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White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*

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A saturating genetic dissection of 'blind' mutants in *Neurospora crassa* has identified a total of two non-redundant loci (*wc-1* and *wc-2*) each of which is required for blue-light perception/signal transduction. Previously, we demonstrated that WC1 is a putative zinc finger transcription factor able to bind specifically to a light-regulated promoter. Here, we present the cloning and characterization of the *wc-2* gene. We demonstrate using mutation analysis and *in vitro* DNA-binding assays that WC2, the second partner of this light signal transduction system, encodes a functional zinc finger DNA-binding protein with putative PAS dimerization and transcription activation domains. This molecular genetic dissection of the second of two components of this light signal transduction system has enabled us to devise a model whereby WC1 and WC2 are proposed to interact via homologous PAS domains, bind to promoters of light-regulated genes and activate transcription. As such, this study provides the first insight into two co-operating partners in blue-light signal transduction in any organism and describes the molecular tools with which to dissect this enigmatic process.

Keywords: blue light/*Neurospora crassa*/signal transduction/*white collar*/zinc finger

Introduction

The capacity of sensing and responding to environmental light signals is widespread throughout the biological kingdom. In higher plants, there are at least three different families of photoreceptors, the phytochromes (red and far-red light absorption), blue-light receptor(s) and ultraviolet receptor(s) (Deng, 1994). Although the best studied signalling pathway in plants involves phytochrome, considerable research has been carried out in the past decades in order to unravel the blue-light perception and signal transduction pathway (Kaufman, 1993; Short and Briggs, 1994). Research on blue-light signal transduction in plants has been complicated by the fact that phytochrome absorbs blue and UV light to a certain degree in addition to red/far-red light. Furthermore, the light signalling pathways of higher plants appear to be extremely complex. They include general signal transduction components such as G proteins, cGMP and Ca^{2+} and seem to be regulated by cross-talk and feedback regulation (Kaufmann, 1993; Bowler and Chua, 1994). Only recently has the first candidate for a blue-light photoreceptor in plants been cloned (CRY1; Ahmad and Cashmore, 1993). The CRY1 protein reveals a close homology to bacterial DNA photolyase and was also shown to bind flavin, although its mode of action and interacting partners are as yet unknown (Lin *et al.*, 1995). Although putative mutants affected in blue-light signal transduction events have been identified in plants, nothing is yet known about the genes coding for the protein components of the blue-light signalling system (Liscum and Hangarter, 1994). Furthermore, numerous genes coding for DNA-binding proteins have been cloned in plants, but none of these proteins has been assigned unequivocally a function in light-induced transcription (Terzaghi and Cashmore, 1995).

The fungus *Neurospora crassa* remains a paradigm for molecular genetic studies on blue-light signal transduction. In contrast to plants, in *Neurospora* only blue light is perceived and has been shown genetically and physiologically to regulate many different developmental processes, e.g. mycelial carotenogenesis (Harding and Turner, 1981), formation of conidia (Lauter and Russo, 1991) and phototropism of peritecial beaks (Harding and Melles, 1984). Blue-light-regulated transcription has also been observed for many genes in *Neurospora* such as the carotenoid biosynthesis genes *al-1*, *al-2* and *al-3* (Nelson *et al*., 1989; Schmidhauser *et al.*, 1990, 1994), the conidiation genes *con-5* and *con-10* (Lauter and Russo, 1991), the circadian clock gene *frequency* and the clock-controlled genes *ccg-1* and *ccg-2* (Loros, 1995).

Several classes of *Neurospora* mutants have been isolated and characterized which appear to be affected in blue-light signal transduction (Harding and Shropshire, 1980; Paietta and Sargent, 1981, 1983; Carattoli *et al.*, 1995). In the two riboflavin mutants *rib-1* and *rib-2*, flavin deficiency is correlated with a reduced sensitivity to blue light, implicating a role for flavin in light perception (Paietta and Sargent, 1981). By contrast, the *wc-1* and *wc-2* mutants are completely blind to blue light based on their dark-grown phenotypes observed even after light induction (Harding and Turner, 1981). In spite of a saturating screen, no additional *wc* loci other than *wc-1* and *wc-2* could be isolated, suggesting that these are the two central and non-redundant components of blue-light signal transduction in *N.crassa* (Degli-Innocenti and Russo, 1984; Linden *et al.*, 1997). The *white collar* mutants each have pigmented conidia whereas the mycelia are white due to a specific deficiency in light-induced carotenoid synthesis in mycelia. In addition, most blue-light-regulated genes are unable to respond to light in a *wc-1* or *wc-2* mutant background. As the *wc-1* and *wc-2* mutants are defective only in blue-light-induced processes but not generally affected in their growth behaviour or in the expression of non-light-regulated genes, their gene products are proposed to be dedicated to blue-light signal transduction. Furthermore, it has been suggested that the *wc*

mutants correspond to two components of a common signal transduction pathway (Ballario *et al.*, 1996). Using a molecular genetic approach, we have begun to examine the role of WC1 and WC2 in blue-light signal transduction of *N.crassa*. The *wc-1* gene has been cloned and characterized to encode a putative transcription factor containing a zinc finger DNA-binding domain and a putative transcription activation domain (Ballario *et al.*, 1996). As the WC1 fusion protein was shown to bind *in vitro* to the promoter of blue-light-regulated genes, it is proposed that WC1 is a transcription factor that affects the induction of light-regulated genes.

Here, we present the isolation and characterization of the *wc-2* gene coding for the second central regulator of blue-light responses. The *wc-2* gene represents the first example of a second component of a blue-light signal transduction pathway that has been characterized in any organism. The *wc-2* gene, which was isolated by use of an integrational mutagenesis approach, encodes characteristic features of transcription factors such as a putative zinc finger domain, putative transcription activation domains and a putative dimerization domain. Therefore, both WC proteins are putative transcription factors specifically involved in blue-light regulation. Due to a novel PAS dimerization domain present in both WC polypeptides, an interaction of WC1 and WC2 is proposed. A model is put forward in which a light-induced heterodimerization of WC1 and WC2 results in binding and transcriptional activation of light-induced genes.

