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Activation of AP-1-Dependent Transcription by a Truncated Translation Initiation Factor†

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Int6/eIF3e is a highly conserved subunit of eukaryotic translation initiation factor 3 (eIF3) that has also been reported to interact with subunits of the proteasome and the COP9 signalosome. Overexpression of full-length Int6 or a 13-kDa C-terminal fragment, Int6CT, in the fission yeast *Schizosaccharomyces pombe* causes multidrug resistance that requires the otherwise inessential AP-1 transcription factor Pap1. Here we show for the first time that Int6CT acts to increase the transcriptional activity of Pap1. Microarray hybridization data indicate that Int6CT overexpression resulted in the up-regulation of 67 genes; this expression profile closely matched that of cells overexpressing Pap1. Analysis of the upstream regulatory sequences of these genes showed that the majority contained AP-1 consensus binding sites. Partial defects in ubiquitin-dependent proteolysis have been suggested to confer Pap1-dependent multidrug resistance, but no such defect was seen on Int6CT overexpression. Indeed, none of the previously identified interactions of endogenous Int6 was required for the activation of Pap1 transcription described here. Moreover, Int6CT-induced activation of Pap1-responsive gene expression was independent of the ability of Pap1 to undergo a redox-regulated conformational change which mediates its relocalization to the nucleus and expression of oxidative stress response genes. Int6CT therefore activates Pap1-dependent transcription by a novel mechanism.

AP-1-dependent transcription is important in a wide variety of biological processes and has been implicated in tumor multidrug resistance, a phenomenon that frequently hinders effective chemotherapy (7, 14). The *Schizosaccharomyces pombe int6* gene was identified through a screen for cDNAs that caused multidrug resistance when overexpressed (10). This screen also identified cDNAs encoding the previously described multidrug resistance determinant Pap1 (33), an AP-1-like transcription factor, and a partial *int6* cDNA encoding the last 121 amino acids of the protein (Int6CT). Int6-induced multidrug resistance is dependent on Pap1 and was previously shown to be associated with the up-regulation of several known Pap1-dependent transcripts (10).

Closely related to the *y*east *AP*-1-like transcription factor (Yap1) in *Saccharomyces cerevisiae*, Pap1 is central to the oxidative stress response of *S. pombe*. Following oxidative stress, Pap1 accumulates in the nucleus (34). Under nonstressed conditions, the Crm1 nuclear export factor binds a C-terminal nuclear export sequence within Pap1 and mediates its nuclear export. Upon oxidative stress, Pap1 undergoes a redox-dependent conformational change, preventing interaction between the nuclear export sequence and Crm1 (8, 36), resulting in an accumulation of Pap1 in the nucleus and activation of Pap1-dependent gene expression. Cells defective in Crm1 function exhibit Pap1-dependent multidrug resistance (20, 35), owing to the increased abundance of nuclear Pap1. In addition, *S. pombe* conditional mutants defective in various subunits of the 19S regulatory particle of the proteasome have been shown to exhibit drug resistance (15, 16, 26) that was suggested to be Pap1-dependent (26).

The highly conserved Int6 protein was identified independently in human cells as the fifth largest subunit (eIF3e) of eukaryotic translation initiation factor 3 (eIF3) (3) and has been shown to interact with the core components of this multisubunit initiation factor (1). However, *int6*Δ strains do not exhibit a dramatic defect in bulk translation initiation (5, 10), suggesting that the role of Int6/eIF3e within eIF3 may be to regulate the translation of specific transcripts under certain conditions. This view was reinforced by a recent study indicating that distinct eIF3 complexes either containing or lacking Int6/eIF3e associate with distinct mRNA subpopulations (40). Int6/eIF3e also interacts with subunits of the COP9 signalosome and the 26S proteasome (18, 38, 39) and has been proposed to regulate proteasome assembly via an interaction with Ras (39).

In this study, we have compared the transcriptional profile induced by Int6CT with that seen on overexpression of Pap1 and have investigated the mechanism of activation of AP-1-dependent transcription by this truncated translation initiation factor.

MATERIALS AND METHODS

General fission yeast methods. *S. pombe* manipulations were carried out as described elsewhere (25) using EMM2 (Edinburgh minimal medium 2) containing, where necessary, leucine and uracil at 225 μg/ml. Strains used in this study are listed in Table 1. Strains were transformed by electroporation (gene pulser; Bio-Rad, Richmond, CA) with derivatives of the vector pREP3X or pREP4X

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† Supplemental material for this article may be found at http://ec.asm.org/.

TABLE 1. *S. pombe* strains used in this study

Strain no.	Genotype	Reference and/or source
CJN18	*h⁻ leu1-32*	Laboratory stock
TP108-3C	*h⁻ leu1-32 ura4-D18 pap1::ura4*	33
CJN255	*h⁻ leu1-32 mts2-1*	15
CJN270	*h⁻ leu1-32 ade6-704*	Laboratory stock
CJN278	*h⁻ leu1-32 mts3-1*	16
HE622	*h⁻ leu1-32 ura4-294 prp4-73*	29
EHH14	*h⁻ leu1-32 ura4-D18 pap1::nmt41-pap1-GFP-leu1*	8
EHH14.C278A	*h⁻ leu1-32 ura4-D18 pap1::nmt41-pap1-C278A-GFP-leu1*	8
CJN391	*h⁻ leu1-32 ade6-704 obr1-GFP*-reporter	This study

containing the last 121 codons of *int6* (encoding Int6CT), a full-length *pap1* cDNA, or no insert (herein referred to as "vector"); inserts in these vectors are under the control of the thiamine-repressible *nmt1* promoter (23). Drug resistance was assayed after derepression of pREP3X gene expression by growth in the absence of thiamine for 17 h and plating appropriate dilutions from mid-log phase cultures onto EMM2 agar containing 10 μg/ml methyl benzimidazole-2-yl carbamate (MBC) for *pap1*$^+$ strains and 20 μg/ml MBC for *nmt41 pap1*$^+$ strains; the latter exhibit slightly higher background drug resistance than the wild type, due to increased levels of Pap1 protein (our unpublished data). Plates were incubated at 30°C for 3 to 4 days.

***obr1*-GFP reporter construction and flow cytometry.** The *sup3-5* gene (encoding a mutant tRNA that suppresses the *S. pombe ade6-704* nonsense mutation) was cloned into pCRScript SK(+) using XhoI and HindIII. The minimal *obr1* promoter was obtained by PCR from *S. pombe* genomic DNA. The sequence encoding green fluorescent protein (GFP) was obtained by PCR from pFA6a-GFP (4). A total of 20 bp of sequence from the *cdc2* 5′ untranscribed region (UTR) was inserted upstream of the GFP open reading frame (ORF). The complete construct was obtained by PCR from a mixture of the above two PCR products. This was then cloned into pCRScript SK(+) *sup3-5* using NotI and BamHI. Details of all oligonucleotide sequences are available from the authors on request.

The reporter was integrated at the *sup3* locus; the resulting strain was transformed by electroporation with pREP3X, pREP3X-*int6CT*, or pREP3X-*pap1*. Transformants were grown at 30°C for 17 h and kept either repressed by the presence of 10 μg/ml thiamine in the medium or derepressed by washing out the thiamine prior to culturing. Cells in log phase from each culture were analyzed by flow cytometry (FACScan; Becton Dickinson). The machine was calibrated such that the autofluorescence was contained within the first log decade of the detection scale. Green fluorescence data from 10,000 cells of each sample were acquired in triplicate. Data were analyzed using CellQuest software (Becton Dickinson).

Immunoblotting. Whole cell extracts were prepared as described previously (25), fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred electrophoretically to nitrocellulose membranes (Optitran BA-S 85; Schleicher & Schuell). Membranes were subsequently incubated with the appropriate primary antibodies as follows: rabbit polyclonal antibodies against Int6 (CN28) (10) used at a 1:1,500 dilution, rabbit polyclonal antibodies against Pap1 (kindly provided by Mark Toone) used at a 1:1,000 dilution, mouse monoclonal Y100 antibodies against Cdc2 (kindly provided by J. Gannon) used at a 1:1,500 dilution, and rabbit polyclonal antiubiquitin (FL-76), catalog no. sc-9133 (Santa Cruz), used at a 1:1,000 dilution. Horseradish peroxidase-conjugated secondary antibodies (Sigma) were used at a 1:1,000 dilution. Protein bands were detected using enhanced chemiluminescence (ECL; Amersham Biosciences) and quantified using Labworks software (UVP BioImaging Systems).

Microarray analysis. Global transcriptional profiles for the transformants and culture conditions shown in Table 2 were determined (all strains were *h⁻ leu1-32*). MBC was added, where appropriate, for the last 2 h of growth. Each condition was tested in triplicate.

