

# Distinct regions of ATF/CREB proteins Atf1 and Pcr1 control recombination hotspot *ade6-M26* and the osmotic stress response

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## ABSTRACT

The Atf1 protein of *Schizosaccharomyces pombe* contains a bZIP (DNA-binding/protein dimerization) domain characteristic of ATF/CREB proteins, but no other functional domains or clear homologs have been reported. Atf1-containing, bZIP protein dimers bind to CRE-like DNA sites, regulate numerous stress responses, and activate meiotic recombination at hotspots like *ade6-M26*. We defined systematically the organization of Atf1 and its heterodimer partner Pcr1, which is required for a subset of Atf1-dependent functions. Surprisingly, only the bZIP domain of Pcr1 is required for hotspot activity and tethering of Atf1 to *ade6* promotes recombination in the absence of its bZIP domain and the Pcr1 protein. Therefore the recombination-activation domain of Atf1-Pcr1 heterodimer resides exclusively in Atf1, and Pcr1 confers DNA-binding site specificity *in vivo*. Atf1 has a modular organization in which distinct regions affect differentially the osmotic stress response (OSA) and meiotic recombination (HRA, HRR). The HRA and HRR regions are necessary and sufficient to activate and repress recombination, respectively. Moreover, Atf1 defines a family of conserved proteins with discrete sequence motifs in the functional domains (OSA, HRA, HRR, bZIP). These findings reveal the functional organization of Atf1 and Pcr1, and illustrate several mechanisms by which bZIP proteins can regulate multiple, seemingly disparate activities.

## INTRODUCTION

Eukaryotes use stress-activated protein kinase (SAPK) pathways to respond to a wide variety of intracellular and extracellular conditions collectively referred to as

‘stress’ (1). The SAPK Spc1 (Sty1/Phh1) of fission yeast is a functionally conserved counterpart of mammalian SAPKs p38 and Jun N-terminal kinase (JNK) (2–5). Spc1 regulates many environmental stress response genes, including a subset common to most stresses (6). One of its targets, Atf1, is a basic, leucine-zipper (bZIP) transcription factor related by its bZIP domain to proteins of the ATF/CREB family (7–12). Atf1 is phosphorylated in a Spc1-dependent manner and is required for the majority of Spc1-dependent core stress responses. The sites of phosphorylation are unknown. Because fission yeast is a tractable organism, Atf1 provides a good model to reveal how other bZIP proteins such as ATF2 or c-Jun can affect multiple, diverse pathways in response to different stress conditions.

Atf1 was purified as a subunit of a heterodimeric protein complex (Mts1-Mts2/Atf1-Pcr1) that binds to and activates a meiosis-specific recombination hotspot in the *ade6* gene (11,13). It was independently identified in genetic screens for components of nutritional-sensing pathways (7–10). The Atf1 and Pcr1 proteins each harbor a bZIP domain, which is a bipartite element in which the basic region makes sequence-specific contacts with DNA and the leucine zipper mediates hetero- and homodimerization (14). Each basic region in a bZIP dimer makes contact with its own half site in the DNA. Atf1 and Pcr1 purified from fission yeast form combinatorial hetero- and homodimers that bind to DNA sites similar to the cyclic-AMP responsive-element, CRE (13). The Atf1-Pcr1 heterodimer binds avidly ( $K_d \sim 1$  nM) to a DNA site called *M26* (5'-ATGACGT-3') (13); the binding affinities and site specificities for Atf1-Atf1 and Pcr1-Pcr1 homodimers have not been clearly defined. However, combinatorial dimerization does occur *in vivo*, because while the Atf1-Pcr1-*M26* complex is essential for some biological activities, other Atf1-dependent activities do not require Pcr1 (11,15–17). Although no other bZIP proteins are proven dimerization partners for Atf1, the Pap1, Atf21 and Atf31 proteins are good candidates (18–27).

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Atf1 is required for a wide array of stress responses including those triggered by osmotic stress, UV light, oxidative stress, nutritional starvation, nucleotide pool depletion, DNA damage, toxic cations and thermal stress [(15) and refs therein]. The different effector functions might be explained, in part, by combinatorial formation of bZIP dimers with distinct DNA site affinities and effector domains. However, combinatorial dimerization alone is inadequate to explain diversity of functions, because Atf1-Pcr1 heterodimer itself exhibits dual specificity as an activator and repressor of chromatin compaction and transcription (15,17,28,29). Furthermore, the broad array of Atf1-dependent functions suggests that this protein may have a modular organization in which distinct domains affect distinct processes. However, there have been no reports on the functional organization of Atf1. Moreover, other than for the highly conserved bZIP domain, Atf1 has no other domains of known function, no obvious structural determinants and no clear homologs have been reported. In short, nothing is known about the functional organization of Atf1 and there are no clear landmarks or features that can be used to guide analyses of multifunctionality.

We therefore used systematic mutagenesis and chimeric proteins to define features of Atf1 and its heterodimer partner Pcr1, which is required for a subset of Atf1-dependent activities. This approach revealed details of modular organization, combinatorial bZIP dimerization and determinants for DNA site specificity *in vivo*. We also report that Atf1 defines a family of conserved proteins which share discrete sequence motifs within the functionally important regions. Together, these findings provide the first insight into the functional architecture of Atf1 and its known heterodimerization partner, Pcr1.

## MATERIALS AND METHODS

### *Schizosaccharomyces pombe* strains and genetic methods

Strains were cultured in minimal nitrogen base liquid (NBL) or on nitrogen base agar (NBA) supplemented as necessary with growth factors (amino acids, bases) at 100 µg/ml. Procedures for determining osmosensitivity and meiotic recombinant frequencies were as described (11). Relevant genotypes are indicated in the figures and full genotypes are provided in Table 1.

### Mutation of Atf1 and Pcr1 proteins

Structure–function analyses were conducted using untagged versions of Atf1 and Pcr1 proteins expressed from their own promoters in the context of their natural 5' and 3' DNA sequences. pBluescript KSII(+) plasmids harboring inserts with centrally located *atf1*<sup>+</sup> (*mts1*<sup>+</sup>) or *pcr1*<sup>+</sup> (*mts2*<sup>+</sup>) genes (11) (GenBank U87869, U87870) were subject to PCR-based, oligonucleotide-mediated, site-directed mutagenesis (30) to remove serially, and in-frame, ~50 codons each. Deletion endpoints are listed elsewhere in this paper. To bifurcate the Atf1 protein, standard cloning methods were used to remove sequences encoding amino-terminal residues 14–393 (ΔN) and carboxy-terminal residues 396–566 (ΔC). All clones were

subject to sequencing (including 5' and 3' UTRs) to identify desired clones and to eliminate clones with artifactual mutations. Wild-type and modified *atf1* and *pcr1* genes were liberated from pBluescript KSII(+) by digestion with restriction endonucleases XhoI and SpeI and subcloned between the XhoI and SpeI restriction sites of fission yeast vector pSP1 (31), which contains the *LEU2*<sup>+</sup> marker of budding yeast. Plasmids were transformed into cells (32) and maintained by selection for leucine prototrophy in a *leu1-32* genetic background.

### Generation of chimeric proteins

Several steps of PCR-based, oligonucleotide-mediated, site-directed mutagenesis (30) were used to link in-frame DNA encoding chimeric proteins. The first encoded, in order, the DNA-binding domain of budding yeast Gal4 (residues 1–147; Gal4DBD) (33) and a FLAG epitope (DYKDDDDK). The second encoded Gal4DBD-FLAG-Atf1 (residues 1–566 of Atf1). The third encoded Gal4DBD-FLAG-Atf1<sup>1–395</sup> (residues 1–395 of Atf1), which corresponds to an N-terminal, non-bZIP portion of Atf1 (described above) that is required for recombination (see 'Results' section). After functional assessment of these fusion proteins, eight additional clones were constructed to encode fusion proteins with smaller fragments of Atf1 (residues 1–75, 51–125, 101–175, 151–225, 201–275, 251–425, 301–375, 321–395). Each DNA clone was introduced between the start codon and termination codon of pSP1-*atf1*, thereby replacing the *atf1* ORF and placing the chimeric proteins under regulation of the normal *atf1* promoter and 3' regulatory sequences. The integrity of each of these constructs (including 5' and 3' UTRs) was confirmed by DNA sequencing.