Results

Isolation of a wc-2 mutant by integrational mutagenesis

A selection system was used to isolate regulatory mutants which are either hampered or completely blocked in the transduction pathway of blue-light perception in *N.crassa* (Carattoli *et al.*, 1995). In this selection system, the light-induced *al-3* promoter was fused to the coding region of the *mtr* gene. The mtr protein is responsible for the uptake of neutral, aliphatic and aromatic amino acids and their toxic analogues in *Neurospora* (Stuart *et al.*, 1988). After transformation of an *mtr*$^-$/*trp*$^-$ strain with this construct, the resulting strain 13-1 grows on a medium supplemented with the toxic amino acid analogue *p*-fluoro-phenylalanine (FPA) only in darkness as the *al-3::mtr* gene construct is not expressed. In the light, however, the *al-3::mtr* promoter is induced, causing *mtr* expression and the uptake of the drug which inhibits cell growth (Linden *et al.*, 1997). Therefore, only mutants impaired in blue-light perception or signal transduction will grow in the light in the presence of FPA. This selection system was applied successfully to the isolation of *wc-1* and *wc-2* mutants after UV mutagenesis. Thus, this selection system was also used on insertion mutants to identify tagged *wc-2* mutants and to isolate the gene. In *N.crassa*, transformation occurs by random non-homologous integration of the transforming DNA into the genome (Paietta and Marzluf, 1985). Therefore, a large pool of independent *N.crassa* transformants should include mutants with the foreign DNA integrated into a specific target gene. The powerful *al-3::mtr* screening system was used to identify the *wc-2* mutant alleles in the DNA-tagged *N.crassa* lines, allowing cloning of the *wc-2* gene. Strain 13-1 was transformed with plasmid pES200 carrying

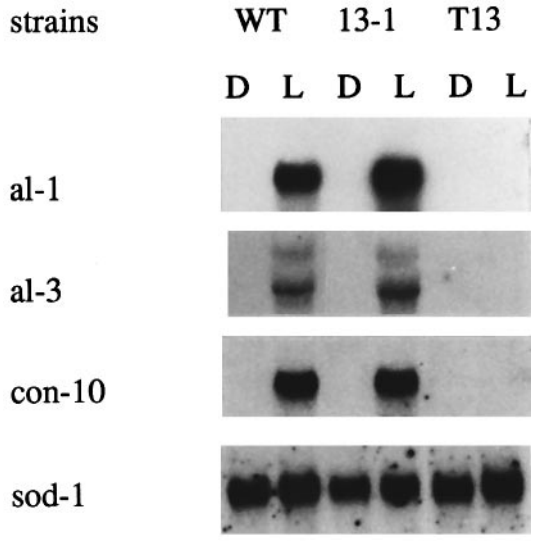

Fig. 1. DNA-tagged *wc-2* mutant T13 shows a specific defect in light-regulated gene expression. Northern blot analysis of wild-type, strain 13-1 and *wc-2* mutant T13. Mycelia were isolated after growth in the dark (D) and after a light induction of 20 min (L). For hybridization, specific probes of *al-1*, *al-3* and *con-10* were used. For normalization, the filter was hybridized with *sod-1*.

markers selectable in both *Escherichia coli* (ampicillin) and *Neurospora* (hygromycin). The transformed sphaeroplasts were grown on solid medium supplemented with hygromycin to allow the formation of homocaryotic conidia. After 8 days' growth, the conidia of transformants were harvested and grown in the presence of FPA to select for light-impaired mutants. Out of ~2×10^5 independent transformants tested, one mutant (T13) was isolated which showed a *white collar* phenotype and was resistant to hygromycin. In genetic crossing experiments, it was shown that the tagged T13 mutant does not recombine with the authentic *wc-2* mutants (alleles ER33 and 234w) which identifies it as a newly created *wc-2* mutant. In backcrosses to *Neurospora* wild-type, the *wc-2* phenotype of T13 co-segregated with the hygromycin resistance conferred by the transforming DNA. It was concluded, therefore, that the insertion of plasmid pES200 had occurred either in or close to the *wc-2* gene.

As previously observed, *wc-2* mutants are impaired in blue-light induction not only of the carotenoid biosynthesis genes *al-1*, *al-2* and *al-3* (Nelson *et al.*, 1989; Schmidhauser *et al.*, 1990, 1994) but also in the induction of other blue-light-regulated genes such as the conidiation genes *con-10* and *con-8* (Lauter and Russo, 1991). To confirm that the T13 allele of *wc-2* was also similarly impaired, the steady-state levels of *al-1*, *al-3* and *con-10* RNA were examined in the T13 mutant grown in darkness and also following light induction (Figure 1). In contrast to the control strains wild-type and 13-1, none of the above genes were inducible by light in the T13 mutant (Figure 1). The region flanking the integrated plasmid was amplified by inverse PCR using oligonucleotide primers complementary to the plasmid sequence and a 0.6 kb fragment containing only *Neurospora* DNA was cloned (indicated in Figure 2). This DNA fragment, presumed to contain a portion of the *wc-2* gene, was used subsequently as a probe in a Southern blot of genomic DNA from the

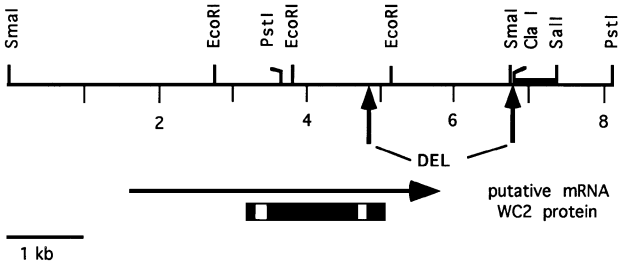

Fig. 2. The *wc-2* gene structure. Restriction sites and length of the genomic DNA sequenced are shown in kb. The black box indicates the predicted WC2 protein composed of 530 amino acids interrupted by the two introns. The horizontal arrow above shows the direction of transcription and the putative *wc-2* transcript of ~4 kb. The integration of pES200 in mutant T13 and the deletion are indicated by vertical arrows (DEL). The *Cla*I–*Sal*I flanking region which was used for the isolation of the *wc-2* genomic clones is shown in bold.

wild-type, strains 13-1 and T13. The results confirmed that the fragment is in fact *Neurospora* DNA flanking the integrated plasmid in the *wc-2* mutant, T13 (data not shown).