Whole-cell RNA was extracted from mid-log phase cultures grown in the absence of thiamine at 30°C for 17 h (21) and purified using the RNeasy mini kit (QIAGEN, Valencia, CA). Total RNA (20 μg) was labeled by directly incorporating Cy-3-dCTP and Cy-5-dCTP 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 *S. pombe* genes (21) (http://www.sanger.ac.uk/PostGenomics/S_pombe/). Microarrays were scanned using a GenePix 4000B laser scanner (Axon Instruments, Foster City, CA) and analyzed with GenePix Pro software. Unreliable signals were filtered out, and data were normalized using a customized Perl script (21). Data were analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA). All processed sets will be available at http://www.sanger.ac.uk/PostGenomics/S_pombe.

To assay global gene expression upon ectopic Pap1 expression, total RNA was extracted and purified as above from *h⁻ leu1-32* transformed with pREP3X or pREP3X-*pap1* and grown to mid-log phase in the absence of thiamine at 30°C for 17 h. These samples were sent to Eurogentec (Brussels) for microarray analysis. Two replicates were used in this experiment. Data were qualitatively compared with those obtained from Sanger Institute *S. pombe* arrays using GeneSpring software. An analogous approach was taken using whole-cell RNA from *S. pombe* TP108-3C (*pap1*Δ) transformed with pREP3X or pREP3X-*int6CT* and grown in the absence of thiamine.

Northern hybridization. Total RNA was extracted as described above, separated by formaldehyde-agarose gel electrophoresis (20 μg per lane), and transferred to Hybond-N+ (Amersham Biosciences) as described previously (10). Probes were amplified by PCR from genomic *S. pombe* DNA (details of oligonucleotide sequences are available from the authors on request). Probes were radiolabeled as described previously (10).

Sequence analysis. Multiple expectation maximization for motif elicitation (MEME; http://meme.sdsc.edu/meme) analysis was carried out on sequences upstream (up to 2,000 bp) of predicted ORFs. High-scoring consensus motifs were then analyzed by a transcription element search system (TESS; http://www.cbil.upenn.edu/tess) to identify potential transcription factor binding sites. Thresholds were set as follows: maximum allowable string mismatch, 20%; minimum element length, 5 bp; minimum log-likelihood ratio score, 8.0.

Tandem affinity purification. Whole-cell extracts were prepared from 10 liters of log-phase culture by grinding cell pellets under liquid nitrogen (Retsch RM100) as a 1:1 suspension in lysis buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4 H_2O, 0.2% IGEPAL, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, and 20% glycerol plus protease inhibitors). Centrifuged lysate was further clarified using a PD10 G-25 desalting column (Amersham Biosciences) according to the manufacturer's protocol. The clarified lysate was incubated with 1 ml of pre-equilibrated immunoglobulin G Sepharose beads on a rotating wheel (16 h, 4°C). After binding, beads were washed in 3× 25 ml IPP50 buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.02% [vol/vol] IGEPAL, 10% glycerol) and packed into a Bio-Rad PolyPrep chromatography column (0.8 by 4 cm). Following 5 column volume washes with IPP50, beads were pre-equilibrated with 5 column volumes of TEV

TABLE 2. Strains and culture conditions tested in microarray analysis[a]

Transformant[b]	Microarray analysis result for:			
	pREP3X insert	Presence of 10 μg/ml thiamine?	*nmt1* promoter status	Medium contained 15 μg/ml MBC?
Vector − thiamine	None	No	Derepressed	No
Vector − thiamine + MBC	None	No	Derepressed	Yes
Int6CT − thiamine	*int6CT*	No	Derepressed	No
Int6CT − thiamine + MBC	*int6CT*	No	Derepressed	Yes
Int6CT + thiamine	*int6CT*	Yes	Repressed	No

[a] The reference sample for each condition tested was vector + thiamine.
[b] −, without; +, with.

cleavage buffer (TCB) (10 mM Tris [pH 8.0], 150 mM NaCl, 0.02% [vol/vol] IGEPAL, 0.5 mM EDTA, 10% glycerol). Beads were incubated in 5 ml TCB and 150 units of TEV protease for three hours at 16°C. One-ml fractions were then mixed with 10 μl 100 mM magnesium acetate.

Immunoblotting was used to determine which fractions contained the cleaved fusion protein. These fractions were pooled and used for the second affinity purification. Calmodulin affinity resin (Stratagene) pre-equilibrated in calmodulin binding buffer (CBB) (10 mM Tris [pH 8.0], 150 mM NaCl, 1 mM magnesium acetate, 2 mM $CaCl_2$, 10% glycerol) was resuspended 1:1 with CBB. Pooled fractions were incubated with 1 ml calmodulin affinity resin and an additional 3 μl 1 M $CaCl_2$ per 1-ml fraction (16 h, 4°C). Beads were washed in 3× 10 ml CBB (containing 0.02% IGEPAL) and packed into a Bio-Rad PolyPrep chromatography column (0.8 by 4 cm) by gravity. Following 5 column volume washes with CBB, the bound proteins were eluted into 0.5-ml fractions with 10 column volumes of calmodulin elution buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.02% Igepal, 1 mM magnesium acetate, 20 mM EGTA) and stored at −80°C.

Pooled, positive fractions (again identified by immunoblotting) were concentrated by trichloracetic acid precipitation, washed in acetone, and resuspended in 15 μl 10 mM Tris (pH 8.0), 150 mM NaCl, and 10% glycerol (16 h, 4°C). A total of 5 μl 4× NuPAGE LDS sample buffer (Invitrogen) was added, and the samples were run on 4 to 12% gradient polyacrylamide gels (Invitrogen). Gels were stained with Sypro Ruby protein stain (Bio-Rad). Bands of interest were excised, washed twice in 100 μl 50 mM ammonium bicarbonate in 50% H_2O, 50% acetonitrile followed by a wash in 100% acetonitrile, dried in a SpeedVac (Thermo Finnigan), and rehydrated with 250 ng trypsin (Promega) in 20 μl 50 mM ammonium bicarbonate followed by 16 h of digestion at 37°C. Samples were acidified with 1 μl formic acid prior to analysis on a Micromass Q-ToF (quadruple time of flight) micromass spectrometer. Data were compared using Mascot (www.matrixscience.com) against the SwissProt database with parameter settings as follows: peptide tolerance, ±0.2 Da; tandem mass spectrometry tolerance, ±0.2 Da; peptide charge state, +1, +2, +3; variable modification, methionine oxidation; no taxonomy restriction.

Analysis of ubiquitin-proteasome pathway. *mts2-1* and *mts3-1* transformants were grown to mid-log phase at 26°C for 18 h in the absence of thiamine. Cultures were split in two with one half remaining at 26°C for a further 4 h and the other being shifted to 36°C for 4 h, after which cells were harvested and processed for immunoblotting with antiubiquitin antibodies. Wild-type cells were derepressed at 26°C for 22 h and were not subjected to a temperature shift.

RESULTS

Induction of Pap1-dependent drug resistance by the C-terminal region of Int6/eIF3e. The minimal region of Int6/eIF3e known to be able to induce multidrug resistance is Int6CT (10). To investigate whether Int6CT-induced resistance like that caused by overexpression of full-length Int6/eIF3e was dependent on Pap1, *S. pombe* strains CJN18 (wild type) and TP108-3C (*pap1*Δ) were transformed with the thiamine-repressible vector pREP3X or pREP3X-*int6CT*, grown in the absence of thiamine and tested for their resistance to MBC. Int6CT-induced drug resistance was indeed dependent on the presence of Pap1 (Fig. 1A).

In line with its role as a subunit of a translation initiation factor, Int6 is a cytoplasmic protein in *S. pombe* (10). As a first step towards characterization of the mechanism by which Int6CT induces Pap1-dependent drug resistance, we determined the subcellular localization in live cells of an Int6CT-GFP fusion expressed from pREP3X (Fig. 1B). The fusion protein, which conferred a level of MBC resistance comparable to that induced by untagged Int6CT (our unpublished results), was uniformly distributed throughout the cell, suggesting that Int6CT-induced drug resistance could involve interaction with nuclear and/or cytoplasmic components.

