA repair gene *rad21*$^+$ was also found to be induced threefold after radiation of G2-synchroninzed cultures.

DISCUSSION

IR-Response Genes

The global gene expression responses to ionizing radiation in *S. pombe* were characterized using microarray technology.

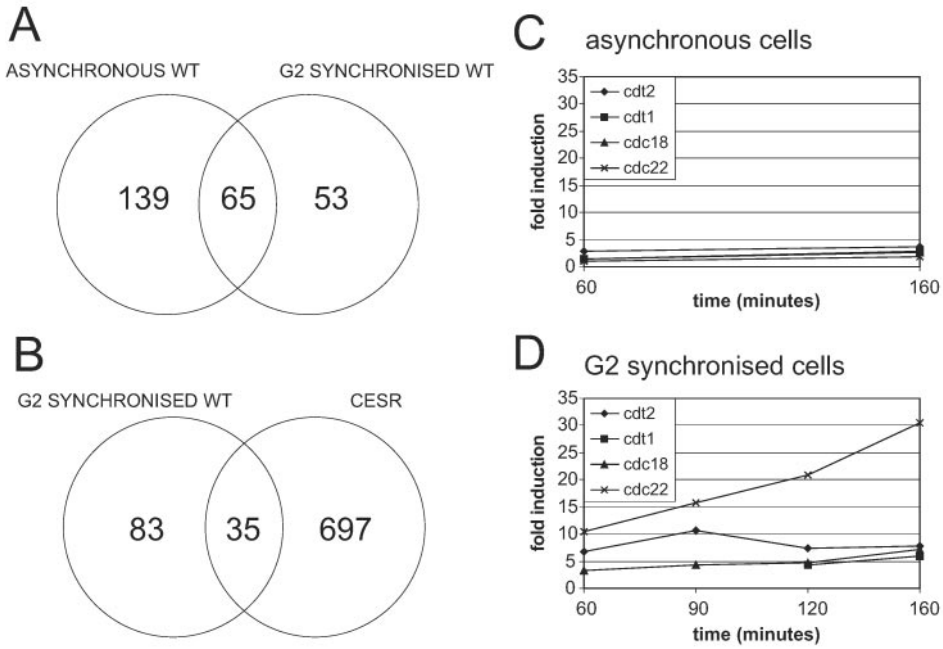

Figure 3. Comparison of gene expression patterns in asynchronous and G2 synchronized cells after exposure to 500 Gy of gamma IR. (A) Venn diagram comparison of genes induced twofold or more in asynchronous wild-type cells (asynchronous WT) with genes induced twofold or more in G2-synchronized wild-type cells (G2 synchronized WT) in response to 500 Gy of gamma IR as determined by microarray analysis. The number of common genes within these groups is shown within the overlapping circular regions. (B) Venn diagram comparison of genes induced twofold or more in G2-synchronized wild-type cells (G2 synchronized WT) in response to 500 Gy of gamma IR (this study) with CESR genes, each induced twofold or more in WT cells in response to a variety of environmental stresses (Chen *et al*., 2003). The number of common genes within these groups is shown within the overlapping circular regions. Gene identities are available from our Website. (C) Expression profiles of *cdc10*$^+$ target genes *cdt1*$^+$, *cdt2*$^+$, *cdc18*$^+$, and *cdc22*$^+$ after irradiation of asynchronous cells and (D) the expression of these genes in G2-synchronized cells.

After analysis of 99.3% of the characterized *S. pombe* genome by using this approach, a specific subset of ~200 genes was identified that exhibited a greater than twofold change in expression levels in response to IR in asynchronous cells. Independent analysis of specific genes by using QPCR was used to confirm a subset of these results. Genes whose expression was found to be induced in response to 500 Gy of gamma IR fall into a variety of predicted functional categories, including DNA damage response, cell cycle control, signal transducers, stress-response genes, and genes involved in carbohydrate, lipid, and protein metabolism. The largest category was a subset of >100 CESR genes, indicating that exposure to ionizing radiation induces a general stress response in *S. pombe*.

Comparative analyses of the ~50 induced IR genes not present in the CESR with non-CESR genes whose expression was modulated in response to H_2O_2 and MMS revealed potentially both IR-specific and DNA damage response gene expression profiles. Although the majority of these genes have yet to be characterized in fission yeast, comparative sequence analysis indicates that these IR-specific and DNA damage-specific genes are likely to function in a variety of cellular processes. An overview of IR-specific and DNA damage-specific expression profiles revealed that several genes increase induction levels at the later time point. Such transcriptional up-regulation could function to confer an adaptive response to IR and DNA damage.

IR-Response Pathways

Our data indicate an important role for the Sty1-MAP kinase in coordinating gene-expression responses to IR. A primary role would seem to be the regulation of CESR responses, where 39% of CESR genes induced at an early time point were found to be modulated in response to IR in a Sty1-dependent manner. However, it seems that Sty1 has less of a role in the transcriptional response to IR than with other stresses, where 70–90% of CESR genes were found to be Sty1-dependent (Chen *et al*., 2003). As expected, genes were identified in this study whose expression was found to be Sty1-dependent specifically in response to IR, and not other stresses, as determined by Chen *et al.* (2003), and are likely to be involved in responding to IR-specific lesions and/or stress.