Isolation of genomic DNA clones, subcloning of the wc-2 gene and complementation of the wc-2 mutant phenotype

The cloned DNA insert proposed to contain a portion of the *wc-2* gene was used as a hybridization probe for the screening of a *Neurospora* genomic DNA cosmid library, and four cosmid clones were isolated. Transformation of the *wc-2* mutant alleles ER33, 234w and T13 with each of these four cosmids resulted in the complementation of the *wc* phenotype in each mutant. Using the same flanking DNA fragment, a 4.5 kb *Pst*I–*Pst*I fragment of *Neurospora* DNA was subcloned (Figure 2). This was used as a probe for subcloning of several restriction fragments from one of the cosmid clones. The subclones subsequently were used in complementation experiments. After transformation of a *wc-2* mutant with plasmid pCBWC2A containing a *Sma*I–*Sma*I 6.75 kb restriction fragment (Figure 2), complementation of the *wc-2* mutant phenotype was observed. Northern blot analysis indicated that the transformed *wc-2* mutants had regained the capacity of blue-light-induced transcriptional activation (data not shown). Thus, it was concluded by complementation that a functional *wc-2* gene was present on this plasmid.

Homology-induced gene inactivation independently confirms the identity of the wc-2 gene

In the above section, we showed that plasmid pCBWC2A contained the *wc-2* gene by using functional complementation of a *wc-2* mutant. To confirm this designation independently, we showed that the pCBWC2A plasmid could be used to create a *wc-2* mutant by homology-induced gene inactivation. In *N.crassa*, two different phenomena of transgene-induced gene silencing have been described: 'quelling' and 'RIPing' which occur during the vegetative and sexual cycle, respectively (Selker, 1990; Romano and Macino, 1992). Both 'quelling' and 'RIPing' affect not only the exogenous but also the endogenous copy of a duplicated gene, and have, therefore, been used to confirm the identity of cloned genes by the ability of the cloned gene to induce a mutant phenotype (Ballario *et al.*, 1996). Plasmid pCBWC2A was transformed in *Neurospora* wild-type and ~30% of all transformants were shown to have a *wc* phenotype ('quelling'). A similar frequency of 'quelling' was also observed for other non-essential genes such as *al-1* (Romano and Macino, 1992). Several of the transformants which had a wild-type phenotype subsequently were backcrossed to wild-type and ~9% of the progeny revealed a *wc* phenotype ('RIPing'). Therefore, by both 'RIPing' and 'quelling', the pCBWC2A plasmid was able to induce a *wc-2* phenotype by DNA-induced gene inactivation, again indicating that the *wc-2* gene is present on this cloned fragment of *Neurospora* DNA.

Nucleotide sequencing and isolation of wc-2 cDNAs

The entire region of the *Sma*I–*Sma*I fragment which encompasses the putative *wc-2* gene and adjacent regions was sequenced (Figure 3). Using the same DNA fragment of pCBWC2A, a *Neurospora* cDNA library was screened. Approximately 20 positive clones were isolated and examined further by restriction analysis and DNA sequencing. All the isolated cDNAs detected the *wc-2* transcript depicted in Figure 2. The largest cDNA clone contained a 3 kb insert truncated at the 5′ end. This cDNA revealed an open reading frame (ORF) of 1590 bp coding for a protein of 530 amino acids. Stop codons in all three reading frames upstream of the first start codon (nt 3218 in Figure 3) indicate that the entire ORF is present on this cDNA. The first ATG start codon is followed by several additional ATG codons in-frame. It is not known at present which of these is the actual start codon used, although the third ATG (nt 3270) was found to be in accordance with the consensus AGXXATGG for eukaryotic initiation sites described by Kozak (1981). Comparison with the genomic DNA sequence resulted in the identification of two introns with 156 (intron 1) and 86 nucleotides (intron 2). Intron 5′-donor and 3′-acceptor sites were identified which corresponded very well with the consensus sequences described for *N.crassa* (Figure 3, Edelmann and Staben, 1994). Two different polyadenylation sites were found by sequencing of several cDNAs, a situation rather common in *Neurospora* (Bruchez *et al.*, 1993). In comparison with the coding region, a very long mRNA transcript was detected, suggesting that *wc-2* contains a long 5′ non-coding region (Figure 3). Reverse transcriptase PCR was used to identify the approximate length of the 5′ non-coding region. When an oligonucleotide at nt 1929 was used, cDNA synthesis was still observed (results not shown), indicating an unusually long 5′ non-coding region of at least 1290 nucleotides. Long 5′ non-coding regions have been identified in other *N.crassa* genes coding for DNA-binding proteins such as NIT-2 and CPC-1 and have been implicated in the control of RNA translation or stability (Paluh *et al.*, 1988; Fu and Marzluf, 1990).