### Construction of *ade6* alleles

The *ade6*<sup>+</sup> gene was amplified by PCR, a 2.8-kb PvuII–SpeI fragment was ligated into pBluescript KSII(–), and oligonucleotide-directed mutagenesis was used to introduce mutations. Substitutions included those generating a SnaBI restriction site for diagnostic purposes. A 1.7 kb fragment harboring *ura4*<sup>+</sup> was added between the SalI and ClaI sites of each plasmid. Plasmids were linearized by digestion with AatII, which cuts within *ade6*, and the alleles were introduced into the *ade6* locus using pop-in, pop-out gene replacement (34). Desired clones were identified, and their integrity confirmed, by a combination of PCR analyses, SnaBI restriction mapping, and DNA sequence analyses of the entire *ade6* locus. The sequences of the new *ade6* alleles are presented elsewhere in this article.

## RESULTS

We first used scanning deletion mutagenesis to characterize Atf1 and Pcr1 (Figure 1). Untagged proteins were each expressed from their respective, natural promoters and 3' regulatory sequences located within plasmid pSP1 (31). Under these conditions any specific mutations within the ORFs that cause loss-of-function or partial loss-of-function phenotypes identify the protein regions that

**Table 1.** Genotypes of *S. pombe* strains used in this study

Strain	Relevant genotype <sup>a</sup>
WSP 0643	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F</i>
WSP 0644	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F</i>
WSP 0646	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F</i>
WSP 1123	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F pcr1-D1::his3</i>
WSP 1584	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1)</i>
WSP 1586	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1pcr1<sup>+</sup>)</i>
WSP 1590	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1pcr1-D2-15)</i>
WSP 1592	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1pcr1-D16-75)</i>
WSP 1594	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1pcr1-D76-109)</i>
WSP 1596	<i>h</i> <sup>-</sup> <i>ade6-M210 pcr1-D1::his3F (pSP1pcr1-D109-171)</i>
WSP 1598	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1)</i>
WSP 1600	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1pcr1<sup>+</sup>)</i>
WSP 1604	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1pcr1-D2-15)</i>
WSP 1606	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1pcr1-D16-75)</i>
WSP 1608	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1pcr1-D76-109)</i>
WSP 1610	<i>h</i> <sup>+</sup> <i>ade6-M26 pcr1-D1::his3F (pSP1pcr1-D109-171)</i>
WSP 1612	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1)</i>
WSP 1614	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1pcr1<sup>+</sup>)</i>
WSP 1618	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1pcr1-D2-15)</i>
WSP 1620	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1pcr1-D16-75)</i>
WSP 1622	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1pcr1-D76-109)</i>
WSP 1624	<i>h</i> <sup>+</sup> <i>ade6-M375 pcr1-D1::his3F (pSP1pcr1-D109-171)</i>
WSP 1631	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1<sup>+</sup>)</i>
WSP 1632	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D14-393)</i>
WSP 1636	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D7-56)</i>
WSP 1639	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D154-203)</i>
WSP 1640	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D260-310)</i>
WSP 1642	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D311-367)</i>
WSP 1645	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D368-444)</i>
WSP 1649	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D479-539)</i>
WSP 1655	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1<sup>+</sup>)</i>
WSP 1656	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D14-393)</i>
WSP 1660	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D7-56)</i>
WSP 1662	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D154-203)</i>
WSP 1664	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D260-310)</i>
WSP 1666	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D311-367)</i>
WSP 1668	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D368-444)</i>
WSP 1672	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D479-539)</i>
WSP 1677	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D479-539)</i>
WSP 1678	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1<sup>+</sup>)</i>
WSP 1680	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D14-393)</i>
WSP 1684	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D7-56)</i>
WSP 1687	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D154-203)</i>
WSP 1689	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D260-310)</i>
WSP 1691	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D311-367)</i>
WSP 1692	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D368-444)</i>
WSP 2562	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D57-105)</i>
WSP 2566	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D106-153)</i>
WSP 2571	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D204-259)</i>
WSP 2574	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D445-478)</i>
WSP 2586	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D57-105)</i>
WSP 2591	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D106-153)</i>
WSP 2594	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D204-259)</i>
WSP 2600	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D445-478)</i>
WSP 2604	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-atf1<sup>+</sup>)</i>
WSP 2610	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D57-105)</i>
WSP 2615	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D106-153)</i>
WSP 2618	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D204-259)</i>
WSP 2623	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D445-478)</i>
WSP 2665	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1)</i>
WSP 2677	<i>h</i> <sup>+</sup> <i>ade6-M26 atf1-D15::ura4F (pSP1atf1-D396-566)</i>
WSP 2679	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1)</i>
WSP 2690	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1atf1-D396-566)</i>
WSP 2693	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1)</i>
WSP 2704	<i>h</i> <sup>+</sup> <i>ade6-M375 atf1-D15::ura4F (pSP1atf1-D396-566)</i>
WSP 2794	<i>h</i> <sup>+</sup> <i>atf1-D15::his3F</i>
WSP 2901	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::his3F</i>
WSP 2905	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F</i>

(continued)

Table 1. Continued

Strain	Relevant genotype <sup>a</sup>
WSP 2907	<i>h</i> <sup>+</sup> <i>ade6-gal4Control atf1-D15::his3F</i>
WSP 2922	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 2924	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 2926	<i>h</i> <sup>+</sup> <i>ade6-gal4Control atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 2981	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::his3F (pSP1gal4DBD)</i>
WSP 2988	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>1-395</sup>)</i>
WSP 2991	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD)</i>
WSP 2998	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>1-395</sup>)</i>
WSP 3001	<i>h</i> <sup>+</sup> <i>ade6-gal4Control atf1-D15::his3F (pSP1gal4DBD)</i>
WSP 3008	<i>h</i> <sup>+</sup> <i>ade6-Gal4Control atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>1-395</sup>)</i>
WSP 3011	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD)</i>
WSP 3018	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>1-395</sup>)</i>
WSP 3052	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::ura4F pcr1-D1::his3</i>
WSP 3059	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::ura4F pcr1-D1::his3</i>
WSP 3068	<i>h</i> <sup>+</sup> <i>ade6-gal4Control atf1-D15::ura4F pcr1-D1::his3</i>
WSP 3141	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD)</i>
WSP 3143	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD)</i>
WSP 3145	<i>h</i> <sup>+</sup> <i>ade6-gal4Control atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD)</i>
WSP 3147	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD)</i>
WSP 3149	<i>h</i> <sup>+</sup> <i>ade6-K87* atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 3151	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 3153	<i>h</i> <sup>+</sup> <i>ade6-gal4control atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 3155	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F pcr1-D1::his3 (pSP1gal4DBD-<i>atf1</i><sup>+</sup>)</i>
WSP 3250	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD)</i>
WSP 3252	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>1-75</sup>)</i>
WSP 3254	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>51-125</sup>)</i>
WSP 3256	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>101-175</sup>)</i>
WSP 3258	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>151-225</sup>)</i>
WSP 3260	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>201-275</sup>)</i>
WSP 3262	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>251-325</sup>)</i>
WSP 3264	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>301-375</sup>)</i>
WSP 3266	<i>h</i> <sup>+</sup> <i>ade6-gal4BS atf1-D15::his3F (pSP1gal4DBD-<i>atf1</i><sup>321-395</sup>)</i>
WSP 3298	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD)</i>
WSP 3300	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>1-75</sup>)</i>
WSP 3302	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>51-125</sup>)</i>
WSP 3304	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>101-175</sup>)</i>
WSP 3306	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>151-225</sup>)</i>
WSP 3308	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>201-275</sup>)</i>
WSP 3310	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>251-325</sup>)</i>
WSP 3312	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>301-375</sup>)</i>
WSP 3314	<i>h</i> <sup>-</sup> <i>ade6-M210 atf1-D15::ura4F (pSP1gal4DBD-<i>atf1</i><sup>321-395</sup>)</i>

<sup>a</sup>All strains were also *ura4-D18 his3-D1 leu1-32*.

