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 yeast 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 Crn1 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 Crn1 (8, 36), resulting in an accumulation of Pap1 in the nucleus and activation of Pap1-dependent gene expression. Cells defective in Crn1 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, int6Δ 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.

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

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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 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 nmt1-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.

| Strain no. | Genotype | Reference and/or source |
|-----------|----------|-------------------------|
| CJN18     | h<sup>+</sup>-leu1-32 | Laboratory stock |
| TP108-3C  | h<sup>+</sup>-leu1-32 ura4-D18 pap1::ura4 | 33 |
| CJN255    | h<sup>+</sup>-leu1-32 his3-2-1 | 15 |
| CJN270    | h<sup>+</sup>-leu1-32 ade6-704 | Laboratory stock |
| CJN278    | h<sup>+</sup>-leu1-32 ade6-704 | 16 |
| EHH14     | h<sup>+</sup>-leu1-32 ura4-D18 pap1::nmt41-pap1-GFP-leu1 | 8 |
| EHH14.C278A | h<sup>+</sup>-leu1-32 ura4-D18 pap1::nmt41-pap1-C278A-GFP-leu1 | 8 |
| CJN391    | h<sup>-</sup>-leu1-32 ade6-704 ob1-GFP-reporter | This study |

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

### TABLE 2. Strains and culture conditions tested in microarray analysis

| Transformant<sup>a</sup> | pREP3X insert | Microarray analysis result for: Presence of 10 μg/ml thiamine? | nmt1 promoter status | Medium contained 15 μg/ml MBC? |
|-------------------------|--------------|------------------------|----------------------|-------------------------------|
| Vector, no thiamine     | None         | No                     | Derepressed          | No                             |
| Vector, thiamine + MBC  | None         | No                     | Derepressed          | Yes                            |
| Int6CT, thiamine        | int6CT       | No                     | Derepressed          | Yes                            |
| Int6CT, thiamine + MBC  | int6CT       | No                     | Derepressed          | Yes                            |
| Int6CT, thiamine        | int6CT       | Yes                    | Repressed            | No                             |

<sup>a</sup> The reference sample for each condition tested was vector + thiamine.

<sup>b</sup> = , without; +, with.
cleavage buffer (TCB) (10 nM 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 CaCl2, 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 CaCl2 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 trichloroacetic 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× NaPAGE 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, treated by trichloracetic acid precipitation, washed in acetone, and resuspended in acidified with 1 M CaCl2 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.

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 CJ18 (wild type) and TP108-3C (pap1Δ) were transformed with the thiamine-repressible vector pREP3X or pREP3X-int6CT, grown in the absence of thiamine and tested for their resistance to MBC. Cultures were split in two with one half remaining at 26°C for a further 4 h and 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.

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 CJ18 (WT; pap1Δ) or TP108-3C (pap1Δ) 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 CJ18 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 1011 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 CJ18 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,
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).

**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.

| Gene name | Annotation |
|-----------|-----------|
| thi2      | (nmt2)    | Thiazole biosynthetic enzyme |
| bsu1      |          | MFS efflux transporter |
| SPBC30.07c|          | Hypothetical protein |
| thi3      | (nmt1)   | Pyrimidine precursor biosynthesis enzyme |
| SPB87.18c |          | 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 |
| SPB87.30c |          | Putative transcriptional regulator |
| SPBC18IB5.05c|        | Thiamine-repressible acid phosphatase precursor |
| SPCC15B5.05c|         | Pyrimidine kinase involved in thiamine biosynthesis |
| zym1      |          | Metallothionein |
| SPBC36.02c|          | Membrane transporter of unknown specificity |
| snz1      |          | Involved in pyridoxine metabolism |

* Annotations are from Gene DB (http://www.genedb.org/genedb/pombe/index.jsp).
TABLE 4. Genes up-regulated due to Int6CT overexpression