The WC2 protein reveals features of a transcription factor and contains a putative PAS dimerization domain

The WC2 protein deduced from the nucleotide sequence is composed of 530 amino acids. WC2 has a calculated

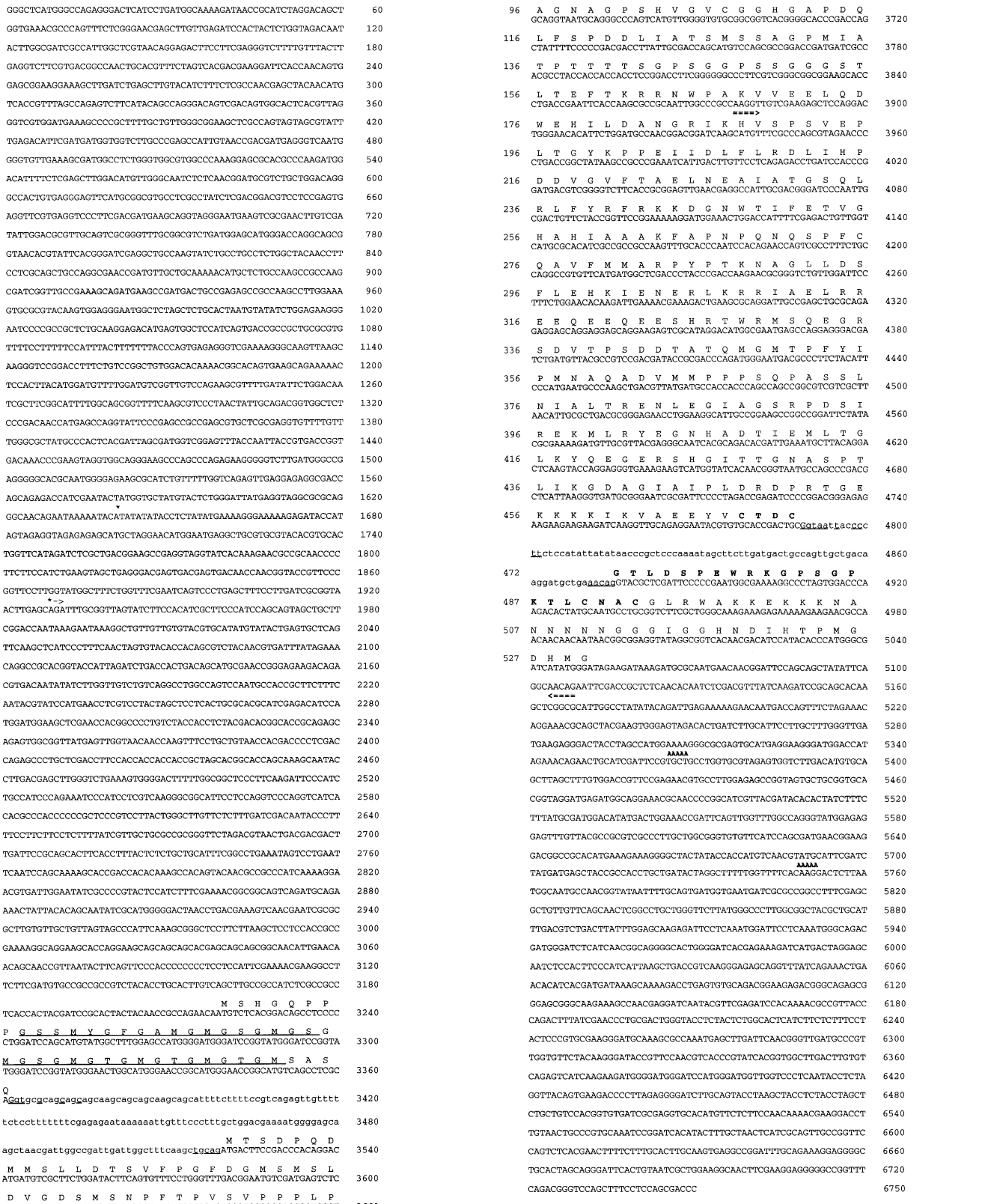

Fig. 3. Nucleotide sequence of the 6.7 kb *Sma*I–*Sma*I fragment containing the *wc-2* gene and flanking sequences. The translated amino acid sequence of the predicted WC2 protein is shown above the nucleotide sequence. Bold letters indicate the putative zinc finger domain. Underlined amino acids indicate the Thr/Gly, Met/Gly and Ser/Gly repeats. Lower case letters correspond to intron 1 (157 bp) and intron 2 (86 bp). Underlined nucleotides in this region show residues in agreement with intron donor and acceptor sites. The largest cDNA in the 5′ direction found by RT–PCR with different oligonucleotides is indicated by an asterisk and an arrow above the sequence (nt 1928). Use of an oligonucleotide further upstream (indicated by an asterisk, nt 1628) did not result in any cDNA synthesis. The two horizontal arrows beneath the sequence show the region which was used for the overexpression of the GST–WC2 fusion protein in *E.coli*. (E) As above, the sequence indicates two different polyadenylation sites of the *wc-2* transcript.