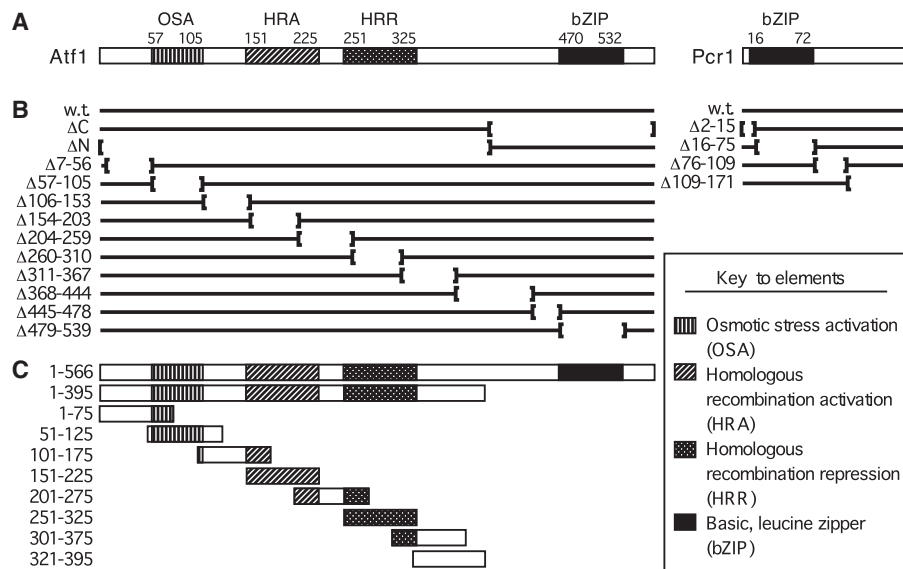
are required for protein stability or function. We focused upon activation of meiotic recombination hotspot *ade6-M26*, which requires Atf1-Pcr1 heterodimer; and upon the osmotic stress response, which is regulated by Atf1 protein independent of Pcr1 (11).

### Two regions of Atf1 are essential for the osmotic stress response

We compared the plating efficiency of cells on medium that lacked or contained 0.8 M NaCl (Figure 2). Wild-type cells plated efficiently on hyper-osmotic medium. In contrast, the *atf1-D15* (null) mutants were sensitive to osmotic stress, as reported (8). The Atf1 protein contains a highly conserved bZIP domain involved in sequence-specific DNA-binding activity (the basic region) and in protein dimerization (the leucine zipper), but otherwise lacks detectable domains of known function. Cells expressing the bZIP region alone ( $\Delta$ N) or all other portions of Atf1 except the bZIP region ( $\Delta$ C) were sensitive to osmotic stress. These results indicate that the

osmoregulatory functions of Atf1 require at least two distinct regions, one of which contains a domain required for protein dimerization and DNA binding (bZIP), the other of which presumably contains at least one activation domain. Alternatively, the deletion boundary between  $\Delta$ N and  $\Delta$ C might span a single essential region.

To further define the functional organization of Atf1 we introduced serial, scanning deletion mutations into the *atf1*<sup>+</sup> gene such that each construct encoded an Atf1 protein in which a segment of ~50 amino acids in length was missing. For eight of 10 serial deletions the proteins supported wild-type plating efficiencies on hyper-osmotic medium (Figure 2). We conclude that those eight mutated Atf1 proteins are each functional and that, at the resolution of our assay, the regions deleted are dispensable for osmoregulation (a modest effect might escape detection with this assay). One of the dispensable regions (residues 368–444) encompasses the boundary between the  $\Delta$ N and  $\Delta$ C deletions, disproving the hypothesis that the  $\Delta$ N– $\Delta$ C junction might span a required element. For two of 10 serial deletions the cells were as sensitive to



**Figure 1.** Functional architecture of Atf1 and Pcr1. (A) Summary of findings. The maximal extents of experimentally defined, functionally important elements (OSA, HRA, HRR, bZIP) are indicated. (B, C) Schematic diagram of constructs used in the study. (B) Cells expressing untagged proteins with the indicated deletions (brackets) were tested for resistance to osmotic stress (Atf1 protein) and for their ability to activate meiotic recombination hotspot *ade6-M26* (Atf1 and Pcr1 proteins). (C) The indicated, overlapping fragments of Atf1 were tethered artificially to *ade6* to test whether they are sufficient to regulate homologous recombination.

hyper-osmotic conditions as *atf1-D15* null mutant cells. One of these deletions (residues 479–539) encompasses the bZIP domain. The other deletion (residues 57–105) defines a non-bZIP region of Atf1 essential for osmoregulation. This same deletion protein supported wild-type levels of other Atf1-dependent functions (see next section). We therefore refer to this portion of Atf1 as an osmotic stress activation (OSA) region. This region occupies about 10% of the non-bZIP portions of Atf1, raising the possibility that the remaining 90% of non-bZIP portions might be used for other activities of this multifunctional protein.

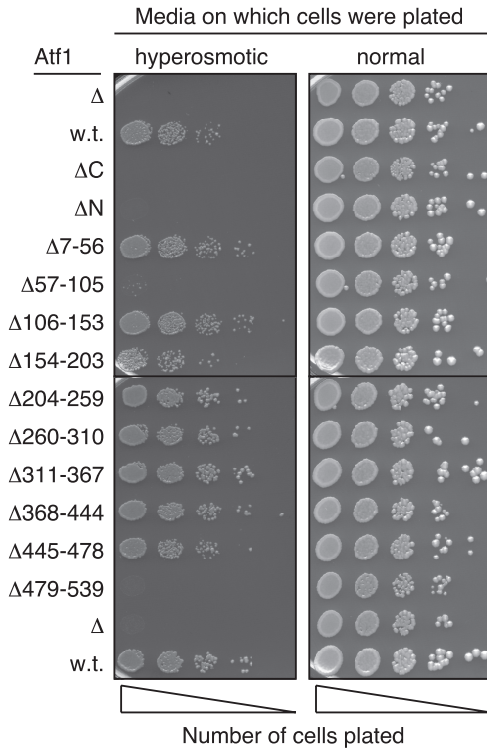
#### Multiple regions of Atf1 positively and negatively affect meiotic recombination hotspot *ade6-M26*

Binding of Atf1-Pcr1 heterodimer to DNA of the *ade6-M26* allele activates a meiotic recombination hotspot (11,13). To determine whether the hotspot is active, we measured recombination between two sets of *ade6* alleles (Figures 3 and 4A). Crosses between strains harboring the *ade6-M375* and *ade6-M210* alleles reveal basal (control) recombination levels, while crosses between strains harboring the *ade6-M26* and *ade6-M210* alleles reveal hotspot recombination levels (11,35). An elevated ratio of recombinants (*M26:M375*) demonstrates that the hotspot is active.

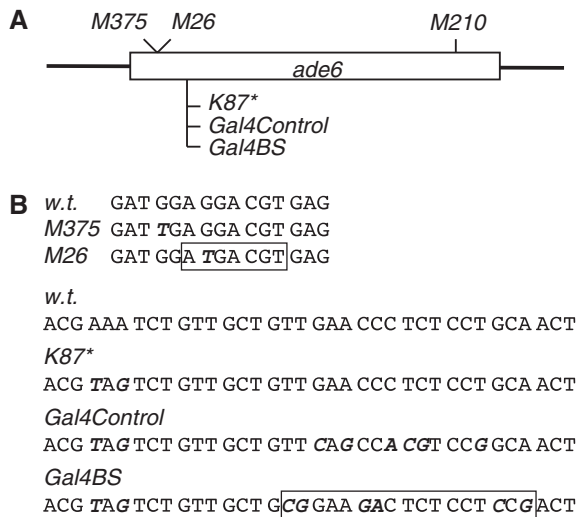
In cells expressing wild-type Atf1 protein the frequency of recombinants from crosses harboring the hotspot (*M26*) allele was about 9-fold higher than that from crosses harboring the control (*M375*) allele (Figure 4B). In *atf1-D15* (null) mutants the control (*M375*) recombinant frequency was similar to that of wild-type cells and the *M26* DNA site-dependent increase in recombination was abolished, confirming that Atf1 is essential for hotspot activity (11). Cells expressing only the bZIP region of Atf1

( $\Delta N$ ) or all other portions of Atf1 except the bZIP region ( $\Delta C$ ) also failed to activate the recombination hotspot. We infer that Atf1 contains at least two distinct regions required to promote *M26*-dependent recombination, or that the deletion boundary between  $\Delta N$  and  $\Delta C$  spans one essential region.

