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Characterization of the pH Signal Transduction Pathway Gene *palA* of *Aspergillus nidulans* and Identification of Possible Homologs

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We have cloned the *palA* gene of *Aspergillus nidulans*, one of six genes participating in ambient pH signal transduction in a regulatory circuit mediating pH regulation of gene expression. The derived 798-residue PalA protein is 29.4% identical over its entire length to a hypothetical protein from the nematode *Caenorhabditis elegans* and also has possible yeast homologs.

In the filamentous fungus *Aspergillus nidulans*, as in many other microorganisms, the syntheses of a number of permeases, secreted enzymes, and exported metabolites are appropriately regulated by ambient pH (3). For example, acid phosphatase and the acid-pH-optimum γ-aminobutyrate permease are synthesized preferentially in acidic media, whereas alkaline phosphatase and penicillin are synthesized preferentially in alkaline media (3, 7, 18). pH regulation of gene expression is mediated by the zinc finger transcription factor PacC, whose 73-kDa full-length translation product is proteolyzed to yield a 29-kDa N-terminal fragment able to activate transcription of genes expressed at alkaline pH and prevent transcription of genes expressed at acid pH (15, 17, 19). The products of six genes, *palA*, *-B*, *-C*, *-F*, *-H*, and *-I*, form a signal transduction pathway through which alkaline ambient pH is able to elicit the conversion of the full-length form of PacC to the functional proteolyzed form (1, 3, 5, 15, 19). Loss-of-function mutations in these *pal* genes prevent this proteolytic conversion and mimic the effects of growth at acidic pH (1, 3, 5, 7, 15, 18).

The mechanism of ambient pH signal transduction is an intriguing subject of scientific and biotechnological importance. We have previously shown that the *palB* gene product is likely to be a cysteine protease of the calpain family, albeit not the protease responsible for the final conversion of PacC to its functional form (5). Here, we report the cloning and sequence of *palA*. Amino acid sequence similarity strongly suggests that the *palA* gene product has a homolog in the nematode *Caenorhabditis elegans*. It also has possible homologs in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*.

The *palA* gene was cloned with linkage group III (containing *palA*) clones from a chromosome-allocated wild-type *A. nidulans* cosmid library (2) and pILJ16 (11) and by identification of *palA*$^+$ cotransformants by their ability to grow on pH 8 medium (3–5) among *argB*$^+$ transformants of a strain of genotype *biA1 yA2 wA3 argB2 areA49 palA1*. *palA1*-rescuing activity was found in cosmid W28H12 and further localized to a 5-kb *Xba*I-*Xho*I fragment (Fig. 1A). To confirm that this fragment contains the *palA* gene itself rather than a gene associated with a fortuitous suppressor activity, strains carrying either of two

