units (IU) pregnant mare's serum gonadotropin (PMSG) on the first day, transferred to 15°C and injected with 50 IU PMSG on the second day, and injected with 400 IU PMSG plus 200 IU human chorionic gonadotropin on the third day. Eggs were expressed from the females by gentle pressure on their ovisacs 30 to 36 hours after the last injection. Males were killed and the vas deferens was placed in a moist chamber. For fertilization, eggs were expressed into a petri dish. A small piece of vas deferens was cut out and used to apply sperm to the eggs. After 5 min, the eggs were flooded with 20% Steinberg's solution. As a control for true parthenogenesis, eggs from each female were expressed from the cloaca, flooded, and treated as above but not painted with sperm.

12. The heart, spleen, and a portion of skeletal muscle were dissected from the adults or juveniles and frozen with an equal volume of deionized water in 1.5-ml Eppendorf microcentrifuge tubes. The electrophoretic procedure was the same as used previously [J. P. Bogart, L. E. Licht, M. J. Oldham, S. J. Darbyshire, *Can. J. Zool.* **63**, 340 (1985)]. This study concentrated on those electromorphs that were distinctly different in the males and females. The following loci were examined: Acon-1, Adh, Ck-1, Ck-2, Got-1, Got-2, Idh-1, Idh-2, Ldh-1, Ldh-2, Mdh-1, Mdh-2, Mdh-3, Mpi, Pgi, 6Pgd, Pgm-1, Pgm-2, and Sod-1. Some loci (for example, Adh, Sod-1, and Sod-2) could not be resolved in larvae. Genomes were identified by the diagnostic electromorphs [J. P. Bogart, *NY State Mus. Bull.* **466**, 209 (1989)].
13. The females were injected with 0.5 ml of colchicine solution (1 mg/ml) 2 days before being processed for chromosomes and electrophoresis. Chromosomes from the offspring were obtained from tail tips of larvae (*7*) or gut epithelial lining of transformed individuals (*8*). Some larvae were used for initial chromosome analyses and electrophoresis. These larvae were put in 0.01% colchicine solution for 24 hours, and their tails were squashed. Larvae were frozen for later electrophoresis.
14. Undeveloped or dead eggs are common in ponds where hybrids occur. Such eggs were noted early in the century by W. H. Piersol [*Am. Nat.* **44**, 732 (1910)]. The low percentage of developing eggs that hatched in this study (27%) is consistent with other studies of hybrid female *Ambystoma* [W. Clanton, *Occas. Pap. Mus. Zool. Univ. Mich.* **290**, 1 (1934); L. E. Licht, *NY State Mus. Bull.* **466**, 170 (1989).
15. Eighty-seven offspring were analyzed from 12 crosses involving 9 LJJ females, 4 *A. jeffersonianum* males, 1 *A. laterale* male, and 1 *A. texanum* male. There were 78 tetraploids, 1 pentaploid, and 8 triploids.
16. In an intensive study of *Ambystoma* in southern Ontario, only one tetraploid was reported by D. L. Servage [thesis, University of Guelph, Guelph, Ontario (1979)]. No tetraploids were found by J. P. Bogart (*7*).
17. R. A. Brandon, *Herpetologica* **33**, 133 (1977); C. E. Nelson and R. R. Humphrey, *ibid.* **28**, 27 (1972).
18. G. Fankhauser, *Q. Rev. Biol.* **20**, 20 (1945); R. R. Humphrey and G. Fankhauser, *J. Exp. Zool.* **134**, 427 (1957).
19. J. Rostrand, *C. R. Soc. Biol.* **115**, 1680 (1934); *ibid.* **122**, 1012 (1936).

20. We thank L. Rye, A. Taylor, and C. Zeyl for helping with aspects of this study and D. Sever and K. Mierzwa for supplying specimens of *A. tigrinum* and *A. laterale*. Supported by summer undergraduate grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to A. Taylor and C. Zeyl and by NSERC operating grants to J.P.B., R.P.E., and L.E.L.

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Similarity Between the Transcriptional Silencer Binding Proteins ABF1 and RAP1

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The yeast ARS binding factor 1 (ABF1)—where ARS is an autonomously replicating sequence—and repressor/activator protein 1 (RAP1) have been implicated in DNA replication, transcriptional activation, and transcriptional silencing. The *ABF1* gene was cloned and sequenced and shown to be essential for viability. The predicted amino acid sequence contains a novel sequence motif related to the zinc finger, and the ABF1 protein requires zinc and unmodified cysteine residues for sequence-specific DNA binding. Interestingly, ABF1 is extensively related to its counterpart, RAP1, and both proteins share a region of similarity with SAN1, a suppressor of certain *SIR4* mutations, suggesting that this region may be involved in mediating SIR function at the silent mating type loci.

A CRITICAL FEATURE IN THE LIFE CYCLE of the yeast *Saccharomyces cerevisiae* is that two loci, *HML* and *HMR*, which contain mating type information and the cis elements required for expression, must be maintained in a transcriptionally silent state (*1*). Silencing involves the binding sites for two sequence-specific DNA binding proteins, ABF1 and RAP1, located within regulatory regions (silencers) flanking these silent loci (*2–4*). Four genes, *SIR1* to *SIR4*, are also required for silencing (*5*) and appear to act, at least in part, through ABF1 and RAP1 (*6*). ABF1 and RAP1 are multifunctional proteins implicated not only in transcriptional repression, but also in transcriptional activation (both proteins), telomere function (RAP1), and initiation of DNA replication (ABF1) (*2, 3, 7, 8*).

Eukaryotic cells are capable of regulating the amount of transcription of a given gene over eight to nine orders of magnitude. Of this range, five to six orders of magnitude have been attributed to transcriptional repression (*9*). An important group of cis-acting sequences involved in repressing transcription of specific genes, often in a developmentally regulated manner, has been delineated (*7, 10, 11*). These sequences can repress transcription in an orientation- and distance-independent manner and, by analogy to transcriptional enhancers, have been termed "silencers" (*7*). Understanding how these silencers function may be important for understanding the full breadth of transcriptional control in eukaryotes.