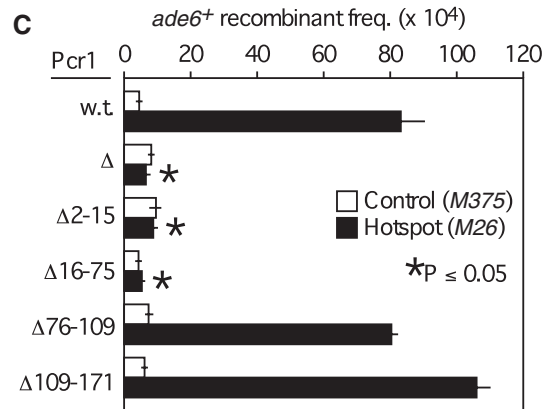
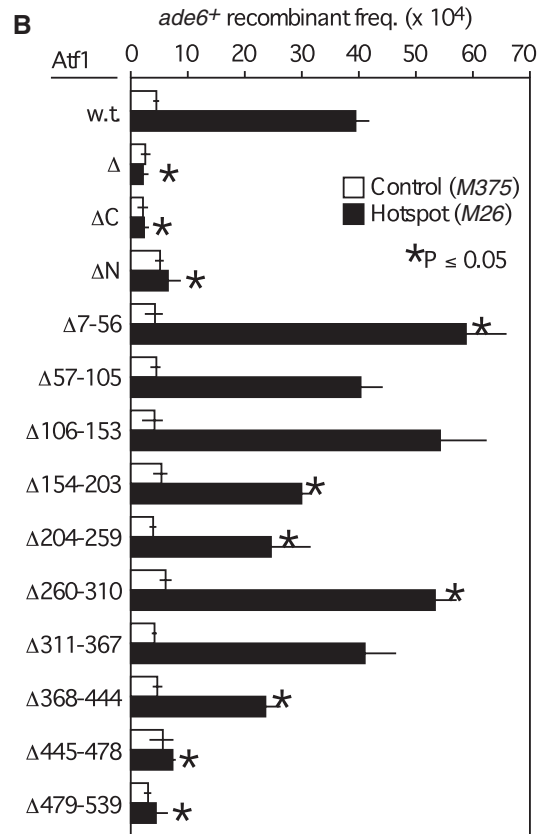
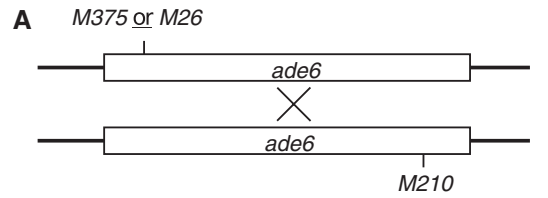
To define the required elements at higher resolution, we measured recombination in strains expressing Atf1 proteins with serial, scanning deletions of about 50 amino acids each (Figure 4B). None of these small deletions significantly affected basal (*M375* control) recombination. However, several of the deletions either attenuated or enhanced the *M26* DNA site-dependent, hotspot recombination. Two deletions (residues 445–478 and 479–539) abolished hotspot activity. The latter contains the bZIP domain and the former overlaps with conserved residues at the amino-terminal end of the bZIP domain. Three deletions (residues 154–203, 204–259 and 368–444) significantly reduced, but did not abolish, hotspot activity. Osmoregulation was unaffected by any of these three deletions (Figure 2). We conclude that there are at least two distinct, non-bZIP regions of Atf1 (residues 154–259 and 368–444) necessary to promote hotspot recombination. Because none of the individual deletions abolishes hotspot activity, these regions may function in a partially redundant or synergistic fashion (see ‘Discussion’ section). Two deletions (residues 7–56 and 260–310) significantly increased recombinant frequencies for the hotspot (*M26*) allele without significantly affecting recombinant frequencies of the control (*M375*) allele (Figure 4B). The corresponding regions of Atf1 might negatively regulate hotspot recombination, but there are alternative explanations for these and the preceding data. The deletions which attenuate or enhance recombination could do so specifically (e.g. by removing functional elements) or



**Figure 2.** Regions of Atf1 protein required for the osmotic stress response. Null mutant (*atf1-D15*) cells were transformed with plasmids expressing no Atf1, full-length Atf1 and truncated versions of Atf1 and were tested for their ability to withstand osmotic stress. Cells from log-phase cultures were spotted onto the indicated media and incubated for three days at 32°C. The WSP strain numbers were, from top-to-bottom, 2665, 1655, 2677, 1656, 1660, 2562, 2566, 1662, 2571, 1664, 1666, 1668, 2574, 1672, 2665 and 1655.



**Figure 3.** Alleles of *ade6* used to measure meiotic recombination. (A) Diagram of *ade6* locus and positions of alleles used. (B) DNA sequences of alleles. Each contains a stop codon (5'-TGA-3' or 5'-TAG-3'). The *ade6-M26* allele also creates a DNA site for binding of Atf1-Pcr1 heterodimer (box), which triggers recombination hotspot activity. The *ade6-Gal4BS* allele contains a DNA-binding site for Gal4 protein (box); its matching control *ade6-Gal4Control* has the same number and type of substitutions, but lacks a Gal4-binding site.



**Figure 4.** Regions of the Atf1 and Pcr1 proteins where deletions positively and negatively affect hotspot recombination at *ade6-M26*. (A) Recombination assay. Strains harboring the indicated *ade6* alleles were crossed and haploid spore colonies were genotyped. (B, C) Cells expressing no protein, full-length protein and truncated proteins were tested for their proficiencies of recombination involving hotspot (*M26*) and control (*M375*) alleles. For this and subsequent figures, the recombinant frequency is (*ade6*<sup>+</sup> titer/viable titer), data are mean ± SD from three or more independent experiments, and *P* values were determined using the student *t*-test. (B) Scanning deletion analysis of Atf1. (C) Scanning deletion analysis of Pcr1.

nonspecifically (e.g. by affecting protein folding). Therefore in a complementary approach, we tested the hypothesis that discrete regions of Atf1 are sufficient to promote or repress recombination (see below).

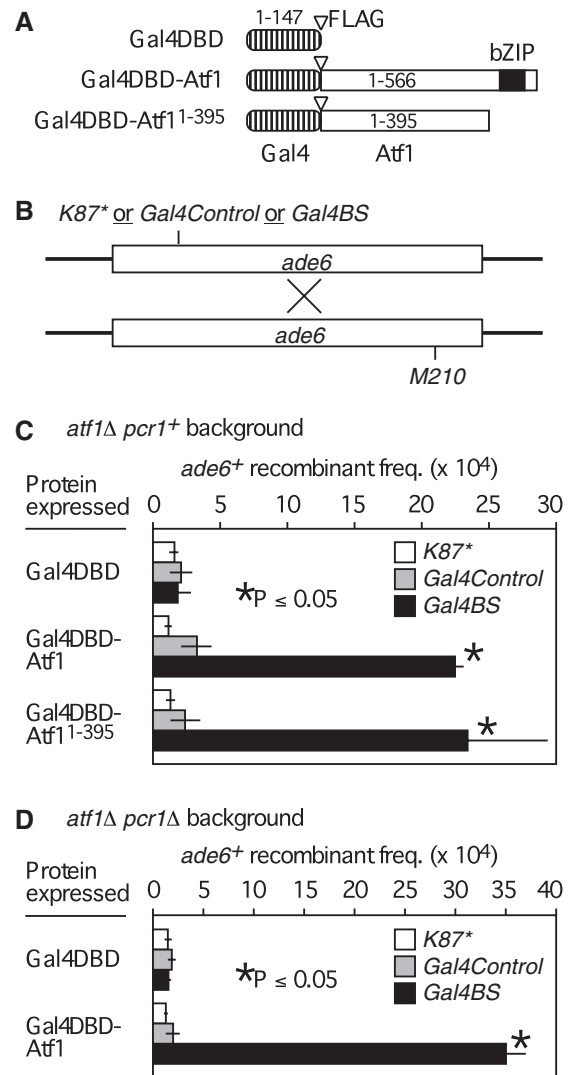
#### Only the bZIP domain of Pcr1 is required to activate meiotic recombination hotspot *ade6-M26*