Tandem affinity purification (TAP) tagging (28) and mass spectrometry were used to compare proteins associated with full-length Int6/eIF3e (expressed as a C-terminal TAP tag fusion from the *int6* locus) with those associated with Int6CT-TAP (expressed from pREP3X at a level sufficient to induce drug resistance). Full-length Int6-TAP interacted with multiple components of the eIF3 complex (Fig. 1C), though no interactions with either COP9 signalosome or proteasome subunits

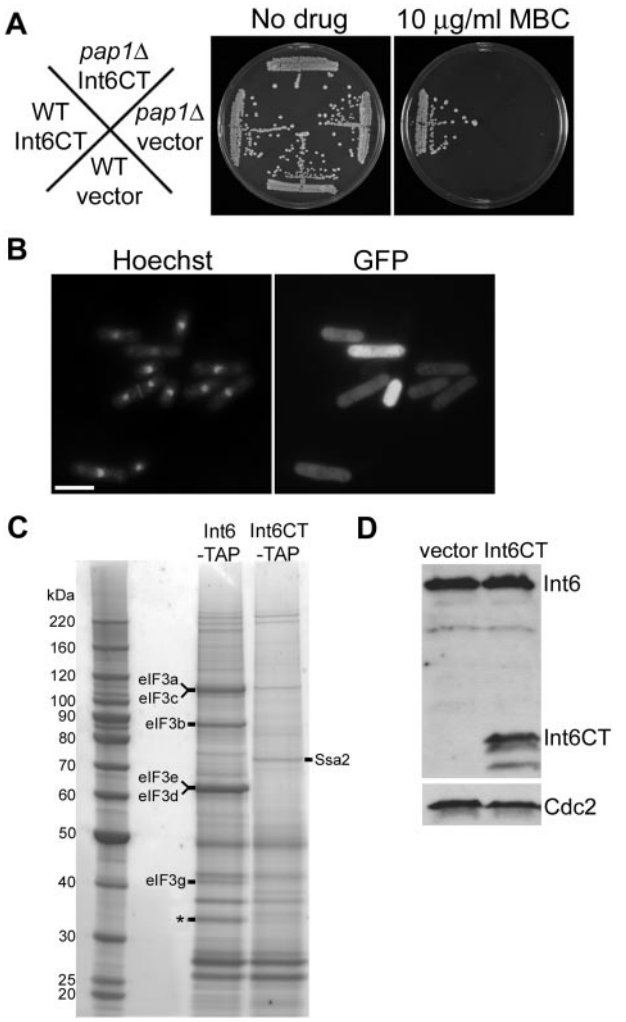

FIG. 1. Overexpressed Int6CT causes Pap1-dependent drug resistance but does not associate with eIF3 subunits or influence endogenous Int6/eIF3e levels. (A) Transformants of *S. pombe* CJN18 (WT; *pap1*$^+$) or TP108-3C (*pap1*Δ) containing either pREP3X (vector) or pREP3X-*int6CT* (Int6CT) were derepressed and streaked onto minimal agar plates with or without 10 μg/ml MBC. Plates were photographed after 4 days of incubation at 30°C. (B) *S. pombe* CJN18 was transformed with pREP3X-int6CT-GFP and grown in EMM2 lacking thiamine at 30°C for 17 h. Live cells harvested by centrifugation were stained with Hoechst 33342 and examined by fluorescence microscopy to reveal DNA and septa (Hoechst, left panel) and GFP fluorescence (right panel). Bar, 10 μm. (C) Lysates of approximately 10^{11} cells of strains expressing either Int6-TAP from the genomic *int6* locus or Int6CT-TAP from the multicopy plasmid pREP3X, as indicated, were subjected to tandem affinity purification. The purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, stained with Sypro Ruby, and identified by mass spectrometry following trypsin digestion. Protein bands identified unambiguously are indicated. The band marked with an asterisk was not identified. (D) Transformants of *S. pombe* CJN18 containing either pREP3X (vector) or pREP3X-*int6CT* (Int6CT) were grown in EMM2 lacking thiamine at 30°C for 17 h. Whole-cell lysates were subjected to immunoblotting using antibodies against Int6 (upper panel) or Cdc2 (loading control; lower panel).

were detected. No interactions with eIF3 subunits were detected with Int6CT-TAP; the only Int6CT-TAP-interacting protein identified by this approach was the Hsp70 homolog Ssa2. These data suggest that the drug resistance induced by Int6CT is unlikely to result from sequestration of proteins that normally bind to full-length Int6/eIF3e. Given that the phenotypes induced by overexpression of Int6/eIF3e and Int6CT were indistinguishable, a further possibility was that Int6CT affected the expression level of endogenous Int6/eIF3e, but immunoblotting showed that this was not the case (Fig. 1D).

Overexpression of Int6/eIF3e causes the up-regulation of several known Pap1-responsive mRNAs (10). As Int6/eIF3e is primarily a component of a translation initiation factor (Fig. 1C), and mutations in genes encoding other such factors have been shown to affect mRNA stability (30), it was important to address the possibility that Int6CT overexpression might increase the stability of Pap1-responsive mRNAs. We therefore measured the half-life of mature mRNA of the SPBC409.13 gene, which is up-regulated in response to both Int6CT and Pap1 overexpression (see below). To make these measurements possible, the production of mature mRNA was shut off using the temperature-sensitive *prp4-73* mutation, which rapidly abolishes pre-RNA splicing following a shift to the restrictive temperature (17). This approach allowed a more complete and specific inhibition of mature mRNA production in *S. pombe* than was possible using other temperature-sensitive mutants or chemical inhibitors of transcription (our unpublished data). Following the induction of either Pap1 or Int6CT in a *prp4-73* strain and the shift to the restrictive temperature, we monitored SPBC409.13 mRNA decay by Northern blotting (Fig. 2A). The rate of decay was similar in cells that had been induced to express SPBC409.13 by Int6CT or Pap1 overexpression (Fig. 2B). Overexpression of Pap1 would be expected to increase target expression at the level of transcription initiation rather than mRNA stabilization; these data therefore indicate that the Int6CT-induced up-regulation of SPBC409.13 mRNA was not due to stabilization of this target transcript.

To investigate further whether Int6CT-induced up-regulation of mRNAs was due to an increase in Pap1 transcriptional activity, a GFP reporter was constructed (Fig. 2C) using the minimal promoter of the Pap1 responsive gene *obr1* (32). The construct was integrated into the genome of *S. pombe* h^- *leu1-32 ade6-704* (CJN270) to create the *obr1-GFP*-reporter strain. This was transformed with pREP3X, pREP3X-*int6CT*, or pREP3X-*pap1*, the plasmid-borne genes were derepressed (or kept repressed), and their overexpression was monitored by immunoblotting (Fig. 2D). Overexpression of Pap1 caused increased expression of GFP (as measured by flow cytometry), presumably due to the increased abundance of active transcription factor. Overexpression of Int6CT also elevated GFP expression, although not as potently as Pap1 overexpression (Fig. 2E). Strains with *int6CT* or *pap1* expression repressed by the addition of thiamine to the growth medium showed no detectable green fluorescence above background. Although Int6CT overexpression activated Pap1-dependent transcription, this was not associated with an increase in the abundance of Pap1 protein (Fig. 2F).

Int6CT overexpression activates a wide variety of genes. Genome-wide expression analyses were performed on *S. pombe* CJN18 transformed with pREP3X or pREP3X-*int6CT*,