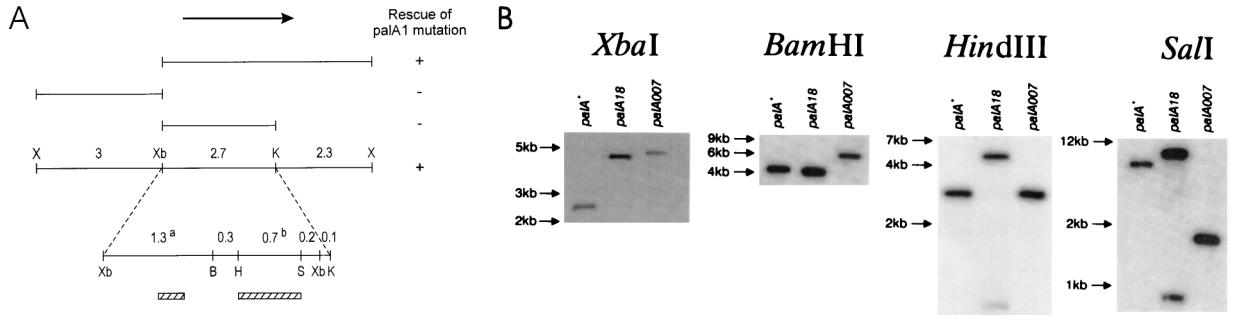

FIG. 1. (A) Rescue of the *palA1* mutation by various restriction fragments, position and orientation of the *palA* transcript, and positions of two *palA*-associated translocation breakpoints. X, *Xho*I; Xb, *Xba*I; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; K, *Kpn*I. The arrow at the top denotes the direction and location of the *palA* transcript relative to the 8-kb *Xho*I fragment in the middle. The ability of various restriction fragments, whose sizes are shown in kilobases, to rescue the *palA1* mutation is indicated to the right. The hatched bars at the bottom indicate the positions of fragments used to obtain cDNA clones and as probes for Northern blots. a, contains the *palA007* translocation breakpoint; b, contains the *palA18* translocation breakpoint. (B) Southern blots localizing the *palA18* and *palA007* translocation breakpoints. From left to right are shown *Xba*I digests probed with the 2.6-kb *Xba*I fragment shown in the expanded portion of the Fig. 1A restriction map, *Bam*HI digests probed with the 1.3-kb *Xba*I-*Bam*HI fragment shown in the expanded portion of the Fig. 1A restriction map, *Hin*dIII digests probed with the 2.7-kb *Hin*dIII fragment extending rightwards from the *Hin*dIII site shown in the expanded portion of the Fig. 1A restriction map, and *Sal*I digests probed with the 0.7-kb *Hin*dIII-*Sal*I fragment shown in the expanded portion of the Fig. 1A restriction map.

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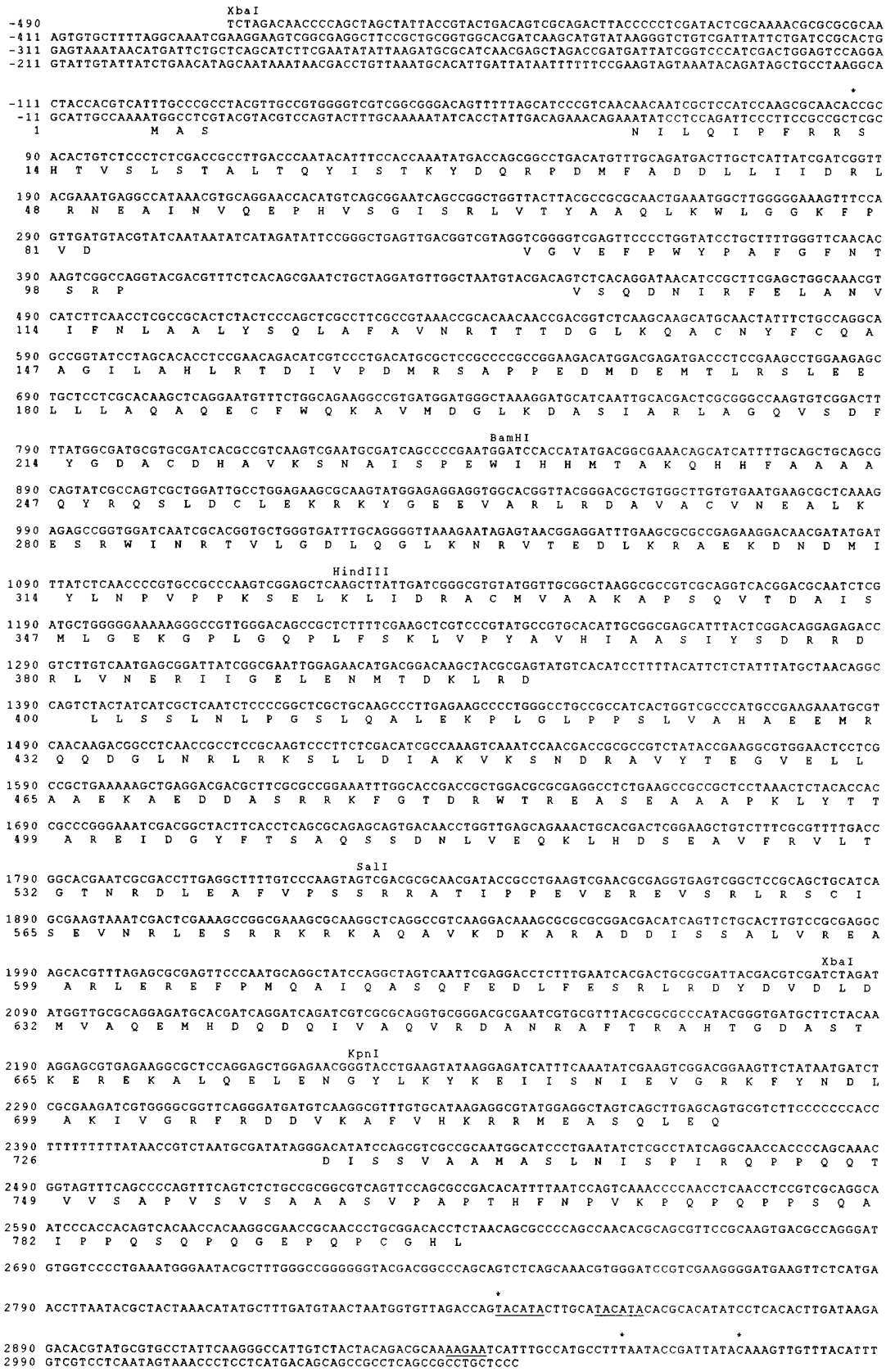