A subset of Atf1-dependent functions, such as hotspot recombination, are carried out by Atf1-Pcr1 heterodimer (11). We therefore conducted scanning deletion analyses of Pcr1 (Figure 4C). In *pcr1-D1* (null) mutants the control (*M375*) recombinant frequency was indistinguishable from that of wild-type cells, demonstrating that Pcr1 is dispensable for basal recombination. However, the *M26* DNA site-dependent increase in recombination was abolished. Thus, activation of the *ade6-M26* hotspot requires Pcr1, as reported (11). Deletions of Pcr1 encompassing the bZIP region abolished the *M26* DNA site-dependent increase in recombination, as expected. Surprisingly, deletions that removed the non-bZIP portions of Pcr1 had no effect upon hotspot activity. Therefore, Pcr1 apparently does not harbor a non-bZIP activation domain involved in promoting hotspot recombination at the *ade6-M26* DNA site. This suggests that the only role of Pcr1 in activating the hotspot is to provide DNA-binding site specificity to the Atf1-Pcr1 heterodimer. This hypothesis has been confirmed by the documentation of Pcr1-independent hotspot activity when Atf1 is artificially tethered to the chromosome by virtue of a heterologous DNA-binding domain (subsequently).

#### An amino-terminal portion of Atf1 is sufficient to promote hotspot meiotic recombination when brought to the chromosome

The deletion experiments identified regions of Atf1 necessary to promote hotspot recombination. To determine whether the amino-terminal (non-bZIP) region of Atf1 is sufficient for activity, we artificially tethered this region to DNA within the *ade6* gene. We chose to use the DNA-binding domain of the budding yeast Gal4 protein for several reasons. First, this DNA-binding domain is well characterized and is of proven utility for targeting fusion proteins to a defined DNA site (36,37). Second, it is a zinc finger domain, and is therefore structurally and functionally distinct from the bZIP DNA-binding domains of Atf1 and Pcr1. Third, there are no recognizable DNA-binding sites for Gal4 within or near to the *ade6* gene.

Three new alleles were created within *ade6* (Figure 3). Each contains a stop codon (*ade6-K87\**), thereby rendering the cells auxotrophic for adenine and providing an allele for the analysis of recombination. The second allele contains the stop codon and substitutions at six nearby base pairs to create a Gal4 DNA-binding site (*ade6-Gal4BS*). Because these additional substitutions might create some type of 'marker effect' [e.g. by affecting mismatch correction during recombination (38,39)], we also created an additional control allele that contains the same number and type of nucleotide substitutions, but that does not generate a Gal4 DNA-binding site



**Figure 5.** Hotspot recombination when Atf1 is targeted to *ade6* by a heterologous DNA-binding domain. (A) The 5' and 3' regulatory sequences of the *atf1* gene were used to drive expression of the indicated, chimeric proteins, which contain the DNA-binding domain of Gal4 (Gal4DBD). These were used to artificially tether the activation domain of Atf1 to a Gal4 DNA-binding site created in *ade6*. (B) Recombination assay. Alleles of *ade6* that contain (*Gal4BS*) or lack (*K87\** and *Gal4Control*) a DNA-binding site for Gal4 were generated (Figure 3). Strains harboring the indicated alleles and chimeric proteins (panel A) were crossed and haploid spore colonies were genotyped. (C) Recombinant frequencies of cells expressing Pcr1 protein. (D) Recombinant frequencies of cells lacking Pcr1 protein.

(*ade6-Gal4Control*). We then expressed three different fusion proteins from the *atf1* promoter (Figure 5A). These contained a Gal4 DNA-binding domain (Gal4DBD), a Gal4DBD coupled to full-length Atf1 protein (Gal4DBD-Atf1) and a Gal4DBD coupled to the amino-terminal 395 amino acids of Atf1 (Gal4DBD-Atf1<sup>1-395</sup>).

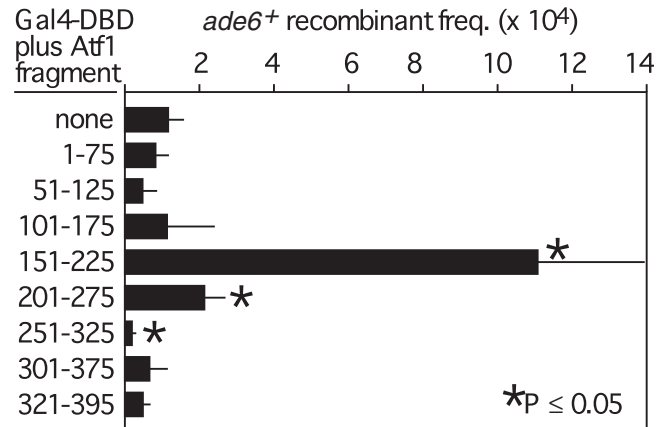
Strains harboring the new *ade6* alleles were crossed to a strain with the tester allele *ade6-M210* and recombinant frequencies were determined (Figure 5B). The recombinant frequencies for the three alleles (*ade6-K87\**, *ade6-Gal4BS* and *ade6-Gal4Control*) were indistinguishable from one-another (Figure 5C) and were similar to those

for the standard negative control allele (*ade6-M375*) (Figure 4). These results demonstrate that the *cis*-acting mutations used to create a DNA-binding site for Gal4 have no significant effect upon recombination within *ade6*. Expression of the Gal4DBD alone had no effect upon recombinant frequencies for the new alleles (Figure 5C), indicating that this heterologous DNA-binding domain alone does not promote recombination at its cognate DNA site in *ade6*. In contrast, expression of Gal4DBD-Atf1 fusion protein conferred robust hotspot activity at its DNA site (*ade6-Gal4BS*) without significantly affecting recombination at either of the control alleles (*ade6-K87\** and *ade6-Gal4Control*). The tethered Atf1 protein was as recombinogenic (11-fold, versus controls) as untagged Atf1-Pcr1 heterodimer was at *ade6-M26* (9-fold, versus controls). Furthermore, a fusion protein harboring an amino-terminal portion of Atf1 but lacking the bZIP domain (Gal4DBD-Atf1<sup>1-395</sup>) was equally proficient at promoting recombination for the *ade6-Gal4BS* allele. These results prove that Atf1 contains at least one homologous recombination activation (HRA) region, located within residues 1–395, that is sufficient to promote recombination. Moreover, these positive results are not subject to concerns about protein stability or folding or localization, which complicate the interpretation of data from deletion studies.

#### Artificial tethering of Atf1 to *ade6* bypasses the requirement for Pcr1 protein in hotspot recombination

Binding of the Atf1-Pcr1 heterodimer is essential for hotspot recombination at *ade6-M26* (11,13). However, scanning deletion analyses of Pcr1 revealed that only its bZIP (DNA-binding/protein dimerization) domain is necessary (Figure 4C). The simplest interpretation is that this bZIP fragment of Pcr1 dimerizes with the bZIP domain of full-length Atf1 to confer DNA-binding site specificity to Atf1-Pcr1 heterodimer. However, the bZIP domain of Pcr1 might have an additional, less-direct role in regulating Atf1-dependent hotspot recombination. For example, the bZIP domain of Pcr1 might help to regulate the expression of other proteins that are required for hotspot activity at *ade6-M26* (17). We therefore measured Gal4DBD-Atf1-dependent recombination in *pcr1-D1* (null) mutants.