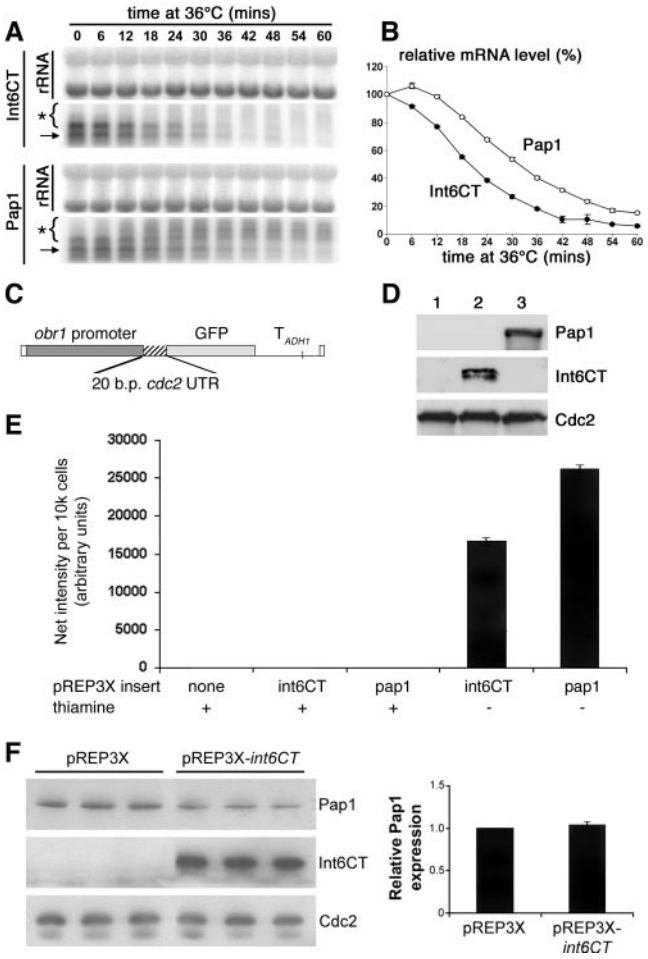

FIG. 2. Int6CT overexpression causes Pap1-dependent drug resistance by activating Pap1-dependent transcription. (A) *S. pombe* h^- *leu1-32 ura4-294 prp4-73* (HE622) was transformed with pREP3X-*int6CT* (Int6CT) or pREP3X-*pap1* (Pap1), and transformants were grown to mid-log phase for 21 h at the permissive temperature, 25°C, in the absence of thiamine. The cultures were shifted to the restrictive temperature, 36°C, and grown for a further hour. A total of 5×10^7 cells were harvested every 6 min from the time of temperature shift and were processed for Northern blotting. The blots were probed to detect SPBC409.13 mRNA. Intensities of bands corresponding to mature SPBC409.13 mRNA (arrow) were normalized to rRNA levels and related to the band intensity at the time of temperature shift. SPBC409.13 pre-mRNA species (asterisks) accumulated after the temperature shift-induced inactivation of Prp4. (B) Quantification of data from panel A from three independent experiments. Error bars show standard deviations. (C) Schematic of *obr1-GFP* reporter construct (not to scale). GFP was placed under the control of the minimal *obr1* promoter, with 20 bp of *cdc2* 5′ UTR upstream of the ATG codon. (D) Expression of Int6CT and Pap1 in the *obr1-GFP*-reporter strain (CJN391) was confirmed by immunoblotting with anti-Int6 and anti-Pap1 antibodies. CJN391 was transformed with pREP3X (lane 1), with pREP3X-*int6CT* (lane 2), or with pREP3X-*pap1* (lane 3). Cdc2 was used as a loading control. (E) Detection of green fluorescence by flow cytometry from the reporter strains used in panel D grown in the presence or absence of 10 μg/ml thiamine (repressed or derepressed, respectively). The error bars represent the standard errors from three independent experiments. (F) Int6CT overexpression does not increase Pap1 protein levels. Levels of Pap1 protein from *S. pombe* CJN18 transformed with pREP3X or pREP3X-*int6CT* and derepressed were assayed by immunoblotting with the antibodies used in panel D (left panel; three independent experiments) and quantified (right panel; error bars represent standard errors from six independent experiments).

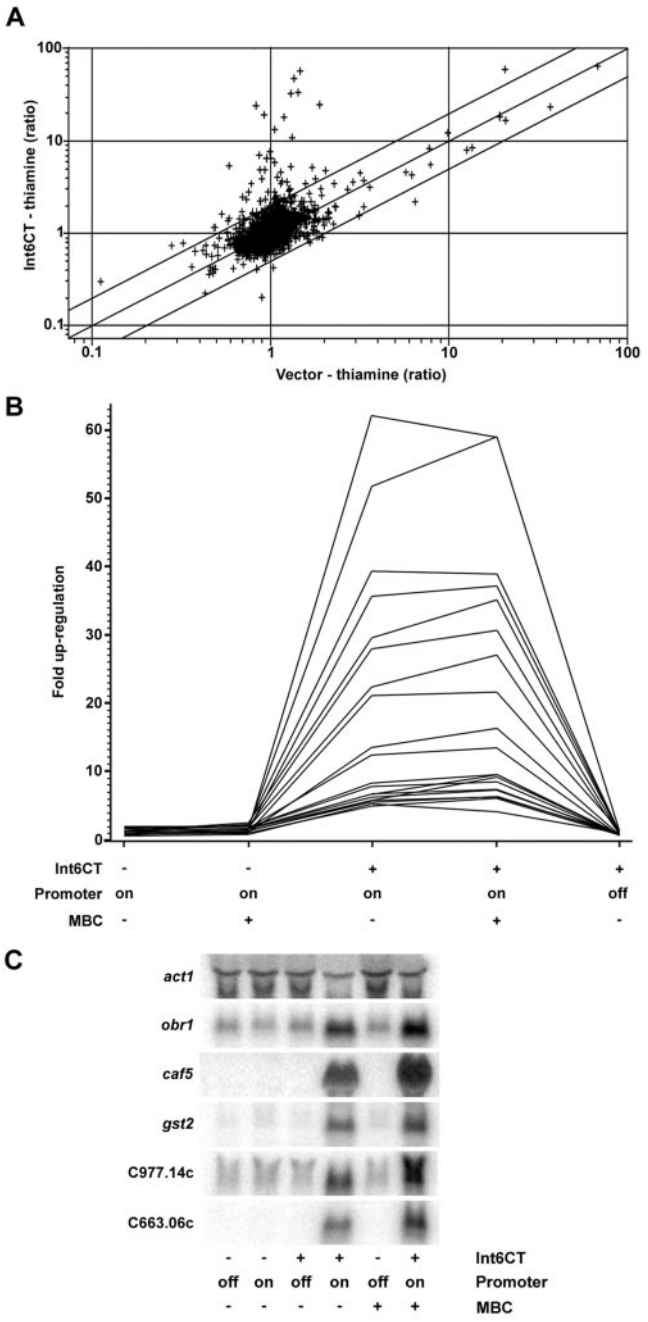

FIG. 3. Overexpression of Int6CT causes up-regulation of a wide variety of stress response genes. (A) Identification of genes up-regulated due to the absence of thiamine from the culture medium. The scatter plot indicates the overlap between the global transcriptional profiles of derepressed (-thiamine) *S. pombe* CJN18 transformed with pREP3X (vector) or pREP3X-*int6CT* (Int6CT). The axes denote the signal ratios for the indicated strains relative to the common reference (vector with thiamine). Each data point represents a single gene and is the mean of three independent replicates. Solid diagonal lines indicate twofold cutoffs. (B) Signal ratios of the 20 genes most highly up-regulated by Int6CT across all conditions tested (presence [+] or absence [−] of Int6CT and MBC; *nmt1* promoter on or off). Each line joins the data for a single gene, with data points being derived as in panel A. (C) Northern hybridization analysis of representative genes identified from microarray experiments, using the culture conditions tested in panel B. Actin mRNA (*act1*) was used as a loading control.

TABLE 3. Genes up-regulated due to thiamine removal from growth medium

Gene name	Annotation[a]
thi2 (*nmt2*)	Thiazole biosynthetic enzyme
bsu1	MFS efflux transporter
SPBC530.07c	Hypothetical protein
thi3 (*nmt1*)	Pyrimidine precursor biosynthesis enzyme
SPBP8B7.18c	Putative thiamine biosynthesis protein
pho4	Thiamine-repressible acid phosphatase precursor
SPAC9.10	Putative amino acid permease
thi4	Probable thiamine biosynthetic bifunctional enzyme
SPBC1604.04	Thiamine pyrophosphate transporter
SPCC162.03	Short-chain dehydrogenase
mfm2	M-factor precursor 2
SPCC31H12.06	Hypothetical protein
SPCC794.03	Putative amino acid permease
SPAC29A4.12c	Hypothetical protein
SPBP8B7.30c	Putative transcriptional regulator
SPBC21H7.03c	Thiamine-repressible acid phosphatase precursor
SPCC18B5.05c	Pyrimidine kinase involved in thiamine biosynthesis
zym1	Metallothionein
SPBC36.02c	Membrane transporter of unknown specificity
snz1	Involved in pyridoxine metabolism

[a] Annotations are from Gene DB (http://www.genedb.org/genedb/pombe/index.jsp).

grown in the presence or absence of thiamine and/or MBC (Table 2). Each growth condition was compared with a common reference, CJN18 transformed with pREP3X and grown in the presence of thiamine.

Upon combining the data from the growth conditions studied, a large number of genes were found to be significantly up-regulated (for details of statistical tests, see reference 21. Some of these were up-regulated due to the absence of thiamine from the growth medium; these genes (along with all others that were unaffected by Int6CT overexpression) exhibited similar expression profiles in "vector without thiamine" and "Int6CT without thiamine" (Fig. 3A) and could be discounted by subtracting the gene list obtained from vector without thiamine from that obtained from Int6CT without thiamine. Twenty genes were found to be significantly up-regulated specifically due to the absence of thiamine (Table 3).