mol. wt of 56 903 and a statistical pI of 7.4. The major amino acids present are glycine (11.9%), proline (8.3%) and serine (8.3%). A hydropathy plot indicates that WC2 is a soluble protein. An acidic region occurring between amino acids 300 and 350 was also identified. In this region, a helical folding structure is predicted. Another region between amino acids 80 and 120 shows a high percentage of prolines (25%). When compared with other proteins of the SwissProt protein sequence database, no overall homology was found, but several notable features were identified in WC2 subdomains: firstly, from residue 467 onwards, a putative zinc-finger-binding domain with the structure C-X2-C-X18-C-X2-C was found (Figure 3). This domain exhibits a high degree of homology with the zinc finger motifs of transcription factors such as WC1 (Ballario *et al.*, 1996) as well as with AREA from *Aspergillus*, GAT1 and SRD1 from yeast and NIT2 from *N.crassa*, all belonging to the group of GATA factors (Figure 4A, Orkin, 1992). Secondly, a putative dimerization region was identified in WC2 (Figure 4B) which showed a homology with PAS (for PER-ARNT-SIM), a dimerization domain present in the period protein (PER) and the *single-minded* gene product of *Drosophila melanogaster* and in both subunits of the mammalian dioxin receptor complex, the aryl hydrocarbon receptor nuclear translocator (ARNT) and the aryl hydrocarbon receptor (AHR) (Huang *et al.*, 1993). This PAS dimerization domain is normally comprised of two direct repeats called PAS A and PAS B. Only one of these subdomains was identified in WC2, which showed a higher similarity to the PAS A repeat in the case of ARNT (39.0%) and AHR (42.9%) and to PAS B in the case of SIM (46.4%) and PER (44.0%). The other blue-light regulatory protein of *N.crassa*, WC1 (Ballario *et al.*, 1996), also contains a previously unidentified putative PAS domain, which exhibits a similarity of 41% with the WC2 PAS domain (Figure 4B). There are only two residues (indicated in Figure 4 by asterisks) which are identical in all PAS A and PAS B domains characterized so far (Wang *et al.*, 1995). These amino acids are also conserved in the PAS domains of both WC2 and WC1 (Figure 4B). In contrast to WC2, a second putative PAS domain was identified in WC1 (amino acids 395–426, data not shown). However, the similarity to other PAS domains is comparably low, and the residues strictly conserved in all other PAS domains could not be found in this domain. The WC2 protein showed an additional similarity of 45.2% over 60 amino acids in the putative PAS domain (amino acids 179–241) to the photoactive yellow protein (PYP, *Ectothiorhodospira halophila*; Baca *et al.*, 1994), although the similarity of the latter protein to the PAS domain of the other proteins was minimal (data not shown). In addition to the homology in the PAS domain, the WC2 protein shares a second region of homology with the PER protein of *D.melanogaster*. This domain was found close to the WC2 N-terminus consisting of Thr/Gly, Met/Gly and Ser/Gly repeats (Figure 3; Jackson *et al.*, 1986). Such a domain was also described in the other circadian clock regulatory protein FRQ from *Neurospora*, although the significance of this domain is not known (McClung *et al.*, 1989).

Binding of WC2 to the al-3 promoter region involved in blue-light regulation

The features of WC2 suggest a role for the protein in transcriptional activation. Using a GST–WC2 fusion protein which contained the putative zinc-finger-binding domain and the PAS dimerization motif (as indicated in Figure 3), *in vitro* DNA-binding experiments were carried out. An *al-3* promoter fragment was used as a putative target gene for WC2 binding for the following reasons: Carattoli *et al.* (1994) identified a promoter region in the *al-3* gene which contained all necessary regulatory elements for blue-light gene induction. This fragment contained two canonical GATA sequences and has been applied successfully in DNA-binding experiments with a WC1 fusion protein (Ballario *et al.*, 1996). Furthermore, in *wc-2* mutants, no light induction of the *al-3* mRNA is observed. In electrophoretic mobility shift assay (EMSA), *Escherichia coli* lysates from cells expressing either the GST–WC2 fusion protein or GST only were incubated with the labelled *al-3* promoter fragment (Figure 5). A band shift was obtained only when the WC2 fusion protein was present (Figure 5A, lane 2) but not when a control GST extract was used (Figure 5A, lane 1). The amount of labelled *al-3* promoter fragment shifted by the GST–WC2 fusion protein was comparatively low. This could be due to incorrect folding of the GST–WC2 fusion protein. To prove that binding of WC2 to the *al-3* promoter fragment was sequence specific, different unlabelled competitor DNAs were used. When increasing amounts of an unlabelled 41mer covering the same region of the *al-3* promoter were added to the reaction, binding of WC2 to *al-3* was competed (Figure 5A, lanes 3–6). By contrast, when mutated GATA motifs were used as a competitor, competition was observed only at higher concentrations tested (25-fold excess of competitor, Figure 5A, lanes 7–10). Addition of an unrelated oligonucleotide revealed no competition at any concentration (Figure 5B, lanes 3–6). When the reaction was performed in the presence of 50 mM EDTA, the binding of the GST–WC2 fusion protein was abolished, indicating that divalent cations such as zinc are required for the binding of WC2 to *al-3* DNA (Figure 5C, lanes 1 and 2). After incubation of equimolar amounts of both WC2 and WC1 fusion proteins with the ^{32}P-labelled *al-3* promoter fragment, no additional gel-shifted bands were observed when compared with bandshifts with WC1 and WC2 incubated separately (data not shown).

Nucleotide sequence analysis of wc-2 mutants reveals that the zinc finger motif is required for WC2 function in vivo

In order to confirm the identity of the isolated *wc-2* gene and to obtain information about important functional domains in the WC2 protein, the *wc-2* coding regions of three different *wc-2* mutants alleles were sequenced and compared with the wild-type *wc-2* sequence (Table I). Several mutations were identified and confirmed by sequencing in both directions of at least two independent PCR reactions. The *wc-2* mutant ER33 obtained from the FGSC showed two independent mutations. One was a nucleotide exchange in intron 2 of the *wc-2* gene (Table I). RT–PCR experiments showed that this mutation did not result in a splicing defect and is, therefore, a neutral mutation. The second mutation in ER33 resulted in the