The artificial tethering of Gal4DBD-Atf1 protein to its cognate DNA site (*ade6-Gal4BS*) bypassed fully the requirement for Pcr1 in promoting hotspot recombination (Figure 5D). We make two important conclusions: First, the recombination-activating region of Atf1-Pcr1 heterodimer does, indeed, reside exclusively within Atf1. Second, the only function of Pcr1 protein in activating hotspot recombination at *ade6-M26* is to confer DNA-binding site specificity to the Atf1-Pcr1 heterodimer, thereby bringing the recombination activation region of Atf1 to the *ade6-M26* DNA site in the chromosome. In addition to confirming that combinatorial bZIP dimerization targets the Atf1-Pcr1 heterodimer to a specific DNA site (11,13,15), these findings provide a molecular mechanism to explain the puzzling observation that some *M26*-like DNA sites promote Atf1-dependent, Pcr1-independent hotspot recombination (40).



**Figure 6.** Regions of Atf1 that are sufficient to activate and repress homologous recombination when targeted to *ade6*. The Gal4DBD alone and fused to the indicated fragments of Atf1 protein were tested for their effects upon recombination at the *ade6-Gal4BS* allele. See Figure 5 for experimental details.

#### Two small, distinct regions of Atf1 (HRA and HRR) activate and repress homologous recombination, respectively

The first 395 amino acids of Atf1 are sufficient to promote recombination when brought to *ade6* (Figure 5C). We sought to define at higher resolution the minimal sufficient elements within this region. Fragments of Atf1 were fused to the Gal4DBD and tested for their effects upon meiotic recombination at *ade6-Gal4BS*. Each fragment contained a unique stretch of 25 amino acids and a 25-amino acid overlap with each of the two adjacent fragments (Figure 1C).

One fusion protein, Gal4DBD-Atf1<sup>151-225</sup>, stimulated recombination ~9-fold relative to the Gal4DBD negative control (Figure 6). This tethered portion of Atf1 is about as recombinogenic as the full-length Gal4-DBD-Atf1 fusion protein (11-fold, versus controls) and as unmodified Atf1-Pcr1 heterodimer bound to *ade6-M26* (9-fold, versus controls). Therefore amino acids 151–225 of Atf1 define a minimal HRA region that is sufficient to promote recombination. The overlapping fragment to the left of HRA had no significant effect upon recombination, and the overlapping fragment to the right of HRA conferred only a modest increase in recombinant frequencies (1.7-fold). So within the sufficient HRA region (75 amino acids), the left-most 25 amino acids and the right-most 25 amino acids are apparently not sufficient for activation.

One fusion protein, Gal4DBD-Atf1<sup>251-325</sup>, reduced recombinant frequencies to 17% of control levels ( $P = 0.0012$ ) (Figure 6). We conclude that amino acids 251–325 of Atf1 are sufficient to repress recombination, and thereby define a homologous recombination repression (HRR) region. Overlapping protein fragments to the left and right of HRR did not reduce recombinant frequencies, indicating that the left-most 25 amino acids and the right-most 25 amino acids of the HRR region (75 amino acids) are not sufficient for repression.

The conclusions from these add-back experiments are not affected by concerns about protein stability or folding or localization, which complicate the interpretation of data



from the deletion studies. In addition, the sufficient elements identified by the add-back experiments are congruent with those required elements identified by the scanning deletion studies, because two deletions which attenuate recombination map to the HRA (activation) region, and one deletion which enhances recombination maps within the HRR (repression) region (Figures 4 and 6).

#### **Atf1 proteins are conserved and contain sequence motifs that are associated with functional regions**

All proteins of the bZIP family share extensive homology of amino acid sequence within the bZIP domain. However, previous reports have noted no extensive homology of any proteins to Atf1 outside of its bZIP domain. Moreover, Atf1 lacks obvious homology to any other domains of known function and there are no available structures. We therefore sought to identify specific features of Atf1 which may exert the region-specific activities defined above.

The non-bZIP portions of Atf1 are characteristic of acidic activator domains ( $pI = 4.6$ ), contain stretches of low sequence complexity, and are enriched for destabilizing and polar amino acids (9–12% each of Pro, Gln, Asn and Ser). Prediction algorithms (41) suggest that ~76% of the non-bZIP portions of Atf1 are disordered. While this may or may not be the case *in vivo*, there is a paucity of even predicted protein structural elements (e.g.  $\alpha$ -helices or  $\beta$ -sheets) against which to compare the functional data. Gapped Blast searches of protein databases (42) identified hundreds of proteins, in 56 species, with homology to Atf1. The bulk of these were identified due to the presence of the highly conserved bZIP domain. However, 25 proteins from diverse phylogenetic classes in phylum Ascomycota (fungi) exhibited detectable colinearity of homology with Atf1 outside of the bZIP domain. Therefore, Atf1 defines a family of proteins that are conserved within the broadest phylum in eukaryotes (43). Ten representative sequences from distinct genera were aligned using T-Coffee (44), which revealed that the homology is clustered within blocks (Figure 7). To refine these blocks, we analyzed the 20 Atf1-colinear homologs with the highest Blast scores using Multiple Sequence Compassion by Log Expectation (MUSCLE) (45). This revealed clusters of conserved residues which, when coupled with the results on structure–function analyses, identify discrete sequence motifs which likely affect osmoregulation and homologous recombination (Figure 8).

## **DISCUSSION**

We report five main findings from our analyses of multifunctional, stress-activated, bZIP transcription factor Atf1 and its known bZIP heterodimerization partner, Pcr1. First, Atf1 protein defines an evolutionarily ancient family of conserved proteins. Second, Atf1 has a modular organization in which distinct regions affect differentially hotspot meiotic recombination and the osmotic stress response. Third, a minimal fragment of Atf1 (HRA) is sufficient to promote homologous recombination.