Cell type in the yeast *S. cerevisiae* is determined by the expression of either one of two alleles, α or **a** from the *MAT* locus near the centromere of chromosome III. Mating type information is also contained at two other loci, *HML* and *HMR*, which are found near the telomeres of chromosome III and which serve as stores of α and **a** mating type information, respectively, during mating type switching. *HML* and *HMR* are actively maintained in an inert state by four short sequences flanking each silent locus (*12*). Each of these sequences can function as an autonomously replicating sequence (ARS), implicating the initiation of DNA replication in silencing. It is within these sequences that ABF1 or RAP1, or both, binds and appears to act in silencing with the products of the four *SIR* genes.

Like general transcriptional repression (*9*), the repressed state of *HML* and *HMR* is stably propagated during DNA replication and, in fact, requires passage through S phase for its establishment (*13*). Furthermore, silencing involves the generation of a chromatin structure reminiscent of heterochromatin (*14*). Since most cellular heterochromatin is located at the periphery of the nucleus, subjacent to the nuclear lamina, the recent observation that a functional domain of the SIR4 protein is closely related to the human nuclear lamins in a region involved in lamin-lamin interactions may provide a link between transcriptional silencing, heterochromatin formation, and subnuclear localization of cis-acting sequences (*15*). The role of transcriptional activators like ABF1 and RAP1 in the establishment and maintenance of the repressed state is important, and analysis of the genes encoding these proteins is likely to offer insights into this mechanism.

We have previously described the purification of ABF1 to homogeneity as a polypeptide with an apparent molecular mass of 135 kD (*3*). A polyclonal rabbit antiserum to purified ABF1 protein was affinity-purified (Fig. 1A, lane 1) and used to screen

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2×10^6 recombinant phage from a yeast genomic λGT11 expression library (*16*). A single strong candidate (λABF1) was obtained and was purified through two more screenings.

This phage produced full-length ABF1, as shown by several tests. First, antibody from crude antiserum to ABF1 was affinity-purified from λABF1 plaques and used in an immunoblot to probe both a crude yeast extract and purified ABF1 (Fig. 1A, lanes 2 and 3). In both cases, a 135-kD band was specifically recognized, demonstrating that λABF1 produces a protein antigenically related to ABF1. Second, immunoblots of SDS-polyacrylamide gels were obtained by using extracts from *Escherichia coli* infected

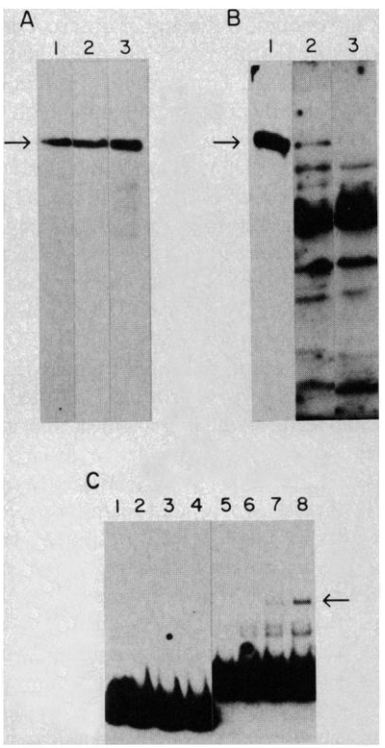

Fig. 1. Demonstration that the recombinant phage λABF1 encodes the full-length *ABF1* gene. (**A**) Immunoblot analysis of yeast cell proteins with affinity-purified antibodies. An immunoblot of 50 μg of whole-cell extract of crude yeast (lanes 1 and 2) or 100 ng of purified ABF1 (lane 3) was probed with an antibody affinity-purified from yeast ABF1 protein (lane 1) or λABF1 phage plaques (lanes 2 and 3). (**B**) Immunoblot analysis of phage-infected *E. coli* extracts with crude antiserum to ABF1. An immunoblot of purified ABF1 (lane 1), extract from λABF1-infected *E. coli* (lane 2), and extract from λGT11-infected *E. coli* (lane 3). The crude serum also recognizes a number of *E. coli* proteins thereby demonstrating the need to use affinity-purified antibody in the cloning. (**C**) Gel retention analysis of extracts from λABF1-infected *E. coli*. Lanes 1 to 4 and lanes 5 to 8 contain 0, 1, 3, and 5 μl of extract (*30*) with a labeled probe containing pBR322 sequences (lanes 1 to 4) or ARS1 sequences (lanes 5 to 8). The positions of ABF1-specific bands in all three panels are indicated by arrows.

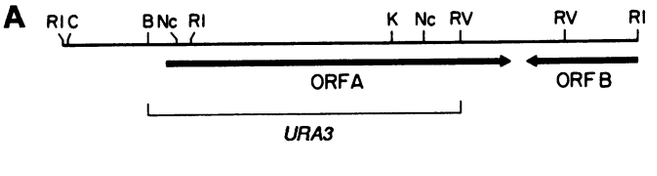

Fig. 2. (**A**) Restriction map of λABF1 insert and position of two open reading frames (ORF A and ORF B). Restriction sites are: RI (Eco RI), C (Cla I), B (Bgl II), Nc (Nco I), K (Kpn I), and RV (Eco RV). The region of ORF A replaced by *URA3* is indicated in the bottom of the figure. After transplacement into the yeast strain W303-1, 21 tetrads were analyzed. Nineteen contained only two viable spores, and two contained only one viable spore. All viable spores were *ura*$^-$. Another construct in which *URA3* was inserted into the Eco RV site within the *ABF1* coding sequence was also transplaced into W303-1. In this case, 17 tetrads were analyzed. Fifteen contained only two viable spores, and two contained only one viable spore. Again, all viable spores were *ura*$^-$. Though ORF B is on the same side and orientation as β-galactosidase transcription, it is not in frame with the β-galactosidase coding sequence and, therefore, cannot make a fusion protein. (**B**) DNA sequence of the λABF1 insert. The two Eco RI fragments in the insert were subcloned into the plasmid pUC118 in both orientations. A nested set of deletions was generated with exonucleases III and VII (*31*). Single-stranded DNA was generated by superinfection with helper phage M13KO7 and sequenced (*32*). The amino acid sequence of ORF A is shown below the nucleotide sequence. The GenBank, EMBL, and PIR databases were scanned by the method of Pearson and Lipman (*33*) and both predicted proteins were unique and not represented in the databases. The positions of all cysteine residues and the histidine residues within the putative metal binding domain of ABF1 are boxed and the asparagine-rich regions are underlined.