The remaining 67 genes were up-regulated specifically as a result of overexpression of Int6CT (Table 4). The up-regulation of these genes only occurred if *int6CT* was present in the vector and derepressed by thiamine removal, as indicated in the graphical representation of the behavior of the 20 most highly up-regulated genes across all conditions tested (Fig. 3B). Of the 67 genes, 23 have functions potentially related to the multidrug resistance phenotype induced by Int6CT overexpression, such as roles in drug efflux, antioxidant, and detoxification pathways. Of the remaining 44 genes, 6 may function in signal transduction and transcriptional regulation, 2 are involved in the regulation of the cell cycle and spore formation, 24 are involved in cellular metabolism, and 10 are yet to be characterized. Twenty-four of the 67 genes are also induced in the core environmental stress response (CESR) (see Table S1

TABLE 4. Genes up-regulated due to Int6CT overexpression

Gene name	Annotation[a]	Pap1-responsive[b]
Transporters		
caf5	MFS multidrug efflux transporter	+
nic1	High-affinity nickel transport protein	+
bfr1	Brefeldin A resistance protein; ABC transporter	+
SPCC417.10	Putative allantoate permease; MFS transporter	
pmd1	ABC transporter family	+
SPBC1683.03c	MFS transporter of unknown specificity	+
SPAC11D3.05	MFS membrane transporter of unknown specificity	
Antioxidant and detoxification		
gst2	Glutathione *S*-transferase II	+
gst1	Glutathione *S*-transferase I	+
SPCC1281.04	Pyridoxal reductase homolog	
SPCC1281.07c	Putative glutathione *S*-transferase	+
tpx1	Thioredoxin peroxidase	+
pgr1	Glutathione reductase	+
SPCC737.06c	Putative glutamate-cysteine ligase regulatory subunit	+
gsa1	Glutathione synthetase large chain	+
trr1	Thioredoxin reductase	+
Flavin metabolism		
SPBC23G7.10c	Putative NADH-dependent flavin oxidoreductase	+
SPAC869.02c	Probable flavohemoprotein	+
SPBC2A9.02	Putative dyhydroflavanol-4-reductase	+
obr1	Flavodoxin	+
SPBC409.13	6,7-Dimethyl-8-ribityllumazine synthase synthase family	+
SPAP27G11.09	Putative GTP cyclohydrolase, possible riboflavin biosynthesis	+
Metal detoxification		
ccs1	Copper chaperone for Sod1 (superoxide dismutase)	+
Signal transduction and transcriptional regulation		
SPAC22G7.08	Probable serine-threonine protein kinase; ion homeostasis	+
hhp2	Serine/threonine protein kinase	+
SPBC16G5.02c	Putative ribokinase	+
SPBC2D10.04	Conserved hypothetical; arrestin family	+
SPBC1271.05c	Zinc finger ZF-AN1 protein	+
SPBC651.09c	RNA polymerase II associated Paf1 complex	+
Cell cycle and differentiation regulation		
cut1	Separase	
isp7	Sexual differentiation protein	
Other metabolism		
SPCC663.06c	Putative short-chain dehydrogenase protein	+
SPCC663.08c	Putative short-chain dehydrogenase protein	+
SPCC24B10.20	Hypothetical short-chain dehydrogenase	+
SPBC215.11c	Putative oxidoreductase; aldo-keto family	+
SPAC977.14c	Putative oxidoreductase	+
SPAC513.07	Putative cinnamoyl-CoA reductase	+
SPAC2E1P3.01	Putative zinc binding dehydrogenase	+
plr1	Pyridoxal reductase	+
SPBC16A3.02c	Putative quinone oxidoreductase	+
SPAC26F1.04c	Zinc binding dehydrogenase (predicted)	+
SPAC5H10.05c	NADHDH_2 domain protein	+
SPAC513.06c	Probable dimeric dihydrodiol dehydrogenase	+
SPAC186.03	L-asparaginase precursor	
SPAC9E9.09c	Aldehyde dehydrogenase (predicted)	+
SPBC4F6.16c	ER disulfide oxidoreductase	+
SPBC115.03	Oxidoreductase	
SPAC1952.08c	Pyridoxamine 5′-phosphate oxidase (predicted)	+
SPAC1782.01	Similar to yeast Ecm29 cell wall stucture/biosynthesis protein	+
itt1	Conserved hypothetical C3HC4-type zinc finger protein; Ub E3 ligase (predicted)	
pdf1	Palmitoyl-protein thioesterase	+
SPBC725.01	Aspartate aminotransferase, mitochondrial	+

Continued on following page

TABLE 4—*Continued*

Gene name	Annotation[a]	Pap1-responsive[b]
SPBC21D10.11	Aminotransferase class V; probable cysteine desulfurase	
SPBC1773.03c	Aminotransferase	
zwf1	Glucose-6-phosphate 1-dehydrogenase	+
Unknown function		
SPAC27D7.10c	Hypothetical protein; pombe specific	+
SPAC6G10.03c	Hypothetical protein	+
SPAC977.01	Hypothetical protein	
SPAC17D11.03	Hypothetical protein	+
SPAC688.03c	Hypothetical protein	+
SPBC337.10c	Conserved hypothetical protein	+
SPAC14C4.05c	Hypothetical protein	+
SPCC550.01c	Hypothetical protein	+
SPCC188.09c	Predicted cell surface-localized, serine/threonine-rich	
Sequence orphan		
SPBC1289.06c	Hypothetical protein	+
Pseudogenes		
SPAC750.01	Oxidoreductase pseudogene	+
SPCC663.07c	Short-chain dehydrogenase pseudogene	+

[a] Annotations are from Gene DB (http://www.genedb.org/genedb/pombe/index.jsp). ER, endoplasmic reticulum; NADHDH_2, NADH dehydrogenase 2; Ub, ubiquitin.
[b] +, also up-regulated on Pap1 overexpression.

in the supplemental material), with 50% of these being involved in cellular metabolism. It should be noted that the definition of the CESR stipulated the induction in at least four of the stress conditions tested (9); it is possible that additional Int6CT-inducible genes are involved in multiple stress responses but were excluded by this strict definition of the CESR.

Genes representative of the various families up-regulated by Int6CT overexpression were selected as follows: *obr1*, encoding a flavodoxin; *caf5*, a multifacilitator superfamily (MFS) drug transporter; *gst2*, glutathione *S*-transferase II; SPAC977.14c, a putative oxidoreductase, and SPCC663.06c, a putative short chain dehydrogenase. The transcripts of these genes were examined by northern hybridization across the various conditions tested in the microarray analysis. Consistent with the microarray data, induction of these genes was dependent on Int6CT overexpression (Fig. 3C). The basal level of expression for several of these genes was very low, in line with the huge fold increases in expression indicated by the microarray analysis.

In contrast to the results of a previous study (9) in which more than 400 genes were found to be repressed in response to at least three types of cellular stress (the repressed CESR genes), only 3 genes were found to be reproducibly down-regulated upon Int6CT overexpression, none of which was previously identified as a repressed CESR gene. Int6CT overexpression therefore caused induction of stress response genes, without the concomitant reduction in the expression of genes usually repressed following cellular stress.

To investigate whether *S. pombe* mounted a transcriptional response to the presence of MBC, the drug was added to the growth medium for the last 2 h of growth before cells were harvested, and the total RNA from these cells was also subjected to microarray analysis. In both the presence and the absence of overexpressed Int6CT, exposure to MBC did not cause a significant difference in global transcription profiles (Fig. 3B).

Most Int6CT-induced genes have upstream AP-1-like sites. The sequences upstream and downstream of the ORFs of the 67 genes induced by Int6CT were analyzed in order to ascertain whether they contained any common regulatory elements. Because the UTRs of most *S. pombe* genes have not yet been mapped, sequences of up to 2 kb flanking each ORF were extracted from the *S. pombe* genome database (http://www.sanger.ac.uk/PostGenomics/S_pombe). These were grouped according to position relative to the ORF (5′ or 3′) and to the effect of Int6CT overexpression on the gene and then subjected to MEME analysis. Several common motifs were revealed (see Table S2 in the supplemental material), and in order to identify potential transcription factor binding sites within the motifs, they were subjected to further sequence analysis using the transcription element search system TESS. The only known binding site identified by this process was located upstream (but not downstream) from the initiator ATG codons of up-regulated genes and was the consensus AP-1 site TTAGTCA to which Pap1 has been shown to bind (31). The sequences upstream of the ORFs of the 20 most highly up-regulated genes were examined for the presence of this site. In addition, because Pap1 can bind to AP-1 sites other than TTAGTCA (19), the frequency of the binding site for the *S. cerevisiae* Pap1 ortholog Yap-1 (TTAGTAA) in these upstream sequences was also investigated.