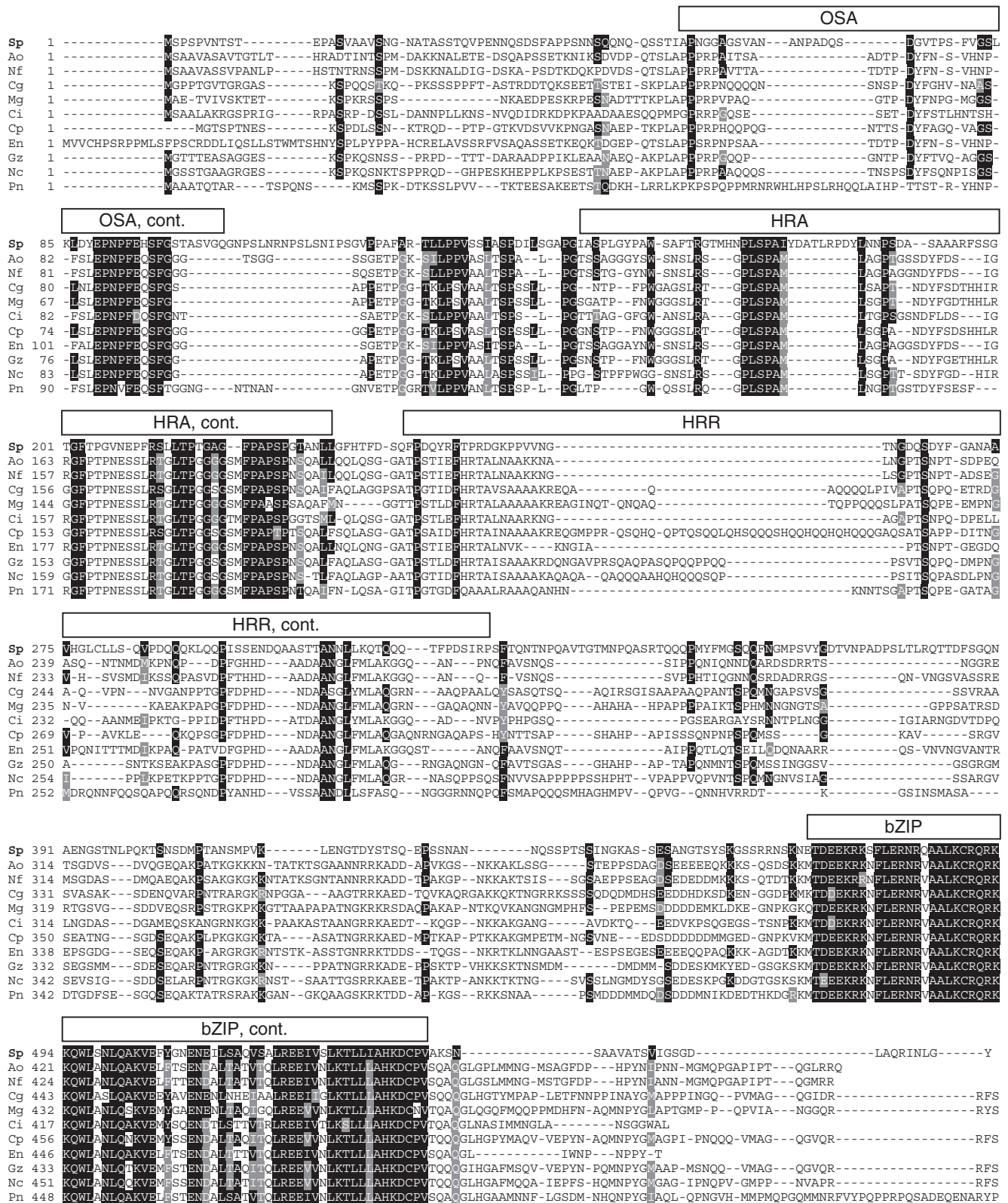
Fourth, a minimal fragment of Atf1 (HRR) is sufficient to repress homologous recombination. Fifth, the only apparent function of Pcr1 in hotspot recombination at *ade6–M26* is to confer DNA-binding site specificity to Atf1–Pcr1 heterodimer.

#### **Functional organization and conservation of Atf1 protein**

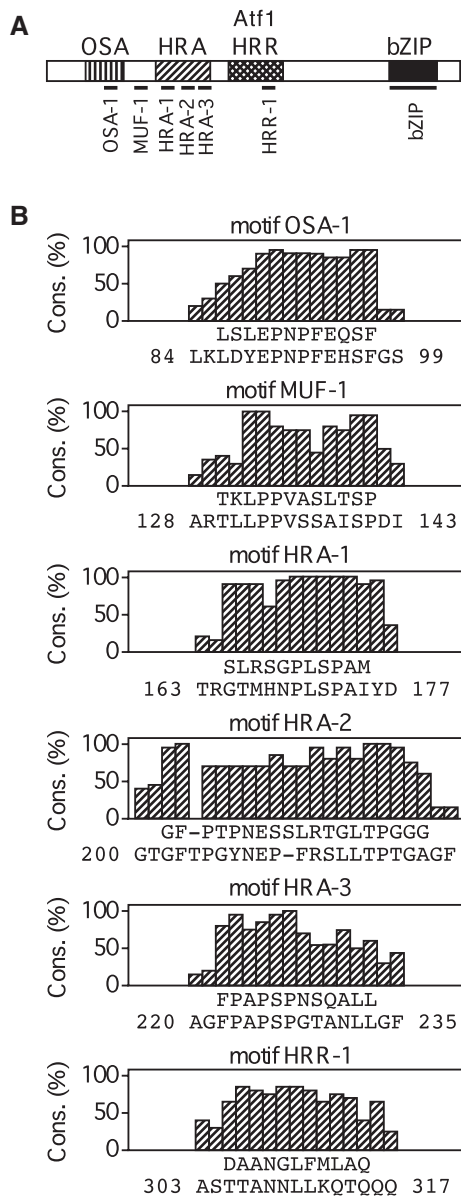
The Atf1 protein is required for a remarkable number of biological activities ('Introduction' section). Part of the mechanism for multifunctionality can now be explained by a modular organization in which distinct, nonoverlapping regions affect different processes (summarized in Figure 1A). Atf1 proteins are conserved (Figure 7) within organisms whose last common ancestor occurred about 1000 million years ago (43). Moreover, while the overall homology is relatively low outside of the bZIP domain, each region of Atf1 defined to be functionally important contains at least one well-conserved sequence motif (Figure 8). These findings suggest that the multifunctional, modular organization of Atf1 is conserved.

*The bZIP domain.* The bZIP domain is a highly conserved, well-defined, bipartite element with two activities (14). The leucine zipper mediates dimerization between bZIP protomers, and each basic region within a bZIP dimer makes sequence-specific contacts to its half-site in DNA. As expected, the bZIP domain of Atf1 is essential for the osmotic stress response (Figure 2) and for hotspot recombination at *ade6–M26* (Figure 4B); and the bZIP domain of Pcr1 is essential for hotspot activity (Figure 4C). In addition, the requirements for the bZIP domain of Atf1 and for the Pcr1 protein in hotspot recombination can be bypassed when a heterologous DNA-binding domain and cognate DNA site are substituted (Figure 5). We discuss below some mechanisms by which combinatorial dimerization of bZIP protomers contribute to multifunctionality.

*The OSA region.* Atf1 is required to regulate the expression of about 100 osmotic stress responsive genes controlled by the stress-activated kinase Spc1 (6). The bZIP dimerization partner of Atf1 required for osmoregulation is unknown. It is not possible to identify the region of Atf1 that is sufficient for osmoregulation, as this would presumably require the successful tethering of fragments of Atf1 (and perhaps its yet-unidentified bZIP dimerization partner) to the promoter regions of all (or almost all) of the Atf1-regulated genes required for the osmotic stress response. However, only one non-bZIP region of Atf1 (OSA), defined by deletion of residues 57–106, is essential for osmoregulation (Figure 2). This is a very specific requirement, because the corresponding Atf1 deletion protein supports wild-type levels of hotspot recombination (i.e. it must be expressed, folded properly, stable, localized to the nucleus and proficient for some DNA-binding-dependent functions) (Figure 4B). So while we cannot eliminate the possibility that other non-bZIP portions of Atf1 have a role in osmoregulation that is below the limit of detection, the bulk of this function can be ascribed to the OSA region (residues 57–106).



**Figure 7.** Conservation of Atf1 protein. Proteins with apparent colinearity of homology to fission yeast Atf1 (Sp) outside of the highly conserved, bZIP domain were identified and ten representatives were aligned using T-Coffee (44). Boxes indicate functional elements defined in this study. Residues are shaded to indicate only those for which at least 50% of family members have conserved (black) or conservative (gray) residues at the corresponding positions in fission yeast Atf1. Protein sequences and accession numbers are: Sp, *Schizosaccharomyces pombe* (BAA12194.1); Ao, *Aspergillus oryzae* (BAE57832.1); Nf, *Neosartorya fischeri* (XP\_001263330.1); Cg, *Chaetomium globosum* (XP\_001219853.1); Mg, *Magnaporthe grisea* (XP\_362629.1); Ci, *Coccidioides immitis* (XP\_001244257.1); Cp, *Claviceps purpurea* (CAD21519.1); En, *Emericella nidulans* (AAN75015.1); Gz, *Gibberella zeae* (XP\_390318.1); Nc, *Neurospora crassa* (CAC18235.2); and Pn, *Phaeosphaeria nodorum* (EAT876741.1). The last three amino acids from Pn Atf1 (PKY) were omitted from the figure to save space.