| Gene name | Annotation                                      | Pap1-responsive |
|-----------|------------------------------------------------|----------------|
| 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 | +              |
| Transporters                                      |                |
| gst2      | Glutathione S-transferase II                  | +              |
| gst1      | Glutathione S-transferase I                   | +              |
| SPCC1281.04 | Pyridoxal reductase homolog                  | +              |
| SPC1281.07c | Putative glutathione S-transferase          | +              |
| tpx1      | Thioredoxin reductase                         | +              |
| pgr1      | Glutathione reductase                         | +              |
| SPCC737.06c | Putative glutamate-cysteine ligase regulatory subunit | + |
| gaal      | Glutathione synthetase large chain            | +              |
| trr1      | Thioredoxin reductase                         | +              |
| Antioxidant and detoxification                     |                |
| Flavin metabolism                                   |                |
| SPBC23G7.10c | Putative NADH-dependent flavin oxidoreductase | +              |
| SPA8C69.02c | Probable flavohemoprotein                    | +              |
| SPBC2A9.02 | Putative dihydroflavanol-4-reductase          | +              |
| obr1      | Flavodoxin                                    | +              |
| SPBC409.13 | 6,7-Dimethyl-8-ribityllumazine synthase synthase family | + |
| SPAPG7G11.09 | Putative GTP cyclohydrolase, possible riboflavin | + |
| Metal detoxification                                |                |
| ccs1      | Copper chaperone for Sod1 (superoxide dismutase) | +             |
| Signal transduction and transcriptional regulation  |                |
| SPAC22G7.08 | Probable serine-threonine protein kinase      | +              |
| lhp2      | Serine/threonine protein kinase                | +              |
| SPBC16G5.02c | Putative ribokinase                          | +              |
| SPBC2D10.04c | 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      | Separaase                                     |                |
| isp7      | Sexual differentiation protein                |                |
| Other metabolism                                   |                |
| SPCC663.06c | Putative short-chain dehydrogenase protein   | +              |
| SPCC663.08c | Putative short-chain dehydrogenase protein   | +              |
| SPBC4B10.20 | Hypothetical short-chain dehydrogenase       | +              |
| SPBC215.11c | Putative oxidoreductase; aldo-keto family    | +              |
| SPAC977.14c | Putative oxidoreductase                      | +              |
| SPAC513.107 | Putative cinnamoyl-CoA reductase             | +              |
| SPAC2E1P3.01 | Putative zinc binding dehydrogenase         | +              |
| pdr1      | Pyridoxal oxidase                             | +              |
| SPBC16A3.02c | Putative quinone oxidoreductase              | +              |
| SPAC2F1.04c | Zinc binding dehydrogenase (predicted)       | +              |
| SPAC51H0.05c | NADHDH_2 domain protein                      | +              |
| SPAC513.06c | Probable dimeric dihydrodiod dehydrogenase  | +              |
| SPAC186.03 | l-asparaginase precursor                     |                |
| SPAC9E1.09c | Aldehyde dehydrogenase (predicted)          | +              |
| SPBC4F1.16c | ER disulfide oxidoreductase                  | +              |
| SPBC215.03 | Oxidoreductase                               |                |
| SPAC952.08c | Pyridoxamine 5'-phosphate oxidase (predicted) | + |
| SPAC1782.01 | Similar to yeast Ecm29 cell wall stucture/biosynthesis protein | + |
| int1      | Conserved hypothetical C3HC4-type zinc finger protein; Ub E3 ligase (predicted) | + |
| pdf1      | Palmitoyl-protein thioesterase              | +              |
| SPBC725.01 | Aspartate aminotransferase, mitochondrial    | +              |
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; gtr2, 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 TTAAGTCA 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 TTAAGTCA (19), the frequency of the binding site for the 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

### TABLE 4—Continued

| Gene name          | Annotationa | Pap1-responsiveb |
|--------------------|-------------|------------------|
| SPBC21D10.11       | Aminotransferase class V; probable cysteine desulphurase | + |
| 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.
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 (pap1Δ) 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/H11002* (our unpublished results), indicating that neither the COP9 signalosome nor Ras is essential for Int6CT-induced drug resistance.

**Int6CT overexpression does not influence bulk polyubiquitylation.** 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 polyubiquitylated proteins. The temperature-sensitive mutants *mts2-1* and *mts3-1* were used as positive controls as they had already been shown to accumulate polyubiquitylated 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 control strain was transformed with pREP3X or pREP3X-int6CT. Immunoblotting clearly showed the accumulation of polyubiquitylated proteins in the *mts* mutants after growth at the restrictive temper-
nature (Fig. 5). Overexpression of Int6CT did not cause an accumulation of polyubiquitylated 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 re-evaluated 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 H2O2 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.

**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 Ccm1 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/elf3e, 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/elf3e is cytoplasmic in S. pombe (10) and that the protein is relocated 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 translation 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 TAF130-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/elf3e 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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