B

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GAA TTC GGT ATG CTG TCA ACG CTT TTC ATC GAT GTA TAT AAG GAA GAA TAC CTC ATT ATA      60
ATA CAT CAA AAC CTT CAT TTG CGC GAT GTT ACG CGT CGC GTT TCC TTC CTA TAA AAA ATA
CAT AAG CTA GTG AAT AAA AGC ATT AAA CGC GTT AGG GGG ATC TGC AAT TAA AGT AAA TAA     180
ATG AAA TAA ATG AAA AAC TGG TTA CAC TAA TTA TAA CGT TAA TAA AAC TAG TTG ATA ATC
AAA ATA TTG CCA TAG CTA AGC CAA TAG TAC AGC GTT GGC TTT CTA TCA TAA CTT ACC AGC     300
CGG GTG CCC AAT CGG GTA TAT CTG TCA CCG AAA GTC ACT GTT AAC GAG GCG TGA TTC AGC
CGG GTA CTA GTC TAG CAT TTT TTT TCG CCT GTT TTT TTC GTG AAA AAC GAA AAG TGG CAA     420
GCA AAC TTA TAT CAC TTT CTA CTT CTT TCC CTG TTC TTT TTG TTT ATT GGC AAT GGC CAC
TCA CCA CAT CCC CAT TAA CGA AAG TCA CCA AAG TGT GTC ATT TAA TAC TAC GGT TAG TGA     540
CAG CAT TTT GTT TAG ATC TCA ACT AGA GAG GAT TAT CAA CTG CTA AGC CCT TTA CAC GTT
ATC TGT GAT CCT GCT GCA CTA GGA AAC GTT TCA AAT CAC CAT ATT TGC AAT TTC ACA AGG     660

ATG GAC AAA TTA GTC GTG AAT TAT TAT GAA TAC AAG CAC CCT ATA ATT AAT AAA GAC CTG     720
MET Asp Lys Leu Val Val Asn Tyr Tyr Glu Tyr Lys His Pro Ile Ile Asn Lys Asp Leu

GCC ATT GGA GCC CAT GGA GGC AAA AAA TTC CCC ACC TTG GGT GCT TGG TAT GAT GTA ATT     780
Ala Ile Gly Ala His Gly Gly Lys Lys Phe Pro Thr Leu Gly Ala Trp Tyr Asp Val Ile

AAT GAG TAC GAA TTT CAG ACG CGT TGC CCT ATT ATT TTA AAG AAT TCG CAT AGG AAC AAA     840
Asn Glu Tyr Glu Phe Gln Thr Arg Cys Pro Ile Ile Leu Lys Asn Ser His Arg Asn Lys

CAT TTT ACA TTT GCC TGT CAT TTG AAA AAC TGT CCA TTT AAA GTC TTG CTA AGC TAT GCT     900
His Phe Thr Phe Ala Cys His Leu Lys Asn Cys Pro Phe Lys Val Leu Leu Ser Tyr Ala

GGC AAT GCT GCA TCC TCA GAA ACC TCA TCT CCT TCT GCA AAT AAT AAT ACC AAC CCT CCG     960
Gly Asn Ala Ala Ser Ser Glu Thr Ser Ser Pro Ser Ala Asn Asn Asn Thr Asn Pro Pro

GGT ACT CCT GAT CAT ATT CAT CAT CAT AGC AAC AAC ATG AAC AAC GAG GAC AAT GAT AAT    1020
Gly Thr Pro Asp His Ile His His His Ser Asn Asn Met Asn Asn Glu Asp Asn Asp Asn

AAC AAT GGC AGT AAT AAT AAG GCT AGC AAT GAC AGT AAA CTT GAC TTC GTT ACT GAT GAT    1080
Asn Asn Gly Ser Asn Asn Lys Ala Ser Asn Asp Ser Lys Leu Asp Phe Val Thr Asp Asp

CTT GAA TAC CAT CTG GCG AAC ATT CAT CCG GAC GAC ACC AAT GAC AAA GTG GAG TCG AGA    1140
Leu Glu Tyr His Leu Ala Asn Ile His Pro Asp Asp Thr Asn Asp Lys Val Glu Ser Arg

AGC AAT GAG GTG AAT GGG AAC AAT GAC GAT GAT GCT GAT GCC AAC AAC ATT TTT AAA CAG    1200
Ser Asn Glu Val Asn Gly Asn Asn Asp Asp Asp Ala Asp Ala Asn Asn Ile Phe Lys Gln

CAA GGT GTT ACT ATC AAG AAC GAC ACT GAA GAT GAT TCG ATA AAT AAG GCC TCT ATT GAC    1260
Gln Gly Val Thr Ile Lys Asn Asp Thr Glu Asp Asp Ser Ile Asn Lys Ala Ser Ile Asp

CGG GGA TTG GAC GAC GAG AGC GGT CCT ACT CAT GGT AAT GAC AGC GGT AAC CAC CGT CAC    1320
Arg Gly Leu Asp Asp Glu Ser Gly Pro Thr His Gly Asn Asp Ser Gly Asn His Arg His

AAC GAG GAG GAT GAC GTC CAT ACC CAA ATG ACG AAA AAC TAT TCT GAC GTA GTG AAC GAT    1380
Asn Glu Glu Asp Asp Val His Thr Gln Met Thr Lys Asn Tyr Ser Asp Val Val Asn Asp

GAA GAC ATC AAC GTT GCC ATT GCC AAT GCT GTT GCA AAT GTA GAT TCT CAA TCA AAC AAT    1440
Glu Asp Ile Asn Val Ala Ile Ala Asn Ala Val Ala Asn Val Asp Ser Gln Ser Asn Asn

AAG CAC GAT GGA AAA GAC GAT GAT GCC ACT AAC AAC AAT GAT GGC CAA GAT AAT ACT AAT    1500
Lys His Asp Gly Lys Asp Asp Asp Ala Thr Asn Asn Asn Asp Gly Gln Asp Asn Thr Asn