Of the 67 genes induced by Int6CT (Table 4), 25 possessed the consensus AP-1 binding site upstream of their ORFs. Of the 20 most highly up-regulated genes, 11 possessed at least one AP-1 consensus site and 13 possessed at least one Yap1-binding site (Fig. 4A). These sites are both 7 bp long and so would be expected to occur by chance once in approximately every 16 kb. The sequences upstream of 20 of the genes shown to be up-regulated due to thiamine removal (Table 3) were

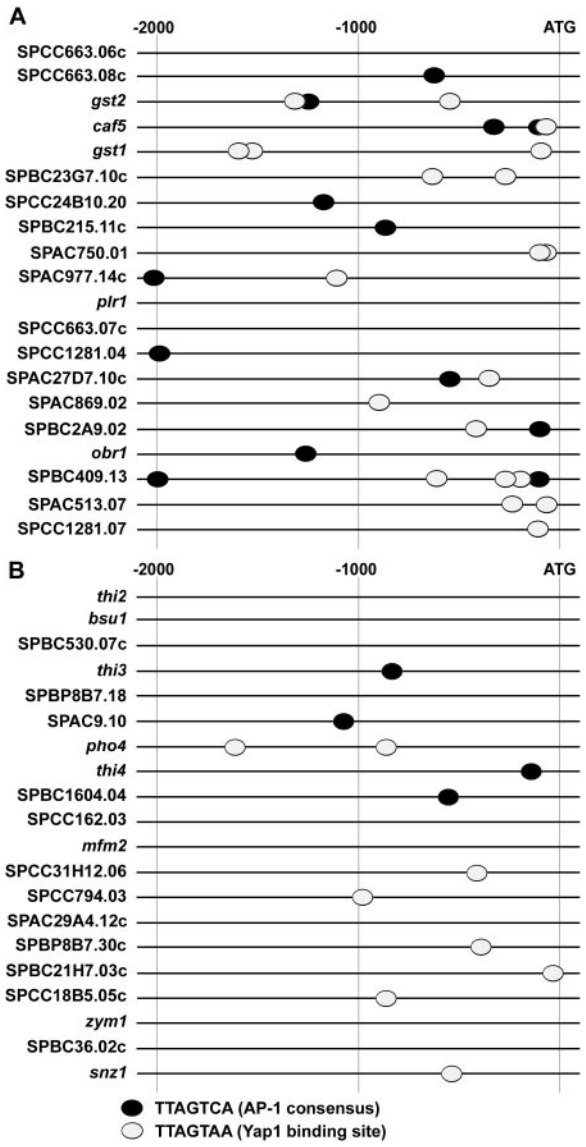

FIG. 4. Genes up-regulated on Int6CT overexpression have an overrepresentation of AP-1-like sites in their putative upstream regulatory regions. (A) Position and frequency of Pap1 and Yap1 consensus-binding sites in sequences up to 2 kb upstream of the initiator codon of the top 20 genes up-regulated due to Int6CT overexpression. (B) Position and frequency of the same sites in sequences up to 2 kb upstream of the initiator codon of the genes up-regulated due to the absence of thiamine.

then searched to see if these sites were equally abundant. Only four genes possessed one AP-1 consensus site, and seven genes possessed one or more Yap-1 binding sites within sequences up to 2 kb upstream of their ORFs (Fig. 4B). This indicated that the high density of AP-1-like sites shown in Fig. 3A is nonrandom and suggested that these genes are likely to be activated by an AP-1-like transcription factor, presumably Pap1.

Pap1 overexpression and Int6CT overexpression induce similar transcript profiles. Due to the high frequency of potential AP-1 binding sites in the regulatory regions of genes activated by Int6CT and the Pap1-dependence of Int6CT-induced multidrug resistance, further microarray experiments were performed to investigate whether the drug resistance caused by Pap1 overexpression (33) could be attributed to a similar pattern of gene expression. *S. pombe* h^- *leu1-32* was transformed with pREP3X or pREP3X-*pap1*, and transformants were grown in the absence of thiamine to allow comparison of the presence or absence of overexpressed Pap1 protein. The expression profile upon Pap1 overexpression was determined using two replicates, allowing semiquantitative comparison with the effects of Int6CT overexpression.

Of the 67 genes induced by Int6CT (Table 4), 55 were also up-regulated in response to Pap1 overexpression, with 19 of the top 20 genes responding similarly in terms of relative signal ratios. Pap1 overexpression appeared to be slightly more potent in up-regulating these common targets, consistent with the results of the *obr1*-GFP reporter assay (Fig. 2E).

The Int6CT-induced transcriptional profile is entirely Pap1-dependent. Although it was known that the multidrug resistance phenotype associated with Int6CT overexpression was Pap1-dependent (Fig. 1A), the transcription of several genes listed in Table 4 appeared to be affected by Int6CT overexpression but not by Pap1 overexpression. We therefore investigated whether or not Int6CT overexpression could activate any genes in the absence of Pap1. *S. pombe* TP108-3C (*pap1*Δ) was transformed with pREP3X or pREP3X-*int6CT*, and transformants were grown in the absence of thiamine. Whole-cell RNA was harvested and used for microarray hybridization. None of the 67 genes, including those unaffected by Pap1 overexpression, was affected by Int6CT overexpression in this strain (our unpublished results). These data indicate that all the transcriptional effects seen on Int6CT overexpression in wild-type cells were mediated by activation of Pap1; Int6CT had no apparent influence on other pathways of transcriptional activation.

Int6CT-induced drug resistance is independent of the COP9 signalosome and Ras. Int6 has been reported to interact with subunits of the COP9 signalosome (18, 38) and has also been implicated in the regulation of proteasome assembly via the Ras pathway (39). However, overexpression of Int6CT still caused substantial drug resistance in strains deleted for Csn1, Csn2, Csn4, or Csn5 and in the Ras mutant, *ste5*$^-$ (our unpublished results), indicating that neither the COP9 signalosome nor Ras is essential for Int6CT-induced drug resistance.

Int6CT overexpression does not influence bulk polyubiquitinylation. As several conditional proteasome mutants have been shown to exhibit multidrug resistance, we examined the activity of the proteasome in cells overexpressing Int6CT. Cell extracts were analyzed by immunoblotting for the accumulation of polyubiquitinylated proteins. The temperature-sensitive mutants *mts2-1* and *mts3-1* were used as positive controls as they had already been shown to accumulate polyubiquitinylated proteins at the restrictive temperature (15). It was particularly of interest to see if the level of proteins marked for degradation at the permissive temperature (where the cells are viable and drug resistant) was similar to that in Int6CT overexpressing cells. *S. pombe mts2-1* and *mts3-1* were transformed with pREP3X, and an isogenic *mts*$^+$ control strain was transformed with pREP3X or pREP3X-*int6CT*. Immunoblotting clearly showed the accumulation of polyubiquitinylated proteins in the *mts* mutants after growth at the restrictive temper-

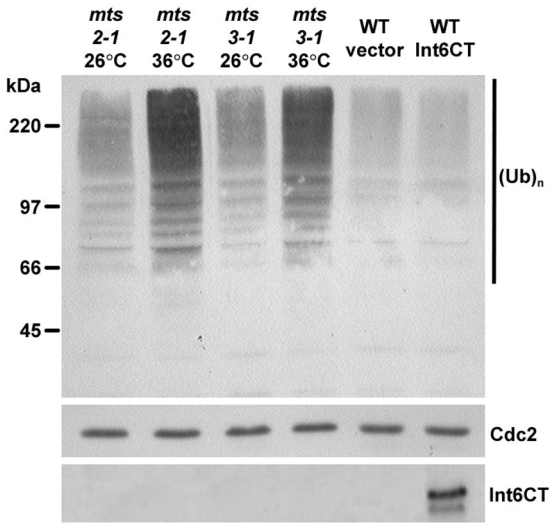

FIG. 5. Int6CT overexpression does not perturb the ubiquitin-proteasome pathway. Abundance of polyubiquitylated proteins [indicated by $(Ub)_n$] in derepressed *S. pombe* CJN18 (WT/*mts*$^+$) transformed with pREP3X (vector) or pREP3X-*int6CT* (Int6CT) and *S. pombe h*$^-$ *leu1-32* CJN255 (*mts2-1*) and CJN278 (*mts3-1*) grown at the permissive temperature (26°C) and the restrictive temperature (36°C) was examined by immunoblotting with antiubiquitin antibodies. Int6CT overexpression was confirmed by immunoblotting with anti-Int6 antibodies; Cdc2 served as a loading control.

ature (Fig. 5). Overexpression of Int6CT did not cause an accumulation of polyubiquitinylated proteins above wild-type levels, and these levels were also lower than those of the *mts* mutants at the permissive temperature. The overexpression of Int6CT had a mild additive effect on the multidrug resistant phenotype of the proteasome mutants (our unpublished results). These data suggest that the mechanism by which Int6CT overexpression causes multidrug resistance is not analogous to that proposed for the *mts* mutants (26).