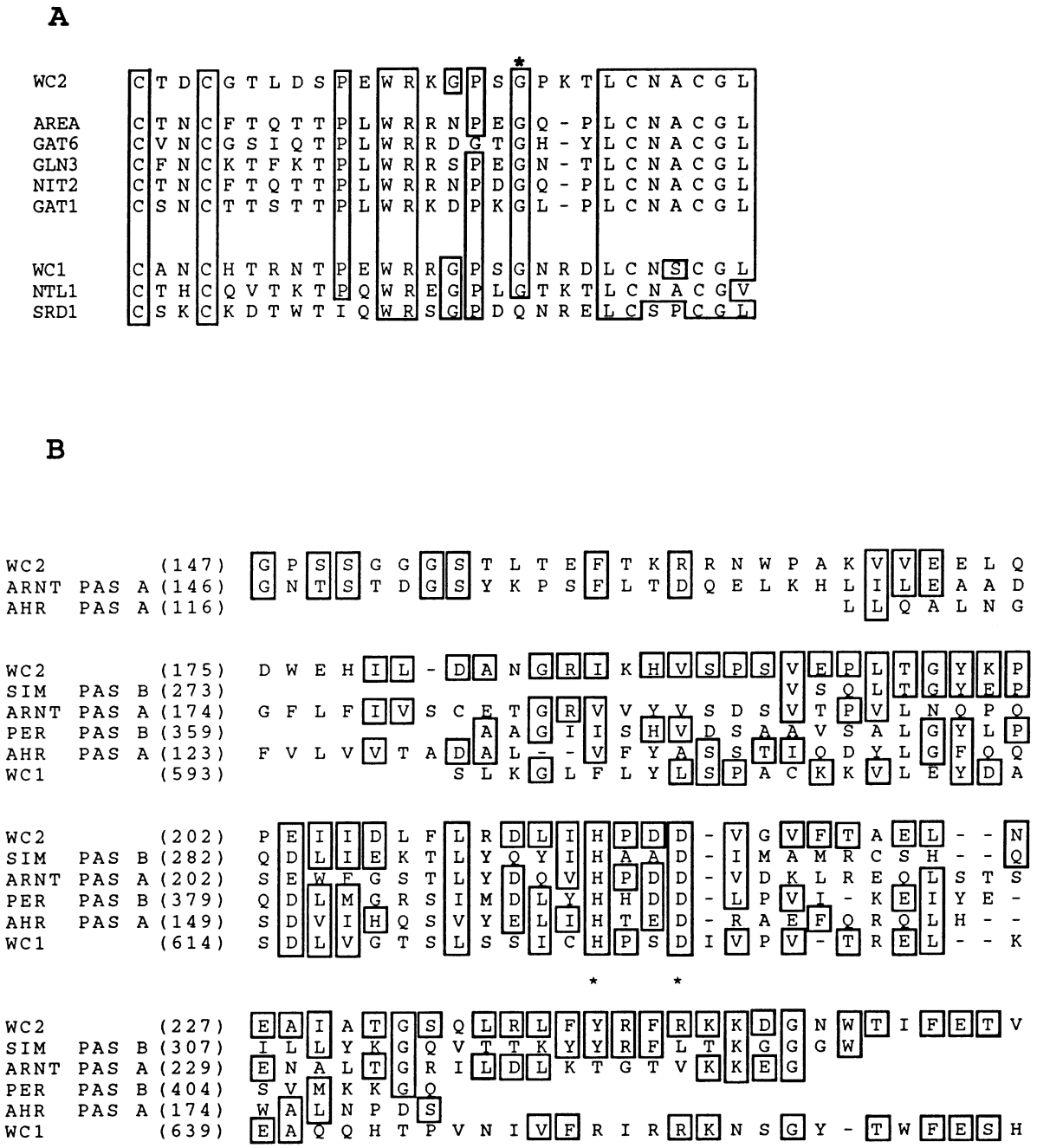

Fig. 4. Multiple alignment of different WC2 protein domains with other polypeptides from the SwissProt protein sequence database. (**A**) Comparison of the putative zinc finger domain of WC2 (amino acids 468–495) with the zinc finger motifs of AREA (*Aspergillus nidulans*, EMBL, X52491), GAT6 (*Rattus norvegicus*, EMBL L22760), GLN3 (*Saccharomyces cerevisiae*, EMBL M35267), NIT2 (*N.crassa*, EMBL M33956), GAT1 (*S.cerevisiae*, EMBL U27344), WC1 (*N.crassa*, EMBL X94300), NTL1 (*Nicotiana tabacum*, EMBL X73111) and SRD1 (*S.cerevisiae*, EMBL X063322). Major identities are boxed. An asterisk indicates the mutated amino acid of the *wc-2* allele ER33. (**B**) Multiple alignment of the putative WC2 PAS domain with the PAS A domain of ARNT and AHR, the PAS B domain of SIM and PER and the putative PAS domain of WC1. References are given in the text. Similar residues between the different proteins and WC2 are boxed. The only two conserved residues in all PAS A and PAS B domains are indicated by asterisks. A hyphen indicates a gap introduced to maximize alignment. The numbers of the first amino acid of each sequence are given in parentheses on the left.

conversion of a conserved glycine to a glutamic acid inside the putative zinc finger domain (Table I, Figure 4A), presumably disrupting this functional domain. Mutant 234w also revealed two mutations; one was a neutral mutation which led to an amino acid exchange close to the amino-terminus of WC2. The other mutation of 234w resulted in a truncated protein of 356 amino acids in which the DNA-binding domain of *wc-2* is absent (Table I). The DNA-tagged *wc-2* mutant T13 also showed a disruption of the zinc-finger-binding domain of WC2 by the integration of the plasmid DNA. It can be presumed, therefore, that the observed *wc-2* phenotype is, at least in some cases, due to the absence or non-function of the putative zinc finger DNA-binding domain.