Our data further indicate a role for the DNA integrity checkpoint pathway in coordinating gene expression responses to IR. Approximately 9% of all fast response-induced genes and 8% of all slow response-induced genes were identified whose expression levels were significantly modulated in a Rad3-dependent manner in response to IR. Such genes included DNA repair *rhp51*$^+$, *rhp54*$^+$, and *dinB*$^+$. This cluster of genes was regulated by Rad3, independently of Chk1 and Cds1 checkpoint kinases in response to IR, suggesting that these genes are controlled by Rad3 through distinct downstream effectors. The expression of many genes was also found to be elevated in *rad3*Δ and *chk1*Δ strains, suggesting a role for these genes in transcriptional repression of these transcripts in response to IR. However, more than one-half of these genes are transcriptionally regulated through the cell cycle, suggesting that the elevated levels of many of these transcripts may have resulted indirectly through inappropriate cell cycle advance in these checkpoint mutants after exposure to IR.

The Rad3-checkpoint and Sty1-stress kinase pathways seem to function largely independently to modulate transcript levels in response to IR. However, a number of unexpected regulatory interactions were identified between the Sty1-MAP kinase pathway and the Rad3-dependent checkpoint pathway. After exposure to IR, a role for the Rad3-dependent checkpoint pathway was identified in coordinating expression of a number of CESR or specific stress-response genes that had previously been demonstrated to be regulated by the Sty1-MAPK pathway. The most striking example was that of *cta1*$^+$, which is transcriptionally induced in response to oxidative stress in a *Sty*1-dependent manner (Wilkinson *et al*., 1996). Surprisingly, we

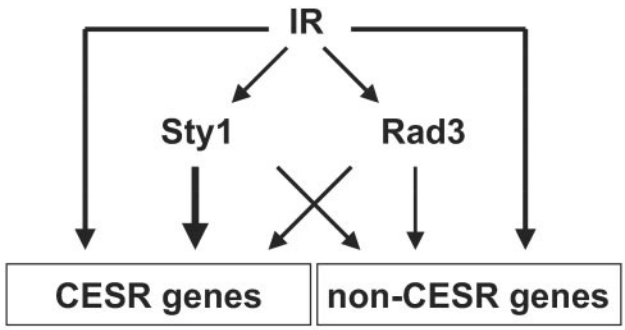

Figure 4. Regulation of gene expression in response to ionizing radiation in fission yeast. The relative contribution of the Sty1 stress response and Rad3 checkpoint kinases in coordinating CESR and non-CESR gene expression responses are indicated. Unknown regulatory pathways are indicated. See text for details.

found *cta1*$^+$ transcript levels to be significantly increased after exposure to IR in a Rad3-dependent manner, and this transcriptional response to IR was largely unaffected in *sty1*Δ cells. However, Rad3 may not be the only regulator as *cta1*$^+$ is still induced ~35-fold in *rad3*Δ cells. These results indicate that *cta1*$^+$ expression levels can be modulated by either Rad3-checkpoint or Sty1-MAPK pathways in response to different stresses.

Although important roles for the Rad3 checkpoint and Sty1-MAP kinases have been identified in modulating transcript levels in response to IR, whether these pathways function to modulate transcription or mRNA turnover is currently unknown. Moreover, the majority of CESR and non-CESR genes responding to IR did so independently of these pathways (Figure 4). Thus, understanding how the expression of these genes is regulated in response to IR remains an important goal.

Eukaryotic IR-Response Genes

An aim of this study was to define a eukaryotic IR-response signature through comparative analysis of the IR responses of fission and budding yeasts. However, the number of genes whose expression levels are altered in response to IR in budding is considerably greater than the numbers recorded in this study for fission yeast (Gasch *et al*., 2001). Thus, the functional significance of the identification of homologous genes in both yeasts whose expression is modulated by IR is unclear. Indeed, both numbers of genes and gene expression profiles may alter in response to exposure to different radiation doses in fission yeast. However, a comparison of the transcriptional responses to a number of DNA-damaging agents has identified a small group of nine genes comprising the DNA damage response signature in *S. cerevisiae* (Gasch *et al*., 2001). Functional homologs of *RAD51*, *RAD54*, *RNR2* and *RNR4*, namely, *rhp51*$^+$, *rhp54*$^+$, and *suc22*$^+$ respectively, were found to be transcriptionally induced in response to IR in fission yeast. Moreover, the IR-induced expression of these genes is regulated by the highly conserved DNA damage checkpoint pathway in both yeasts, suggesting that such conserved responses to IR represent fundamental survival pathways common to all eukaryotes. These studies further identified a role for Rad3 in modulating the expression of CESR genes in response to IR in *S. pombe*. In *S. cerevisiae*, the Mec1 pathway was similarly found to coordinate the induction of a large number of environmental stress response genes after exposure to IR (De Sanctis *et al*., 2001; Gasch *et al*., 2001), suggesting a conserved role for the DNA integrity checkpoint pathway in coordinating environmental stress response genes after exposure to IR. These CESR genes function to mount a protective response to a variety of environmental stresses, including oxidative stress. Both Rad3 and Mec1 are structurally related to the checkpoint kinase ATM, which when absent results in the disease Ataxia telangiectasia (A-T). A-T patients suffer from significant neurodegeneration associated with cerebellar ataxia in addition to immunodeficiency, premature ageing, acute radiosensitivity, and cancer predisposition (for review, see Shiloh, 2001). It has been suggested that many of the A-T features might result from elevated levels of oxidative stress in A-T cells or tissues (for review, see Barzilai *et al*., 2002). Our data suggest that one possible mechanism by which the ATM checkpoint kinase may function to coordinate the cellular response to oxidative stress in mammalian cells is through modulating the transcription of CESR genes. Understanding the underlying mechanisms by which the DNA checkpoints, stress response and other pathways function to maintain homoeostasis in response to ionizing radiation and other stresses will therefore be of significant biomedical interest.

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