Int6CT-induced multidrug resistance is independent of the ability of Pap1 to respond to oxidative stress. *S. pombe* cells that are subjected to oxidative stress relocalize Pap1 to the nucleus to activate Pap1-dependent transcription (34). Previous microscopy data from our laboratory suggested that Pap1 (tagged with GFP) did not accumulate in the nucleus upon Int6 overexpression (10). However, these conclusions were reevaluated in the light of a recent study of Pap1 regulation following oxidative stress (36), which demonstrated that this nuclear relocalization is extremely transient, lasting approximately 5 to 10 min.

Derepression of the *nmt1* promoter takes approximately 14 to 16 h and is therefore not as abrupt a stimulus as the addition of H_2O_2 to culture medium; therefore any potentially analogous effect of Int6CT overexpression on Pap1 localization would be markedly more gradual and difficult to detect by fluorescence microscopy. Fortunately it was possible to address this question using a mutant form of Pap1. Cysteine residue 278 is critically required for the reversible oxidation and nuclear accumulation of Pap1; mutation of this residue to alanine (Pap1-C278A) prevents Pap1 nuclear accumulation in response to oxidative stress (8).

S. pombe EHH14 (*pap1-GFP*) and EHH14.C278A (*pap1-C278A-GFP*) were transformed with pREP4X or pREP4X-*int6CT* and derepressed by growth in the absence of thiamine. Int6CT overexpression was confirmed by immunoblotting (our unpublished results). Resistance to 20 μg/ml MBC was induced by Int6CT overexpression in both *pap1-GFP* and *pap1-C278A-GFP* (Fig. 6), indicating that Int6CT-induced drug resistance is independent of redox regulation of Pap1 nuclear accumulation.

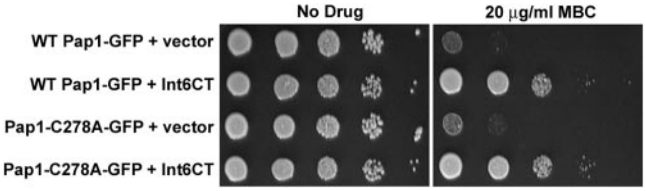

FIG. 6. Int6CT-induced Pap1 activation is independent of redox-regulated Pap1 nuclear accumulation. Tenfold serial dilutions of mid-log phase cultures of derepressed *S. pombe* strains EHH14 (WT Pap1-GFP) and EHH14.C278A (Pap1-C278A-GFP) transformed with pREP3X (vector) or pREP3X-*int6CT* (Int6CT) were spotted onto minimal agar plates with or without 20 μg/ml MBC. Plates were photographed after 4 days of incubation at 30°C.

DISCUSSION

This study demonstrates the activation of AP-1-dependent transcription by the ectopic expression of a truncated translation initiation factor, Int6CT. Earlier studies (1, 10) pointed towards full-length Int6 being a component of the multisubunit translation initiation factor eIF3, a view supported here by our TAP-tagging data (Fig. 1C). While our earlier experiments showed that Int6-induced drug resistance in *S. pombe* was genetically dependent on *pap1*$^+$, at that time it seemed most likely that the resistance was mediated through altered translation (10). For example, Int6 overexpression might have favored the translation of mRNAs transcribed in a *pap1*-dependent manner. We have shown in this study that overexpression of Int6CT caused *pap1*-dependent multidrug resistance in *S. pombe* (Fig. 1A), which resulted from an increase in *pap1*-dependent transcription, rather than the stabilization of *pap1*-responsive mRNAs (Fig. 2). Pap1 overexpression caused the transcriptional up-regulation of a variety of stress response-related genes. Remarkably, Int6CT overexpression induced a transcriptional profile almost identical to that seen upon Pap1 overexpression (Table 4), and a large number of the target genes possess potential AP-1 binding sites in their 5′ flanking regions (Fig. 4). Indeed, in the absence of Pap1, overexpression of Int6CT did not result in the up-regulation of any of the 67 genes shown in Table 4. Thus, genes lacking consensus Pap1 sites in their promoter regions are also likely to be Pap1-dependent. Preliminary studies indicate that Pap1 is also able to bind the Yap1 site in vitro (our unpublished results); by extension, it seems likely that a variety of related sites are bound in vivo.

Since Pap1 is normally activated in response to oxidative stress, it is pertinent to ask whether Int6CT expression simply induces a stress condition to which Pap1 responds. If this were the case, the stress would appear to be quite specific, as the vast majority of *S. pombe* cDNAs (including, for example, a

cDNA encoding only the N-terminal region of Int6) fail to induce Pap1 activation, as judged by the induction of drug resistance (10). Furthermore, a single missense mutation in the sequence encoding Int6CT can abolish Pap1 activation without altering the steady-state level of Int6CT protein (E. Rawson and C. J. Norbury, unpublished data). The failure of Int6CT expression to trigger the pattern of transcriptional repression that characterizes *S. pombe* cells exposed to a variety of stresses (9) also argues against the establishment of a constitutively stressed state. Instead, Int6CT appears able to trigger specifically the Pap1 activation arm of the oxidative stress response in the absence of other aspects of this response.

How then does Int6CT activate Pap1? Previously, Pap1-responsive gene expression has been shown to be activated by a variety of pathways, resulting in a pattern of drug resistance also seen when Pap1 is overexpressed (33). Conditional mutations in subunits of the 19S proteasome regulatory particle cause MBC resistance, and reduced proteolysis of Pap1 has been proposed as the mechanism for this resistance (26). Impairment of Pap1 nuclear export, as a consequence of mutations in Crm1 or Pap1, results in Pap1-dependent drug resistance due to the accumulation of Pap1 in the nucleus (6, 20, 35). Intriguingly, Pap1 activation by Int6CT overexpression does not appear to involve any of these mechanisms; Pap1 protein levels remain unchanged following Int6CT induction (Fig. 2F), and the ubiquitin-proteasome pathway is apparently unaffected (Fig. 5). Int6CT-induced Pap1 activation is also independent of the COP9 signalosome and the Ras pathway (our unpublished data), with which endogenous Int6 has been linked previously (18, 38, 39), and independent of the recently described oxidative regulation of Pap1 localization (36); Fig. 6).

The possibility that Int6/eIF3e, an accessory component of a multisubunit translation factor, might also act to modulate AP-1-dependent transcription is an intriguing one, but at this stage it is unclear whether the activation of Pap1 by Int6CT represents a normal physiological mechanism. Fluorescence microscopy indicates that the majority of Int6/eIF3e is cytoplasmic in *S. pombe* (10) and that the protein is relocalized to granular structures following cellular stress (13). The function of these stress granules, which also contain other translation factors and RNA, is not yet established in fission yeast; however, analogous structures in human cells have been proposed to participate in recovery from stress by determining the fate of stalled translation initiation complexes (2). In the light of these findings, it is conceivable that Int6 directs the selective translation of a Pap1 coactivator under stressed conditions. Direct interaction between Int6 and transcription factors in the nucleus may also be possible; a significant proportion of Int6 is nuclear in human cells (37) and has been found to associate with the human T-cell leukemia virus transactivator Tax (11). It is therefore conceivable that Int6CT interacts directly with Pap1 to influence its activity, but our tandem affinity purification studies of Int6CT and Pap1 have not revealed any interactors other than heat shock proteins, which are common artifacts of this technique (Fig. 1C). We cannot rule out the possibility that a putative Int6CT-Pap1 interaction is either insufficiently stable to withstand the purification process or occurs in an insoluble subcellular fraction. A further possibility is that the selective activation of Pap1 is a consequence of biochemical properties of the Int6CT fragment that do not relate to the physiological role of the full-length Int6 protein. An analogous activity has been ascribed to pathogenic variants of the human huntingtin protein that include long polyglutamine tracts (12); such proteins are capable of inhibiting Sp1 and $TAF_{II}130$-dependent transcription, even though the wild-type huntingtin protein is not considered to function as a transcriptional regulator. Discrimination between the various possible modes of Int6CT-mediated activation of Pap1 will require further study.

Murine *Int-6* was first identified as a gene truncated by integration of mouse mammary tumor virus (MMTV) proviral DNA in mammary cancers and a preneoplastic outgrowth (22). Intriguingly the MMTV-truncated *Int-6* alleles, which can induce malignant transformation in transfection assays (24, 27), encode Int6/eIF3e proteins lacking the C-terminal region corresponding to the Int6CT fragment used in this study. It will now be worthwhile to investigate the possible involvement of transcriptional changes resulting from *Int-6* truncation in these MMTV-induced tumors.