FIG. 2. Nucleotide sequence of a 3,536-bp region containing the *palA* gene and its derived translation product. Restriction sites shown in Fig. 1A are indicated. The 5′ end of all four cDNA clones is starred, as are the 3′ ends of the three polyadenylated cDNA clones. The sequence CAAT is present upstream of the cDNAs at −39 and −189. Sequences playing a role in mRNA 3′-end formation in *S. cerevisiae* (9, 10) are underlined.

FIG. 3. Alignment of the derived PalA sequence with those of a hypothetical *C. elegans* protein (Z29561) and Bro1p of *S. cerevisiae*. Vertical lines indicate identities; dots between sequence lines denote similarities (A/G, D/E, F/Y/W, I/L/M/V, K/R, N/Q, and S/T). Alignments of PalA to Z29561 and Bro1p were carried out individually with Bestfit from the Genetics Computer Group package (6) and then combined and edited by eye.

translocation-associated *palA* mutant alleles were subjected to Southern hybridization analysis along with a wild-type strain. The detection of restriction fragment polymorphisms correlated with chromosomal rearrangement mutations establishes physical linkage between a cloned fragment and a mutationally identified gene (8). By using several probes from the region shown in Fig. 1A and an appropriate set of digests, the chromosome III *palA007* translocation breakpoint was localized to the 1.3-kb *Xba*I-*Bam*HI fragment and the chromosome III *palA18* translocation breakpoint was localized to the 0.7-kb *Hin*dIII-*Sal*I fragment (Fig. 1B). (*palA007* [formerly *npeC007*] is associated with a translocation involving chromosomes II and III [18], whereas *palA18*, whose selection was described previously [1], is associated with a translocation involving chromosomes III and VIII.) The localization of the two *palA*-associated translocation breakpoints within the 5-kb *Xba*I-*Xho*I fragment in conjunction with the ability of this fragment to rescue the *palA1* mutation provides unequivocal evidence that it contains the *palA* gene. In Northern blots, the 2.7-kb *Xba*I-*Kpn*I fragment and the two smaller fragments denoted by hatched bars at the bottom of Fig. 1A hybridized to a single mRNA with a size of ~2.8 kb. Levels of this mRNA do not seem to be influenced by the PacC-mediated pH regulatory system: Northern blots with total RNA from an alkalinity-mimicking *pacC*^{c}*11* mutant or an acidity-mimicking *palF15* mutant showed no appreciable differences in relative *palA* mRNA levels when compared to wild-type levels, in marked contrast to PacC-regulated transcripts in an experiment similar to that shown in Fig. 1 of Tilburn et al. (19) (data not shown). We did consistently find lower relative *palA* mRNA levels in wild-type cells grown at pH 4 than in wild-type cells grown at pH 6.5 or 8 (over which range there is no variation) (data not shown), but this might relate to differential mRNA lability under the growth and/or extraction conditions used. The lack

of influence of extreme mutations in genes of the PacC-mediated pH regulatory system and the lack of effect of changing the growth pH for the wild type between pH 6.5 and pH 8 argues very strongly that the PacC-mediated pH regulatory system, of which the *palA* gene product is a component, does not regulate *palA* expression.