AAT AAC GAT CAC AAC AAT AAC AGC AAC ATC AAT AAC AAC AAT GTC GGT AGC CAC GGC ATT    1560
Asn Asn Asp His Asn Asn Asn Ser Asn Ile Asn Asn Asn Asn Val Gly Ser His Gly Ile

TCC TCC CAC TCA CCA TCC TCC ATA CGA GAC ACG TCT ATG AAT TTA GAC GTC TTC AAT TCT    1620
Ser Ser His Ser Pro Ser Ser Ile Arg Asp Thr Ser Met Asn Leu Asp Val Phe Asn Ser

GCT ACC GAT GAT ATA CCG GGC CCA TTT GTC GTT ACC AAA ATT GAG CCC TAT CAT AGT CAC    1680
Ala Thr Asp Asp Ile Pro Gly Pro Phe Val Val Thr Lys Ile Glu Pro Tyr His Ser His

CCA CTA GAA GAT AAC TTG TCG CTG GGT AAA TTT ATT CTA ACT AAG ATT CCA AAG ATT TTA    1740
Pro Leu Glu Asp Asn Leu Ser Leu Gly Lys Phe Ile Leu Thr Lys Ile Pro Lys Ile Leu

CAA AAC GAT TTA AAG TTT GAT CAA ATA CTA GAA AGC TCT TAC AAT AAT TCT AAC CAT ACA    1800
Gln Asn Asp Leu Lys Phe Asp Gln Ile Leu Glu Ser Ser Tyr Asn Asn Ser Asn His Thr

GTG AGT AAA TTT AAA GTT TCT CAT TAC GTG GAG GAG TCC GGC CTT TTA GAC ATT TTA ATG    1860
Val Ser Lys Phe Lys Val Ser His Tyr Val Glu Glu Ser Gly Leu Leu Asp Ile Leu Met

CAA AGA TAT GGA TTA ACC GCC GAG GAT TTC GAA AAA AGG TTA CTT TCC CAA ATA GCC AGA    1920
Gln Arg Tyr Gly Leu Thr Ala Glu Asp Phe Glu Lys Arg Leu Leu Ser Gln Ile Ala Arg

CGT ATA ACG ACG TAT AAA GCA AGA TTT GTT TTG AAA AAG AAA AAA ATG GGC GAG TAT AAT    1980
Arg Ile Thr Thr Tyr Lys Ala Arg Phe Val Leu Lys Lys Lys Lys Met Gly Glu Tyr Asn

GAT TTA CAA CCT TCT TCA TCT TCC AAT AAC AAC AAT AAC AAC GAT GGT GAG CTT TCT GGC    2040
Asp Leu Gln Pro Ser Ser Ser Ser Asn Asn Asn Asn Asn Asn Asp Gly Glu Leu Ser Gly

ACG AAT TTG AGA AGT AAC TCT ATC GAC TAC GCC AAA CAT CAG GAA ATA TCA AGT GCG GGT    2100
Thr Asn Leu Arg Ser Asn Ser Ile Asp Tyr Ala Lys His Gln Glu Ile Ser Ser Ala Gly

ACC TCG TCG AAC ACA ACC AAA AAT GTG AAT AAT AAC AAG AAT GAC AGT AAT GAC GAT AAT    2160
Thr Ser Ser Asn Thr Thr Lys Asn Val Asn Asn Asn Lys Asn Asp Ser Asn Asp Asp Asn

AAC GGC AAC AAT AAT AAT GAC GCC TCA AAT TTA ATG GAA AGT GTG CTA GAT AAA ACC TCT    2220
Asn Gly Asn Asn Asn Asn Asp Ala Ser Asn Leu Met Glu Ser Val Leu Asp Lys Thr Ser

AGT CAC CGG TAT CAA CCA AAG AAG ATG CCA AGC GTC AAT AAA TGG AGC AAG CCA GAT CAA    2280
Ser His Arg Tyr Gln Pro Lys Lys Met Pro Ser Val Asn Lys Trp Ser Lys Pro Asp Gln

ATA ACT CAT TCA GAC GTG TCC ATG GTT GGC CTA GAT GAA TCA AAC GAT GGC GGT AAT GAA    2340
Ile Thr His Ser Asp Val Ser Met Val Gly Leu Asp Glu Ser Asn Asp Gly Gly Asn Glu

AAT GTC CAC CCA ACC TTG GCT GAA GTA GAC GCT CAA GAA GCT CGT GAA ACT GCT CAG TTA    2400
Asn Val His Pro Thr Leu Ala Glu Val Asp Ala Gln Glu Ala Arg Glu Thr Ala Gln Leu

GCC ATA GAC AAG ATC AAT TCT TAT AAG AGA TCC ATT GAT GAC AAG AAT GGT GAT GGC CAT    2460
Ala Ile Asp Lys Ile Asn Ser Tyr Lys Arg Ser Ile Asp Asp Lys Asn Gly Asp Gly His

AAC AAT TCG TCG AGA AAC GTG GTA GAT GAA AAC TTG ATC AAC GAT ATG GAT TCA GAA GAT    2520
Asn Asn Ser Ser Arg Asn Val Val Asp Glu Asn Leu Ile Asn Asp Met Asp Ser Glu Asp

GCT CAC AAG TCC AAA AGA CAG CAT TTG TCA GAT ATC ACA CTG GAA GAG AGG AAT GAA GAC    2580
Ala His Lys Ser Lys Arg Gln His Leu Ser Asp Ile Thr Leu Glu Glu Arg Asn Glu Asp

GAC AAA CTA CCA CAT GAA GTG GCG GAA CAG TTA AGG TTA CTG TCA TCG CAT TTG AAA GAG    2640
Asp Lys Leu Pro His Glu Val Ala Glu Gln Leu Arg Leu Leu Ser Ser His Leu Lys Glu

GTA GAG AAT CTA CAC CAG AAT AAT GAT GAC GAT GTA ATG GTG GAC GTG GAT GAT GAT    2700
Val Glu Asn Leu His Gln Asn Asn Asp Asp Asp Val Asp Asp Val MET Val Asp Val Asp