**Figure 8.** Conserved sequence motifs within functionally important regions of Atf1. (A) Positions of sequence motifs relative to functionally defined regions. (B) Conserved motifs. Twenty, Atf1-colinear homologs were analyzed using MUSCLE (45). Upper text shows the sequence of each conserved motif and the histogram bars show the degree of conservation at each amino acid position. Lower text displays the sequence of fission yeast Atf1 at the position of the motif. Motifs were identified using sequences whose accession numbers are: BAA12194.1, BAE57832.1, XP\_001271133.1, XP\_001263330.1, XP\_001219853.1, EAT86741.1, AAN75015.1, CAC18235.2, CAD21519.1, XP\_390318.1, XP\_001244257.1, XP\_362629.1, XP\_001213842.1, XP\_961431.1, XP\_754486.1, EAL22789.1, XP\_569310.1, ABD77427.2, EAU84943.1 and XP\_501922.1.

Within the OSA region, Atf1 of fission yeast and its colinear homologs in other organisms share a highly conserved amino acid sequence motif (motif OSA-1, Figure 8) suggestive of functional conservation. Comparison to a less well-conserved protein supports this idea. The Sko1 protein of budding yeast (46) is also a stress-responsive, dual-function activator/repressor, bZIP

protein that binds to CRE-like DNA sites (47–50). However unlike multifunctional Atf1, Sko1 is dedicated predominantly to osmoregulation. Outside of the bZIP domain, Sko1 has a patch of strong homology to Atf1 that overlaps precisely with the osmoregulatory motif OSA-1. This supports the hypothesis that the OSA region is conserved functionally, and this hypothesis might be tested by mutating the OSA region of Sko1.

*The HRA region.* A single region of the Atf1 protein (HRA), located within residues 151–225, is both necessary (Figure 4B) and sufficient (Figure 6) for hotspot activity. This region alone is as ‘hot’ for promoting recombination (relative to controls) as full-length, unmodified Atf1-Pcr1 heterodimer is at *ade6-M26* (11). The left-most and right-most portions of the HRA region are not sufficient for activation (Figure 6). In addition, deletions which remove only the left half or right half of this region each remove about half of the activity (Figure 4B), suggesting that two (or more) elements within the HRA region function synergistically to activate recombination. The sequence composition is consistent with this interpretation: the HRA region of Atf1 is the most highly conserved region, outside of the bZIP domain, and three well-conserved sequence motifs are present (motifs HRA-1, HRA-2 and HRA-3, Figure 8). Two of these motifs, HRA-2 and HRA-3, are contiguous and may define a single element.

*The HRR region.* A single region of Atf1 (HRR), located within residues 251–325, is both necessary (Figure 4B) and sufficient (Figure 6) to repress recombination. This region alone is a potent repressor, as it reduces recombination 83%, relative to controls (6-fold difference). It is spatially and functionally distinct from the HRA region, so HRR and HRA can function both independently, and antagonistically relative to each other. The left-most and right-most portions of the HRR region are not sufficient for repression (Figure 6). As with other functionally important elements defined in this study, there is a conserved sequence motif (motif HRR-1, Figure 8) embedded in the HRR region, though this motif is less well conserved for fission yeast Atf1 than are the other motifs.

*A motif of unassigned function (MUF).* An Atf1 protein missing amino acids 106–153 supports wild-type levels of osmoregulation (Figure 2) and hotspot recombination (Figure 4B). However, within this region is a well-conserved sequence motif (MUF-1, Figure 8). Because conservation of sequence homology suggests conservation of function, this motif is presumably required for additional activities of Atf1 that may be conserved.

*Implications.* The modular organization of Atf1 reveals one basis for its multifunctionality. The functionally important regions and motifs (Figure 8) define, hypothetically, conserved interaction sites for proteins that affect various Atf1-dependent activities. For example, the OSA, HRA and HRR regions may contain, respectively, docking sites for components of the transcription machinery, for chromatin remodeling factors and recombination proteins, and for heterochromatin proteins

(11,15,17,28,29,34,51–53). While beyond the scope of the present study, these and related hypotheses can now be tested using a variety of directly targeted, function/element/motif-specific experimental approaches.

### Combinatorial dimerization of Atf1 confers DNA site specificity *in vivo*: implications for meiotic recombination and stress responses

The *ade6-M26* allele of fission yeast (35) has a single base pair substitution (54) which created fortuitously a 7 bp, CRE-like DNA site (*M26*; 5'-ATGACGT-3') (55) that is bound avidly by Atf1-Pcr1 heterodimer *in vitro* (13) and *in vivo* (12). For this *M26* DNA site in *ade6*, binding of Atf1-Pcr1 heterodimer is essential for recombination hotspot activity (11).

Other, *M26*-like DNA sites that occur naturally in the fission yeast genome or that were created by mutagenesis are recombination hotspots (40,53,56). Surprisingly, while all hotspot-active, *M26*-like DNA sites tested require Atf1, some sites exhibit independence from Pcr1. This puzzling observation can now be explained by the fact that the homologous recombination activation (HRA) region of Atf1-Pcr1 heterodimer resides entirely within Atf1 (Figures 4–6). Atf1 and Pcr1, purified to apparent homogeneity from fission yeast cells, form combinatorial dimers (Atf1-Atf1, Atf1-Pcr1, Pcr1-Pcr1) with subtly different DNA site specificity (13). *In vivo* some *M26*-like DNA sites are demonstrably occupied by Atf1-Pcr1 heterodimer (12), whereas others must be bound by an Atf1 homodimer or heterodimer that does not contain Pcr1 (11). In each case, the HRA region of Atf1 is brought to the respective *M26*-like DNA site. In the latter case (Pcr1-independent binding of Atf1-containing dimers), hotspot activity is independent of Pcr1.

We note that published sequences of hotspot-active, *M26*-like DNA sites (40,55,56) fall into two classes diagnostic for binding of bZIP heterodimers and homodimers (14). About half of the sites contain a nonpalindromic *M26* site (5'-ATGACGT-3') and about half contain the *M26* site embedded within a perfect or near-perfect palindrome (5'-ATGACGTCAT-3'). On average the latter sites are 'hotter'. Furthermore, Pcr1-independent, hotspot-active, *M26*-like DNA sites are palindromic. We therefore suggest that the palindromic DNA sites contain two Atf1 half sites, bind an Atf1-Atf1 homodimer, and recruit two doses of the recombination activating (HRA) region of Atf1. Similarly, the nonpalindromic *M26* DNA sites would be bound by Atf1-Pcr1 heterodimer and recruit only one dose of the HRA region of Atf1. This is the case for *ade6-M26* (11,13).

The Atf1-promoted hotspot recombination provides an example of exquisite DNA site specificity *in vivo*. Atf1-Pcr1 heterodimer and Atf1 dimerized with some protein other than Pcr1 each must bind to necessarily distinct, yet closely related DNA sites to promote recombination *in vivo* [this study and (11–13,40,56)]. This combinatorial assortment and high DNA site specificity *in vivo* has broad implications. We calculate that the 53 unique bZIP proteins in humans have the potential to form 1431 distinct dimers, though it is likely

that fewer are actually made due to structural constraints (14). These various dimers, like Atf1-containing dimers of fission yeast, may be able to identify their 'own' DNA sites *in vivo* with a very high level of discrimination. If so, the actual number of functionally distinct, *cis*-acting regulatory sites for bZIP proteins may approach the number of different bZIP dimers in the cell.

## CONCLUSIONS

We report that the Atf1 protein of fission yeast defines a conserved family of stress-activated bZIP proteins. The multiple functions of Atf1 are regulated, in part, by a modular organization in which distinct regions of Atf1 affect distinct responses. Effector functions are also regulated, in part, by the combinatorial formation of different bZIP homodimers and heterodimers. *In vivo* each subunit of the dimer contributes, by virtue of conferring specificity for its own DNA half-site, to assembly of the dimer onto specific DNA sites (e.g. Atf1-Pcr1 heterodimer at *ade6-M26*). For some functions, such as homologous recombination, the activation or repression domains reside exclusively in one subunit of the bZIP dimer. For others, combinatorial association of functional domains in both protomers of the bZIP dimer would be required. These findings provide the first insight into the functional architecture of Atf1 and its known heterodimerization partner, Pcr1. They also illustrate several distinct mechanisms by which individual bZIP proteins can regulate multiple, seemingly disparate responses to a wide variety of intracellular and extracellular signals.

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