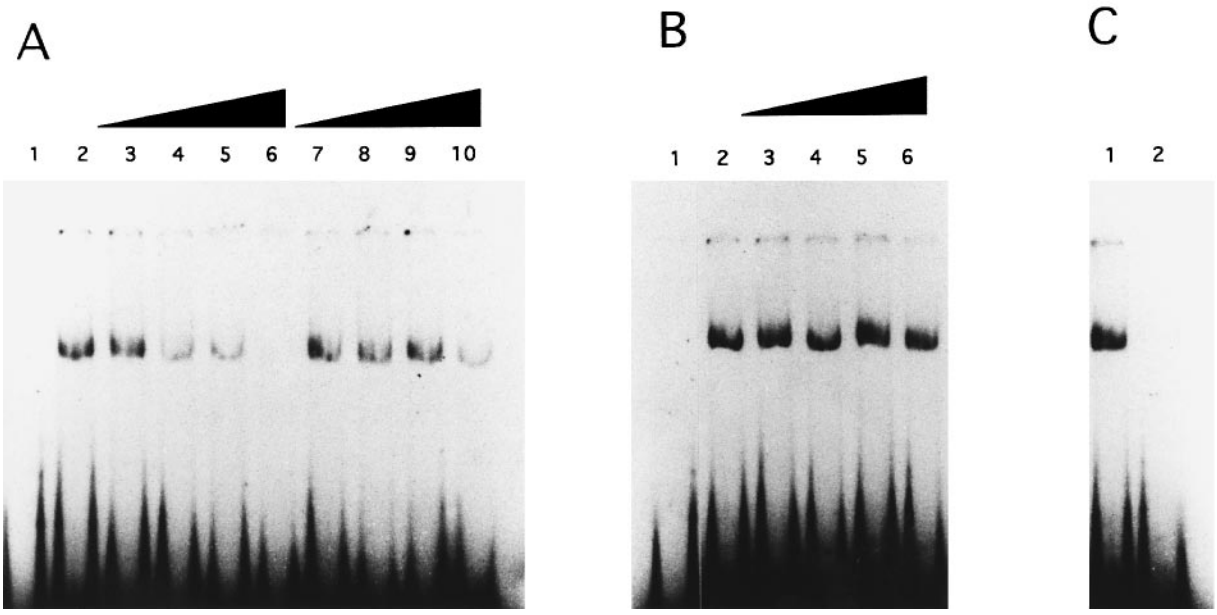

Fig. 5. Binding of an *al-3* promoter fragment containing two GATA elements by a GST–WC2 fusion protein. In an electrophoretic mobility shift experiment, 1 ng of ^{32}P-labelled promoter fragment (78 bp) was incubated with 5 μg of either GST (**A**, lane 1) or GST–WC2 protein extract (A, lane 2); lanes 3–6 are the same as lane 2, but with increasing amounts of an *al-3* 41mer oligonucleotide as an unlabelled competitor (0.5, 2.5, 5.0 and 25.0 ng); lanes 7–10 are the same as lane 2, but with an unlabelled *al-3* oligonucleotide with mutated GATA motifs. (**B**) Lanes 1 and 2 are the same as lanes 1 and 2 in (A), lanes 3–6 are the same as lane 2, but with an unlabelled, unrelated oligonucleotide as competitor (0.5, 2.5, 5.0 and 25.0 ng). (**C**) Lane 1 is the same as lane 1 in (A) and lane 2 the same as lane 1 but in the presence of 50 mM EDTA.

Table I. Mutations in the *wc-2* gene of different *wc* mutants

Neurospora wc-2 mutant strains	Mutation in the nucleotide sequence	Alteration of the *wc-2* amino acid sequence
Allele ER33	t4808 →a	none
	G4915→A	Gly485→Glu
Allele 234W	G3258→A	Gly14→Ser
	Del T^{4440}	change in rf and stop after Pro356
Allele T13	integration of pES200 at C4892 and deletion of 1619 nt downstream	Del of aa sequence after Pro477

The wc-2 mRNA steady-state levels are light regulated and this light regulation occurs also in a wc-1 and wc-2 mutant background

Using RNA from *Neurospora* wild-type and different *wc* mutants, the expression pattern of the *wc-2* gene was examined (Figure 6). A *wc-2* transcript of ~4 kb was detected in the wild-type which showed a small but significant increase in response to light (Figure 6). Mutant T13 revealed a somewhat smaller *wc-2* transcript, which was due to the integration of plasmid pES200 in the *wc-2* gene (Figure 6). All the other *wc* mutants still showed a transcript of the same size as the wild-type. Curiously, light induction of the *wc-2* gene was detected not only in all *wc-2* mutants examined but also in a *wc-1* mutant. When *wc-1*- and *al-1*-specific probes were used for hybridization, light induction of these genes was observed only for the wild-type but not in either a *wc-2* or a *wc-1* mutant background (Figure 6). However, only a 2-fold increase at the most in *wc-2* mRNA steady-state levels was detected after light induction. This is very low compared with the light induction normally observed for other *Neurospora* blue-light-regulated genes such as *wc-1*, *al-1*, *al-3* and *con-10* (Figures 1 and 6).

Discussion

Here, we report the isolation of the *wc-2* gene coding for an essential regulatory protein involved in blue-light signal transduction in the fungus *N.crassa*. The *wc-2* gene was isolated by an insertional mutagenesis approach, a method which has been applied previously to the isolation of other *Neurospora* genes (Kang and Metzenberg, 1993). A prerequisite for this approach, however, is a powerful screening system for the selection of the DNA-tagged mutants. Here, a screening system was applied which already had been shown to be appropriate for the isolation of *wc-1* and *wc-2* mutants after UV mutagenesis (Linden *et al.*, 1997). Confirmation that the gene tagged, cloned and sequenced corresponds to the *wc-2* gene is provided by several independent lines of evidence. Firstly, the DNA-tagged mutant T13 was shown to co-segregate in sexual crosses with the *wc-2* mutation of two previously isolated *wc-2* mutants (Degli-Innocenti and Russo, 1984). The T13 mutant was also impaired in the induction of several blue-light-regulated genes involved in carotenogenesis and conidiation (Figure 1). Secondly, the resistance to hygromycin present on the tagging plasmid strictly co-segregated with the *wc-2* mutant phenotype in backcrosses