GTA GAA TCG CAG TAT AAT AAG AAC ACA AAT CAT CAT AAT AAC CAT CAT AGC CAA CCT CAT    2760
Val Glu Ser Gln Tyr Asn Lys Asn Thr Asn His His Asn Asn His His Ser Gln Pro His

CAC GAT GAA GAA GAT GTT GCT GGA CTA ATA GGG AAA GCC GAT GAT GAA GAA GAC CTT TCT    2820
His Asp Glu Glu Asp Val Ala Gly Leu Ile Gly Lys Ala Asp Asp Glu Glu Asp Leu Ser

GAT GAA AAC ATT CAA CCA GAA TTA AGA GGT CAA TAG ATA CCC AGT TGA GAA GTC GAG CTA    2880
Asp Glu Asn Ile Gln Pro Glu Leu Arg Gly Gln

TTT TTC TCA TGT TTC TTT TGT TTA TTC ACT TTT CTA TAC TTT TTC TTT TCT ATT GAA ATA    2940
TAT ATT ATA TCT TAT GTA TGT TTT TTT GTA TTA TAA ACA AGC AAA TTT CGT CTT GCC ATT
TAC CTT CTG ATA TTC ATC ACA TGT ATA TCT CAA TTC AAT TTG TTA AGA AAA TCA GCC AAA    3060
AAA AGC GGA CCG ATC CTA TCT TGA TCT ATA TCT CTT AGT TTG TTG AAG TTA ATG AAT TGC
CTT TTC AAT CTC TGC AAT TGC GCC AAC GTA ACG ACG TTA CCA ATG GGC AAG TCT ACG AAG    3180
AAG CAA CCA TCA TCA TTG ATA TCG GTA ATC CCT TCG GAA ACA ATG ACT CTT GTT CCG TTA
CTT ACC CCG CCA ATA GAA GTC AGG CTT TTG ATG TGG TTC ATT ATG GTT TTT ATT ATC TTA    3300
CTA GTT TCG ATG TCG AGA ACC TGG ATG AAA TTG GAC TGA GAC GCA GGC TTT AGT ACC GTG
GCT GAG TTC GGT TTT TGT AAG CCC TTA CTC AAC GGA TCA TTG TGT CCA CTG TTT TTA GCC    3420
GAT TTG GAA GTT TGA AAG ACT ACT TTA CAA ATA TCG TCG ATG TAG TCA GTT ATG TTG TCC
TGA GGA GTA AGA ATA GCA AAG AGT GGA GAG TCC CAT CGG TTG TTT GAG TTA GGC TCT TCG    3540
TAT CGT TGA ATC AAT TGA TTC AAT AAC TCG GGT TCC CAA GGG TTC GAG GTC TTA
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AAA TCC GAA TTC                                                                  3672
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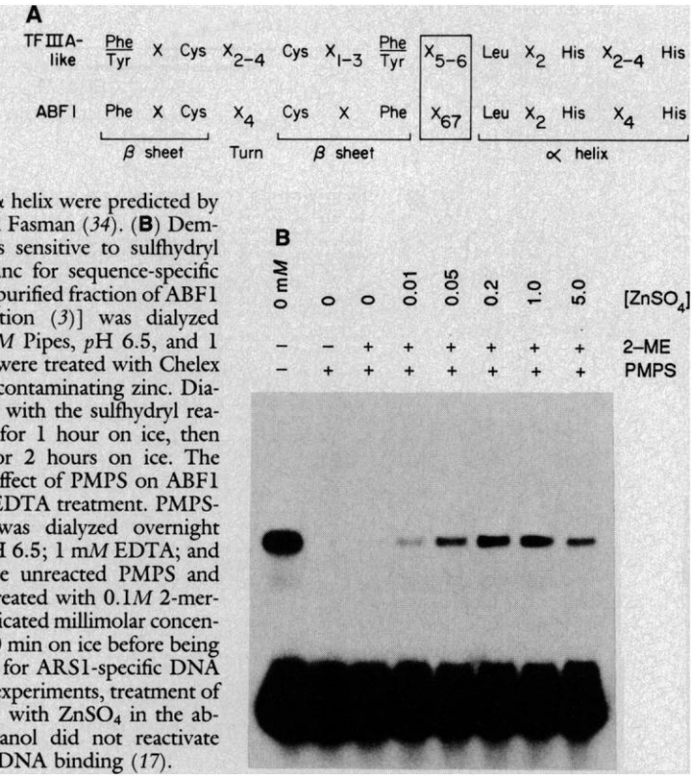

Fig. 3. (**A**) Consensus sequence derived from the nine zinc fingers of *Xenopus* TFIIIA, the yeast ADR1 and human SP1 (*21*) compared with the putative zinc finger of ABF1. Regions of β sheet and α helix were predicted by the method of Chou and Fasman (*34*). (**B**) Demonstration that ABF1 is sensitive to sulfhydryl reagents and requires zinc for sequence-specific DNA binding. A highly purified fraction of ABF1 [phenyl Sepharose fraction (*3*)] was dialyzed overnight against 10 m*M* Pipes, *p*H 6.5, and 1 m*M* EDTA. All buffers were treated with Chelex 100 (Bio-Rad) to limit contaminating zinc. Dialyzed ABF1 was treated with the sulfhydryl reagent PMPS (1 mg/ml) for 1 hour on ice, then with 50 m*M* EDTA for 2 hours on ice. The second lane shows the effect of PMPS on ABF1 binding activity before EDTA treatment. PMPS-EDTA–treated ABF1 was dialyzed overnight against 10 m*M* Pipes, *p*H 6.5; 1 m*M* EDTA; and 20% glycerol to remove unreacted PMPS and EDTA. Portions were treated with 0.1*M* 2-mercaptoethanol and the indicated millimolar concentrations of $ZnSO_4$ for 30 min on ice before being assayed by gel retention for ARS1-specific DNA binding (*3*). In separate experiments, treatment of PMPS-inactivated ABF1 with $ZnSO_4$ in the absence of 2-mercaptoethanol did not reactivate ABF1 sequence-specific DNA binding (*17*).

with λABF1 and control λGT11 in order to determine the size of the ABF1-related antigen. Cells infected with λABF1 produced a polypeptide of 135 kD recognized by an antibody to ABF1 that comigrated with purified ABF1 and was not synthesized in cells infected with λGT11 (Fig. 1B). These results indicate that λABF1 contains the entire *ABF1* coding sequence. Finally, extracts from λABF1-infected *E. coli* were tested for the presence of an activity capable of binding specifically to ARS1 DNA. Gel retention assays showed that λABF1-infected cells produce an ARS1-specific protein complex (Fig. 1C) coincident with that produced by purified ABF1; this protein complex is not found in cells infected with λGT11 (*17*).