A genomic region with a size of 3,536 bp encompassing the 2.7-kb *Xba*I-*Kpn*I fragment hybridizing to the *palA* message and including the fragments in which the *palA*-associated translocation breakpoints occur was sequenced (Fig. 2). Sequencing of cDNA clones from a λ gt10 library (16) revealed the presence of five introns with sizes of (moving from 5′ to 3′) 49, 50, 52, 45, and 50 nucleotides, respectively (Fig. 2). Four cDNA clones, all clearly independent as shown by their differing 3′ ends (vide infra), all have the same 5′ end, which is only 14 bp upstream of the putative initiation codon (Fig. 2). This could mean either that the *palA* mRNA has an exceptionally short leader region or that reverse transcription does not normally proceed beyond this point (perhaps because of stable secondary structure in the mRNA). Three of the four cDNA clones are polyadenylated, each at a different site (Fig. 2). Taking poly(A) tails into account, the length of these polyadenylated cDNAs (longest is 2,641 bp) agrees reasonably well with the 2.8-kb estimate of messenger size, suggesting that their 5′ end is either the transcription start point or quite near to it. There are no potential PacC binding sites (GCCARG [19]) in the 490 bp upstream of the initiation codon, consistent with the absence of PacC-mediated transcriptional regulation.

The derived amino acid sequence of *palA* contains 798 residues and has a calculated molecular mass of 89.4 kDa. If the *palA* protein product PalA is located in the nucleus, the very basic sequence RRKRK beginning at residue 573 is a possible nuclear localization signal or part of one. The C terminus of PalA is exceptionally proline rich, with proline accounting for 16 of the last 59 residues. The positions of the *Xba*I, *Bam*HI, *Hin*dIII, and *Sal*I sites bordering the *palA007* and *palA18* translocation breakpoints (Fig. 1A) in the Fig. 2 sequence should be particularly noted. The fact that all extant *palA* mutations, including *palA007* with its breakpoint in the promoter, 5′ untranslated region, or 5′ portion of the coding region and *palA18* with its breakpoint in the central part of the coding region, have the same phenotype argues very strongly that this is the null phenotype, particularly as it is indistinguishable from that of the *palB* disruption (5) allele.

In Bestfit (6) pairwise alignments (Fig. 3), PalA shows 29.4% identity over 850 residues with an 882-residue hypothetical protein from the nematode *C. elegans* (accession number Z29561), 23.1% identity over 769 residues with a 701-residue hypothetical protein from the fission yeast *S. pombe* (accession number Z54354) (not shown), and 20.8% identity over 852 residues with the 844-residue Bro1p protein (14) of the budding yeast *S. cerevisiae*. Although no function has yet been identified for the probable *C. elegans* PalA homolog (or the possible *S. pombe* homolog), Bro1p is likely to be in a pathway that interacts with (possibly sharing downstream targets with) the protein kinase C–mitogen-activated protein kinase pathway in *S. cerevisiae* (14). Like Bro1p (14), PalA contains putative SH3 domain-binding motifs (consensus PXXP [12, 13, 20]) with PVPP beginning at residue 317 and with PVKP and PQPP beginning at residues 770 and 775, respectively. A very recent database entry (accession number Z75183) for a hypothetical protein encoded by chromosome XV of *S. cerevisiae* has 30.2% identity over 732 residues in a Bestfit alignment with PalA.