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REFERENCES

1. **Akiyoshi, Y., J. Clayton, L. Phan, M. Yamamoto, A. G. Hinnebusch, Y. Watanabe, and K. Asano.** 2001. Fission yeast homolog of murine Int-6 protein, encoded by mouse mammary tumor virus integration site, is associated with the conserved core subunits of eukaryotic translation initiation factor 3. J. Biol. Chem. **276:**10056–10062.
2. **Anderson, P., and N. Kedersha.** 2002. Stressful initiations. J. Cell Sci. **115:** 3227–3234.
3. **Asano, K., W. C. Merrick, and J. W. Hershey.** 1997. The translation initiation factor eIF3-p48 subunit is encoded by int-6, a site of frequent integration by the mouse mammary tumor virus genome. J. Biol. Chem. **272:**23477–23480.
4. **Bahler, J., J. Q. Wu, M. S. Longtine, N. G. Shah, A. McKenzie, III, A. B. Steever, A. Wach, P. Philippsen, and J. R. Pringle.** 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast **14:**943–951.
5. **Bandyopadhyay, A., T. Matsumoto, and U. Maitra.** 2000. Fission yeast Int6 is not essential for global translation initiation, but deletion of $int6^+$ causes hypersensitivity to caffeine and affects spore formation. Mol. Biol. Cell. **11:**4005–4018.
6. **Benko, Z., C. Fenyvesvolgyi, M. Pesti, and M. Sipiczki.** 2004. The transcription factor Pap1/Caf3 plays a central role in the determination of caffeine resistance in Schizosaccharomyces pombe. Mol. Genet. Genomics **271:**161–170.
7. **Bonovich, M., M. Olive, E. Reed, B. O'Connell, and C. Vinson.** 2002. Adenoviral delivery of A-FOS, an AP-1 dominant negative, selectively inhibits drug resistance in two human cancer cell lines. Cancer Gene Ther. **9:**62–70.
8. **Castillo, E. A., J. Ayte, C. Chiva, A. Moldon, M. Carrascal, J. Abian, N. Jones, and E. Hidalgo.** 2002. Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. Mol. Microbiol. **45:**243–254.
9. **Chen, D., W. M. Toone, J. Mata, R. Lyne, G. Burns, K. Kivinen, A. Brazma, N. Jones, and J. Bahler.** 2003. Global transcriptional responses of fission yeast to environmental stress. Mol. Biol. Cell. **14:**214–229.
10. **Crane, R., R. Craig, R. Murray, I. Dunand-Sauthier, T. Humphrey, and C. Norbury.** 2000. A fission yeast homolog of Int-6, the mammalian oncoprotein and eIF3 subunit, induces drug resistance when overexpressed. Mol. Biol. Cell. **11:**3993–4003.
11. **Desbois, C., R. Rousset, F. Bantignies, and P. Jalinot.** 1996. Exclusion of Int-6 from PML nuclear bodies by binding to the HTLV-I Tax oncoprotein. Science **273:**951–953.

12. **Dunah, A. W., H. Jeong, A. Griffin, Y. M. Kim, D. G. Standaert, S. M. Hersch, M. M. Mouradian, A. B. Young, N. Tanese, and D. Krainc.** 2002. Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. Science **296:**2238–2243.
13. **Dunand-Sauthier, I., C. Walker, C. Wilkinson, C. Gordon, R. Crane, C. Norbury, and T. Humphrey.** 2002. Sum1, a component of the fission yeast eIF3 translation initiation complex, is rapidly relocalized during environmental stress and interacts with components of the 26S proteasome. Mol. Biol. Cell. **13:**1626–1640.
14. **Eferl, R., and E. F. Wagner.** 2003. AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer **3:**859–868.
15. **Gordon, C., G. McGurk, P. Dillon, C. Rosen, and N. D. Hastie.** 1993. Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. Nature **366:**355–357.
16. **Gordon, C., G. McGurk, M. Wallace, and N. D. Hastie.** 1996. A conditional lethal mutant in the fission yeast 26 S protease subunit mts3+ is defective in metaphase to anaphase transition. J. Biol. Chem. **271:**5704–5711.
17. **Gross, T., K. Richert, C. Mierke, M. Lutzelberger, and N. F. Kaufer.** 1998. Identification and characterization of srp1, a gene of fission yeast encoding a RNA binding domain and a RS domain typical of SR splicing factors. Nucleic Acids Res. **26:**505–511.
18. **Hoareau Alves, K., V. Bochard, S. Rety, and P. Jalinot.** 2002. Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome. FEBS Lett. **527:**15–21.
19. **Jones, R. H., S. Moreno, P. Nurse, and N. C. Jones.** 1988. Expression of the SV40 promoter in fission yeast: identification and characterization of an AP-1-like factor. Cell **53:**659–667.
20. **Kumada, K., M. Yanagida, and T. Toda.** 1996. Caffeine-resistance in fission yeast is caused by mutations in a single essential gene, crm1$^+$. Mol. Gen. Genet. **250:**59–68.
21. **Lyne, R., G. Burns, J. Mata, C. J. Penkett, G. Rustici, D. Chen, C. Langford, D. Vetrie, and J. Bahler.** 2003. Whole-genome microarrays of fission yeast: characteristics, accuracy, reproducibility, and processing of array data. BMC Genomics **4:**27.
22. **Marchetti, A., F. Buttitta, S. Miyazaki, D. Gallahan, G. H. Smith, and R. Callahan.** 1995. *Int-6*, a highly conserved, widely expressed gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. J. Virol. **69:** 1932–1938.
23. **Maundrell, K.** 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:**127–130.
24. **Mayeur, G. L., and J. W. Hershey.** 2002. Malignant transformation by the eukaryotic translation initiation factor 3 subunit p48 (eIF3e). FEBS Lett. **514:**49–54.
25. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. **194:**795–823.
26. **Penney, M., C. Wilkinson, M. Wallace, J. P. Javerzat, K. Ferrell, M. Seeger, W. Dubiel, S. McKay, R. Allshire, and C. Gordon.** 1998. The Pad1+ gene encodes a subunit of the 26 S proteasome in fission yeast. J. Biol. Chem. **273:**23938–23945.
27. **Rasmussen, S. B., E. Kordon, R. Callahan, and G. H. Smith.** 2001. Evidence for the transforming activity of a truncated Int6 gene, in vitro. Oncogene **20:**5291–5301.
28. **Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin.** 1999. A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. **17:**1030–1032.
29. **Schmidt, H., K. Richert, R. A. Drakas, and N. F. Kaufer.** 1999. spp42, identified as a classical suppressor of prp4-73, which encodes a kinase involved in pre-mRNA splicing in fission yeast, is a homologue of the splicing factor Prp8p. Genetics **153:**1183–1191.
30. **Schwartz, D. C., and R. Parker.** 1999. Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of mRNAs in *Saccharomyces cerevisiae*. Mol. Cell Biol. **19:**5247–5256.
31. **Shimanuki, M., Y. Saka, M. Yanagida, and T. Toda.** 1995. A novel essential fission yeast gene pad1$^+$ positively regulates *pap1*$^+$-dependent transcription and is implicated in the maintenance of chromosome structure. J. Cell Sci. **108:**569–579.
32. **Toda, T., M. Shimanuki, Y. Saka, H. Yamano, Y. Adachi, M. Shirakawa, Y. Kyogoku, and M. Yanagida.** 1992. Fission yeast pap1-dependent transcription is negatively regulated by an essential nuclear protein, crm1. Mol. Cell Biol. **12:**5474–5484.
33. **Toda, T., M. Shimanuki, and M. Yanagida.** 1991. Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. Genes Dev. **5:**60–73.
34. **Toone, W. M., S. Kuge, M. Samuels, B. A. Morgan, T. Toda, and N. Jones.** 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. Genes Dev. **12:**1453–1463.
35. **Turi, T. G., P. Webster, and J. K. Rose.** 1994. Brefeldin A sensitivity and resistance in Schizosaccharomyces pombe. Isolation of multiple genes conferring resistance. J. Biol. Chem. **269:**24229–24236.
36. **Vivancos, A. P., E. A. Castillo, N. Jones, J. Ayte, and E. Hidalgo.** 2004. Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. Mol. Microbiol. **52:**1427–1435.
37. **Watkins, S. J., and C. J. Norbury.** 2004. Cell cycle-related variation in subcellular localization of eIF3e/INT6 in human fibroblasts. Cell Prolif. **37:**149–160.
38. **Yahalom, A., T. H. Kim, E. Winter, B. Karniol, A. G. von Arnim, and D. A. Chamovitz.** 2001. Arabidopsis eIF3e (INT-6) associates with both eIF3c and the COP9 signalosome subunit CSN7. J. Biol. Chem. **276:**334–340.
39. **Yen, H. C., C. Gordon, and E. C. Chang.** 2003. Schizosaccharomyces pombe Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. Cell **112:**207–217.
40. **Zhou, C., F. Arslan, S. Wee, S. Krishnan, A. R. Ivanov, A. Oliva, J. Leatherwood, and D. A. Wolf.** 2005. PCI proteins eIF3e and eIF3m define distinct translation initiation factor 3 complexes. BMC Biol. **3:**14.