A restriction map of the 3.8-kb λABF1 insert was determined (Fig. 2A). One genomic copy of the *ABF1* gene in the diploid yeast strain W303-1 was deleted by homologous recombination with a construct in which the *ABF1* sequences between the Bgl II and the Eco RV sites (Fig. 2A) were replaced with the yeast *URA3* gene or with another construct in which the *URA3* gene was inserted into the Eco RV site within the *ABF1* coding sequence (*18*). Replacement was verified by DNA blot hybridization. Sporulation of both these strains generated only two viable, *ura*$^-$ spores per tetrad demonstrating that the *ABF1* gene is essential for viability.

The sequence of the λABF1 insert was determined (Fig. 2B) and found to contain two open reading frames (ORFs) (Fig. 2A). The first AUG of the largest ORF, ORF A, lies within a good match to the optimal translational start site in yeast, including the highly conserved A residue at position −3 (*19*). ORF A can encode a polypeptide of 81,759 daltons which, though significantly smaller than the apparent molecular mass of ABF1, is by far the largest and only complete ORF in the insert. Thus, we conclude that it represents the *ABF1* coding region and that ABF1 migrates anomolously slowly in SDS-polyacrylamide gels. λABF1 expresses full-length ABF1, and this expression is not inducible by isopropylthiogalactoside (*17*), in agreement with the idea that ABF1 is not expressed as a β-galactosidase fusion protein in this phage.

The *ABF1* coding sequence predicts an unusually high proportion of asparagine (14.8%), aspartic acid (11.4%), and serine (9.7%) residues, and many of the asparagine residues are found in three discrete homopolymeric stretches. The predicted protein has a low isoelectric point (4.59) and a high net negative charge at neutral *p*H (−6.0), consistent with its chromatographic behavior on various ion-exchange resins (*3*). Other transcription factors with high asparagine and serine content and a low isoelectric point, such as the yeast heat shock transcription factor (HSTF) (*20*) and RAP1 (*4*), also migrate slowly in SDS gels.

Examination of the *ABF1* sequence for potential DNA binding motifs revealed an unusual feature. A consensus of the zinc finger motif typified by a large number of transcription factors, including *Xenopus* TFIIIA, yeast ADR1, and mammalian SP1 is shown in Fig. 3A (*21*). This sequence motif can be considered as two halves, one containing two cysteine residues in a region of predicted antiparallel β sheet and the other containing two histidine residues in a region of predicted α helix. Zinc is coordinated by the cysteine and histidine residues, whereas conserved aromatic and leucine residues are thought to form a hydrophobic core between the two halves. These two halves are generally connected by a linker of

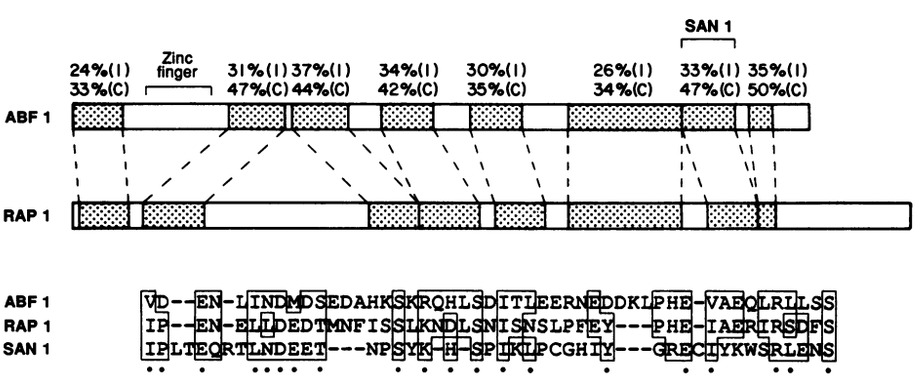

Fig. 4. (**Top**) Comparison of ABF1 and RAP1 sequences. Homologies between ABF1 and RAP1 were detected with the ALIGN program (*27*). Filled regions indicate regions of homology between the two proteins. Numbers above the filled regions indicate percent identity (I) and conservation (C) between these proteins with respect to the ABF1 sequence. It should be noted that the comparisons in these regions contain some short gaps. The actual alignment of these two sequences is available on request. The region of SAN1 homology is indicated. (**Bottom**) Region of homology between ABF1, RAP1, and SAN1 detected as described above. Closed circles beneath the SAN1 sequence indicate amino acid residues of SAN1 identical with either ABF1 or RAP1.

five or six amino acid residues. This basic view of the structure of the zinc finger has recently been confirmed (*22*). The *ABF1* gene predicts virtually perfect matches to each half of the zinc finger in β sheet and α helix; however, the spacing between the two halves is substantially longer (67 amino acid residues).

To determine whether ABF1 requires zinc for sequence-specific DNA binding, we used the reversible sulfhydryl reagent, *p*-(hydroxymercuri)benzenesulfonate (PMPS). This reagent has been successfully used in examining the zinc requirements of several metalloproteins including RNA polymerase (*23*), bacteriophage T4 gene 32 protein (*24*), and *E. coli* topoisomerase I (*25*), and we have devised a modified protocol to examine the role of zinc in the sequence-specific DNA binding activity of ABF1 (Fig. 3B). First, ABF1 can be completely inactivated by treatment with PMPS (Fig. 3B, lane 2) under conditions in which another DNA binding protein, ABF2 (*26*), is not affected (*17*), an indication that ABF1 contains cysteine residues required for binding activity. If PMPS-treated ABF1 is subsequently treated with 50 m*M* EDTA to strip divalent cations from the protein, sequence-specific DNA binding activity can only be restored by reversal of PMPS modification with 2-mercaptoethanol in conjunction with the addition of exogenous zinc. Neither 2-mercaptoethanol (Fig. 3B, lane 3) nor zinc sulfate (*17*) alone was sufficient to restore DNA binding activity. This result shows that ABF1 requires zinc and unmodified cysteine residues for sequence-specific DNA binding activity, suggesting that there is a functional metal-binding domain in ABF1 (ABF1 only contains three cysteine residues). Whether this domain is directly involved in contacting specific DNA sequences and is sufficient for sequence-specific DNA binding is unknown and is currently being determined.