Although the role of PalA in pH signal transduction remains to be elucidated, the identification of a probable homolog in *C. elegans* (29.4% identity over the entire length of PalA) and possible homologs in *S. pombe* and *S. cerevisiae* opens the way to a multiorganism approach to the mechanism of pH signalling or at least its evolutionary origins. The *C. elegans* probable homolog is of particular interest because it raises the enchanting possibility of conservation of pH signal transduction between the fungal and animal kingdoms.

Nucleotide sequence accession number. The EMBL accession number for the *palA* sequence is Z83333.

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REFERENCES

1. **Arst, H. N., Jr., E. Bignell, and J. Tilburn.** 1994. Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. Mol. Gen. Genet. **245:**787–790.
2. **Brody, H. J., J. Griffith, A. J. Cuticchia, J. Arnold, and W. E. Timberlake.** 1991. Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*. Nucleic Acids Res. **19:**3105–3109.
3. **Caddick, M. X., A. G. Brownlee, and H. N. Arst, Jr.** 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. **203:**346–353.
4. **Cove, D. J.** 1976. Chlorate toxicity in *Aspergillus nidulans*. Studies of mutants altered in nitrate assimilation. Mol. Gen. Genet. **146:**147–159.
5. **Denison, S. H., M. Orejas, and H. N. Arst, Jr.** 1995. Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. **270:**28519–28522.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
7. **Espeso, E. A., J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva.** 1993. pH regulation is a major determinant in expression of a fungal biosynthetic gene. EMBO J. **12:**3947–3956.
8. **Green, P. M., and C. Scazzocchio.** 1985. A cloning strategy in filamentous fungi, p. 345–353. *In* J. W. Bennett and L. L. Lasure (ed.), Gene manipulations in fungi. Academic Press, Orlando, Fla.
9. **Guo, Z., and F. Sherman.** 1995. 3′-end-forming signals of yeast mRNA. Mol. Cell. Biol. **15:**5983–5990.
10. **Irniger, S., and G. H. Braus.** 1994. Saturation mutagenesis of a polyadenylation signal reveals a hexanucleotide element essential for mRNA 3′ end formation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **91:**257–261.
11. **Johnstone, I. L., S. G. Hughes, and A. J. Clutterbuck.** 1985. Cloning an *Aspergillus nidulans* developmental gene by transformation. EMBO J. **4:**1307–1311.
12. **Lee, C. H., K. Saksela, U. A. Mirza, B. T. Chait, and J. Kuriyan.** 1996. Crystal structure of the conserved core of HIV-1 Nef complexed with Src family SH3 domain. Cell **85:**931–942.
13. **Lim, W. A., F. M. Richards, and R. O. Fox.** 1994. Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. Nature (London) **372:**375–379.
14. **Nickas, M. E., and M. P. Yaffe.** 1996. *BRO1*, a novel gene that interacts with components of the Pkc1p-mitogen-activated protein kinase pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2585–2593.
15. **Orejas, M., E. A. Espeso, J. Tilburn, S. Sarkar, H. N. Arst, Jr., and M. A. Peñalva.** 1995. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. **9:**1622–1632.
16. **Osmani, S. A., R. T. Pu, and N. R. Morris.** 1988. Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. Cell **53:**237–244.
17. **Sarkar, S., M. X. Caddick, E. Bignell, J. Tilburn, and H. N. Arst, Jr.** 1996. Regulation of gene expression by ambient pH in *Aspergillus*: genes expressed at acid pH. Biochem. Soc. Trans. **24:**360–363.
18. **Shah, A. J., J. Tilburn, M. W. Adlard, and H. N. Arst, Jr.** 1991. pH regulation of penicillin production in *Aspergillus nidulans*. FEMS Microbiol. Lett. **77:** 209–212.
19. **Tilburn, J., S. Sarkar, D. A. Widdick, E. A. Espeso, M. Orejas, J. Mungroo, M. A. Peñalva, and H. N. Arst, Jr.** 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. **14:**779–790.
20. **Yu, H., J. K. Chen, S. Feng, D. C. Dalgarno, A. W. Brauer, and S. L. Schreiber.** 1994. Structural basis for the binding of proline-rich peptides to SH3 domains. Cell **76:**933–945.