Since the ABF1 and RAP1 binding sites are functionally redundant at *HMR E*, we considered that the proteins binding to these sequences might be related. Direct comparison by means of the ALIGN program (*27*) revealed that ABF1 and RAP1 are 30% identical and 40% conserved over 60% of the protein (Fig. 4A). The similarity between ABF1 and RAP1 does not include the putative zinc finger described above, suggesting that although these two proteins are related, they may interact differently with DNA. Regardless, the similarity between these proteins is extensive and likely includes regions not involved in DNA binding.

A recessive mutation called *san1* which suppresses certain weak *sir4*$^-$ mutations has recently been identified and the wild-type *SAN1* gene has been cloned (*28*). Overexpression of this gene in a *SIR*$^+$ background leads to partial derepression of *HMR*, whereas disruption of the *SAN1* gene does not affect mating in a wild-type background. The predicted amino acid sequence of SAN1, like those of ABF1 and RAP1, is rich in asparagine and serine, and Fig. 4B shows that all three proteins share a region of high homology. A biochemical explanation consistent with the genetic and sequence data is that the *SAN1* gene product competes in vivo with ABF1 and RAP1 for interaction with SIR by virtue of this region of homology. Thus, a loss of *SAN1* would alleviate competition for the SIR proteins and overexpression of *SAN1* would exacerbate such competition.

The results presented in this report have three interesting implications. First, the ABF1 protein sequence contains a motif related to but distinct from the canonical zinc finger, which appears to be an important functional domain of the protein because sequence-specific DNA binding activity requires zinc and unmodified cysteines. Second, like the *RAP1* gene (*4*), the *ABF1* gene is essential for viability. Since the cis elements within *HML* and *HMR* are not essential for viability, this result emphasizes the multifunctional nature of these proteins. And third, the ABF1 sequence is related to that of RAP1 and contains a region related to SAN1, which is encoded by a gene implicated in control of the silent loci. All four flanking sequences required for silencing contain either ABF1 or RAP1 binding sites, and these binding sites are redundant at *HMR E*. Since the *SIR* genes appear to act through these protein binding sites, related sequences in ABF1 and RAP1 are likely to be involved in interactions with the *SIR* gene products.

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30. Antibody was generated to purified ABF1 with three injections (10, 5, and 5 μg) into rabbits at 2-week intervals. In order to affinity-purify the antibody to ABF1, a highly purified fraction of ABF1 (phenyl Sepharose) was subjected to electrophoresis in 10% SDS-polyacrylamide gels (*29*), electroblotted to nitrocellulose, stained with 0.2% Ponceau S in 3% trichloroacetic acid (TCA), and washed with water. A strip of nitrocellulose containing the 135-kD ABF1 polypeptide was cut out, blocked with TN (10 m*M* tris, *p*H 7.4, and 150 m*M* NaCl) plus 3% bovine serum albumin (BSA), and incubated overnight with 1 ml of crude antiserum. The strip was washed once with TN, twice with TN plus 0.5% NP-40 and 0.5% sodium deoxycholate, and once with TN. Antibody was eluted by incubating the strip with 0.5 ml of 4*M* $MgCl_2$ containing BSA (0.05 mg/ml) at room temperature for 10 min. This eluate was immediately diluted with 1 ml of 10 m*M* tris, *p*H 7.2, and dialyzed overnight against 10 m*M* tris, *p*H 7.2. Antibody binding in protein immunoblots and plaque lifts was detected with 0.5 μCi of ^{125}I-labeled protein A (ICN Radiochemicals). Plaques (5×10^4) of λABF1 on a small petri dish were used to affinity-purify antibody from crude antiserum to ABF1 as described (*16*). *Escherichia coli* Y1090 cells (5×10^8) were infected at a multiplicity of 2.5 with λABF1. After 60 min at 37°C, cells were chilled, centrifuged, washed, and suspended in 0.5 ml of buffer X (*3*). Fifty microliters of lysozyme (50 mg/ml) was added, and cells were incubated for 5 min at room temperature and lysed by sonication. Ammonium sulfate (4*M*) was added to 10% saturation, and the mixture was incubated for 20 min on ice and centrifuged at 10,000*g* for 10 min. The pellet was suspended in 0.1 ml of Laemmli sample buffer

(*29*), and 20 μl was subjected to electrophoresis in 10% SDS-polyacrylamide gels, electroblotted, and probed with crude antiserum to ABF1 (1:500 dilution). To assay for ABF1-specific DNA binding activity in *E. coli* extracts, we plated 5×10^4 λABF1 on small petri dishes. After plaques first appeared (3 to 4 hours), 3 ml of TN was placed on the dishes and they were incubated for 4 hours at 37°C. Buffer was harvested, centrifuged, and assayed for DNA binding in a gel retention assay with either the 275-bp Bam HI–Sal I fragment from pBR322 (lanes 1 to 4) or the 325-bp Eco RI–Hind III fragment from pARS1.2 (lanes 5 to 8) as described (*3*). All yeast techniques were from *Methods in Yeast Genetics* [F. Sherman, G. R. Fink, J. B. Hicks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)].

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A Nondeletional Mechanism of Thymic Self Tolerance

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T cells become tolerant of self antigens during their development in the thymus. Clonal deletion of thymocytes bearing self-reactive T cell receptors is a major mechanism for generating tolerance and occurs readily for antigens expressed by bone marrow–derived cells. Tolerance to antigens expressed on the radioresistant thymic stromal elements is demonstrated here to occur via a nondeletional mechanism. For minor lymphocyte stimulatory (Mls-1^a) and major histocompatibility complex (MHC) antigens, this alternate form of tolerance induction results in clonal anergy.

IT IS OUR CURRENT VIEW THAT A PRECURSOR T cell entering the thymus will ultimately undergo one of three developmental fates. An immature T cell that does not recognize any component of the thymic stroma will die, presumably via a type of programmed cell death (*1*). An interaction between the thymocyte T cell receptor (TCR) and MHC molecules (or MHC plus peptide) on a radioresistant epithelial element rescues the cell from death and allows differentiation to a functionally mature T cell (positive selection) (*2–4*). In contrast, interactions with bone marrow–derived elements such as macrophages or dendritic cells result in clonal deletion of the self-reactive thymocyte (*5–7*).

Our understanding of thymic tolerance was enhanced by the development of monoclonal antibodies (MAbs) to the particular TCR V_β regions that confer specificity for defined antigens (*8–10*). These MAbs were used to show that clonal deletion of self-reactive cells is a major mechanism for maintaining tolerance to self antigens. This process also occurs in transgenic mice in which the majority of T cells bear a single receptor with autospecificity (*11*).

Deletion occurs at a $CD4^+8^+$ precursor stage of development and involves the CD4 and CD8 accessory molecules (*12*). Although there is evidence that clonal deletion occurs via an interaction with bone marrow–derived cells, it remains unclear whether epithelial elements within the thymus can also promote this event. In radiation bone marrow chimeras, donor T cells may develop functional tolerance to radioresistant thymic epithelial antigens (*3, 13*). We now investigate the mechanism by which the radioresistant elements within the thymus can induce self tolerance.

To manipulate the site of antigen expression, a series of radiation bone marrow chimeras were constructed such that the antigens recognized by specific V_β's were expressed only by the radioresistant host cells and not by bone marrow–derived elements. All of the chimeras were of the parent bone marrow into F_1-irradiated host type ($P \rightarrow F_1$; H-2^s or H-$2^k \rightarrow$ H-$2^{s\times k}$) in which the host (B10.S × AKR)F_1 was always held constant. This host expresses the MHC haplotype H-$2^{s\times k}$, and the minor lymphocyte stimulatory (Mls) antigens, Mls-$1^{a\times b}$. The donor was derived from either H-2^s or H-2^k strains that were Mls-1^b. In all cases the donor and host could be distinguished by their expression of either CD5 or CD45 alleles.

Initially we analyzed the chimeras for clonal deletion of $V_\beta 6$-bearing T cells. T cells bearing $V_\beta 6$ are deleted in Mls-1^a mice if these mice express certain MHC haplotypes such as H-2^k and H-2^d (such haplotypes are referred to as "permissive" for Mls presentation) (*10*). To increase the reliability of the analysis, we enriched thymocytes for mature cells by J11d MAb (*14*) plus complement lysis. This treatment enriches for $CD3^{Hi}$ mature cells (>90%). Two-color flow cytometric (FC) analyses of $V_\beta 6$ versus CD4 or CD8 were performed, and the resulting data were normalized for the number of $CD3^{Hi}$, $CD4^+8^-$, or $CD4^-8^+$ cells (an example of which is in Fig. 1). $V_\beta 6$ cells were present in SJL (H-2^s) mice (Fig. 1A), but deleted in both the $CD4^+8^-$ and

Table 1. Analysis for clonal deletion of $V_\beta 6$ thymocytes in chimeras made with an Mls-1^a–bearing host. Bone marrow chimeras were constructed as in Fig. 1. The (B10.S × AKR)F_1 host is Mls-$1^{a\times b}$ and all donors are Mls-1^b except CBA/J, which is Mls-1^a. This latter chimera serves as a control to indicate the $V_\beta 6$ frequency when the antigen relevant for deletion is on both the host and donor. The FC analysis was performed 35 to 80 days after reconstitution when chimeras were >97% donor type. For FC analysis, thymocytes from individual mice were treated with J11d.2 plus complement to enrich for mature cells. Remaining cells were stained as in Fig. 1. Percentages represent the arithmetic mean and SEM for three to eight individual animals. All values have been normalized to include only $CD3^{Hi}$ cells.

Strains	$V_\beta 6$ thymocytes (%)		
	Total*	$CD4^+8^-$†	$CD4^-8^+$‡
	H-2^s donors		
SJL → (B10.S × AKR)F_1	9.7 (±1.9)	10.1 (±2.0)	8.2 (±1.2)
A.SW → (B10.S × AKR)F_1	5.9 (±0.5)	5.7 (±0.6)	7.2 (±0.8)
SJL	10.6 (±0.6)	8.8 (±0.3)	14.2 (±0.6)
A.SW	7.2 (±0.4)	6.1 (±0.5)	11.2 (±1.5)
(B10.S × AKR)F_1	0.2 (±0.1)	0.1 (±0.1)	0.3 (±0.3)
(SJL × AKR)F_1	0.9 (±0.3)	0.9 (±0.3)	0.6 (±0.3)
	H-2^k donors		
CBA/J → (B10.S × AKR)F_1	0.7 (±0.3)	0.7 (±0.1)	1.9 (±1.5)
CBA/Ca → (B10.S × AKR)F_1	0.5 (±0.3)	0.3 (±0.4)	0.7 (±0.7)
C3H → (B10.S × AKR)F_1	2.2 (±1.0)	2.3 (±1.1)	2.0 (±1.2)
CBA/J	0.6 (±0.3)	0.5 (±0.1)	0.8 (±0.2)
CBA/Ca	10.2 (±1.2)	8.8 (±1.3)	14.5 (±0.6)
C3H	12.2 (±0.6)	9.8 (±1.7)	16.4 (±4.1)

*Values represent total $V_\beta 6^+$ cells divided by total $CD4^-8^+$ (×100). †Values represent $V_\beta 6^+$, $CD4^+8^-$ cells divided by total $CD4^+8^-$ (×100). ‡Values represent $V_\beta 6^+$, $CD4^-8^+$ cells divided by total $CD4^-8^+$ (×100).

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