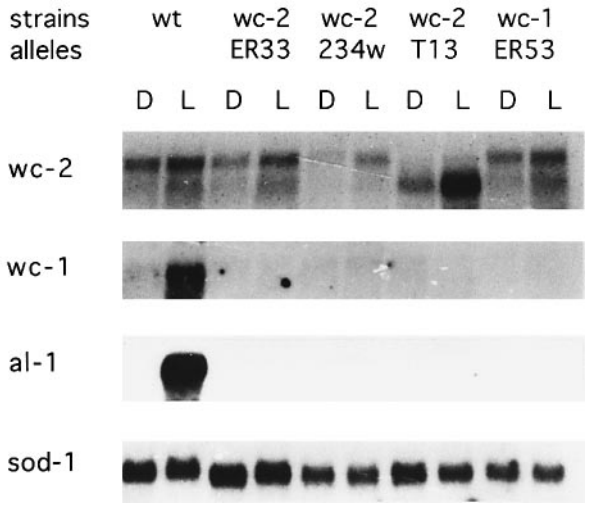

Fig. 6. Expression of the *wc-2* gene in *Neurospora* wild-type, *wc-1* and in different mutant strains of *wc-2*. The mycelia were harvested either after growth in the dark (D) or after a 20 min light induction (L). For hybridization, specific probes of *wc-2* , *wc-1* and *al-1* were used. For normalization, the filter was hybridized with *sod-1*.

of T13 to wild-type. Thirdly, complementation of the *wc* phenotype was achieved by transformation of several *wc-2* mutants using the genomic DNA fragment sequenced. Fourthly, co-suppression of the endogenous *wc-2* gene by 'quelling' and 'RIPing' phenomena was observed after transformation of *Neurospora* wild-type with the cloned *wc-2* gene. Finally, sequencing of three *wc-2* alleles including the tagged mutant was carried out and different mutations were identified resulting in changes in putative functional domains of the amino acid sequence of WC2 (Table I).

The deduced amino acid sequence of WC2 revealed several structural characteristics which indicate a putative role in transcriptional activation in response to blue light (Figures 3, 4 and 7). First of all, proline-rich and acidic regions have been found in the WC2 protein. These domains have been described for many other transcription factors and have been implicated in transcriptional activation (Hope *et al.*, 1988; Mermod *et al.*, 1989). Whether one of these proline-rich or acidic regions of WC2 actually plays a role in transcriptional activation is not known. Furthermore, a putative zinc finger domain was found close to the C-terminus of WC2 which showed similarity to the DNA-binding domain of GATA factors (Figure 4). This sequence was one of the two regions of the WC2 protein which is also shared by WC1, the first *Neurospora* blue-light regulator gene isolated (Ballario *et al.*, 1996). Both WC1 and WC2 proteins contain an 18-amino-acid loop in the zinc finger, in contrast to the other GATA factors which contain a 17-amino-acid finger loop, a characteristic found only in two other putative transcription factors, NTL1 and SRD1 (Daniel-Vedele and Carboche, 1993; Hesse *et al.*, 1994). In contrast to the vertebrate GATA factors which contain two zinc finger domains, the WC1 and WC2 proteins revealed only one putative zinc finger.

The presence of the WC2 zinc finger domain resembling that of WC1 and other transcription factors prompted us to test the hypothesis that WC2 accomplishes its function in blue-light regulation by binding to promoters of light-regulated genes as was observed previously for WC1. Indeed, it was shown in bandshift experiments that a WC2 fusion protein is capable of binding a fragment of the light-regulated *al-3* promoter which had been implicated in blue-light photoinduction (Carattoli *et al.*, 1994) and which contains two GATA motifs (Figure 5). Competitor studies with both related and unrelated, unlabelled oligonucleotides proved the specifity of the interaction. Competition with an oligonucleotide in which the GATA motifs had been mutated occurred only at higher concentrations. This effect, which points to a possible influence of the adjacent regions surrounding the GATA motifs on the WC2 binding, has also been observed for WC1, and was attributed to the unusual structural characteristics of these zinc finger domains in comparison with other GATA factors (Ballario *et al.*, 1996). Moreover, the molecular characterization of the lesion in three *wc-2* mutant alleles ER33, 234w and T13 showed mutation or disruption of the putative zinc-finger-binding domain, supporting the *in vivo* role of WC2 as a transcription factor (Table I). It can be concluded that the *wc* phenotype and the lack of light-induced transcriptonal activation of light-regulated genes observed for *wc-2* mutants is due, at least in some cases, to the inability of the WC2 protein to bind to light-regulated promoters. Therefore, a somewhat surprising finding was that the *wc-2* mRNA steady-state levels were light inducible not only in the wild-type but also in *wc-1* and *wc-2* mutant backgrounds (Figure 6). A different result was observed for *wc-1* regulation, where not only the *wc-1* but also the *wc-2* product were necessary for light induction (Figure 6, Ballario *et al.*, 1996). Normally, all light-regulated *Neurospora* genes described are not inducible in either a *wc-2* or *wc-1* mutant background. Only recently has another exception been reported. Loros (1995) found that photoinduction of the *Neurospora* clock gene *frequency* (*frq*) occurs also in a *wc-2* mutant background. Thus, a different light signalling pathway can be presumed for the light induction of *wc-2* and *frequency* which is independent of functional WC1 and/or WC2 proteins. It was shown that the *wc-2* transcript contains long 5′ and 3′ non-coding regions which suggest a possible post-transcriptional control (Figure 3). Therefore, a light-dependent post-transcriptional regulation of *wc-2* mRNA stability in a *wc* background could be a possible explanation for these results.

Apart from the described features, further interesting domains of WC2 were identified by protein sequence database searches (Figures 4 and 7). A WC2 domain was found which showed a high similarity with the PER-PAS domain. This putative PAS dimerization domain in WC2 which is also present in other regulatory proteins, e.g. in both subunits of the mammalian dioxin receptor (AHR and ARNT, Figure 7B), was shown to represent a protein dimerization motif in the latter proteins (Huang *et al.*, 1993). A prokaryotic PAS domain of the KinA sensor protein (*Bacillus subtilis)* was also described, which indicated a high degree of evolutionary conservation of this motif (Wang *et al.*,1995). However, for the first time, a putative PAS domain is described in WC2 which does not comprise the usual PAS A and PAS B repeats, but consists of only one PAS domain. The identity of this putative PAS domain is confirmed by the high homology and the conservation of specific amino acids present in

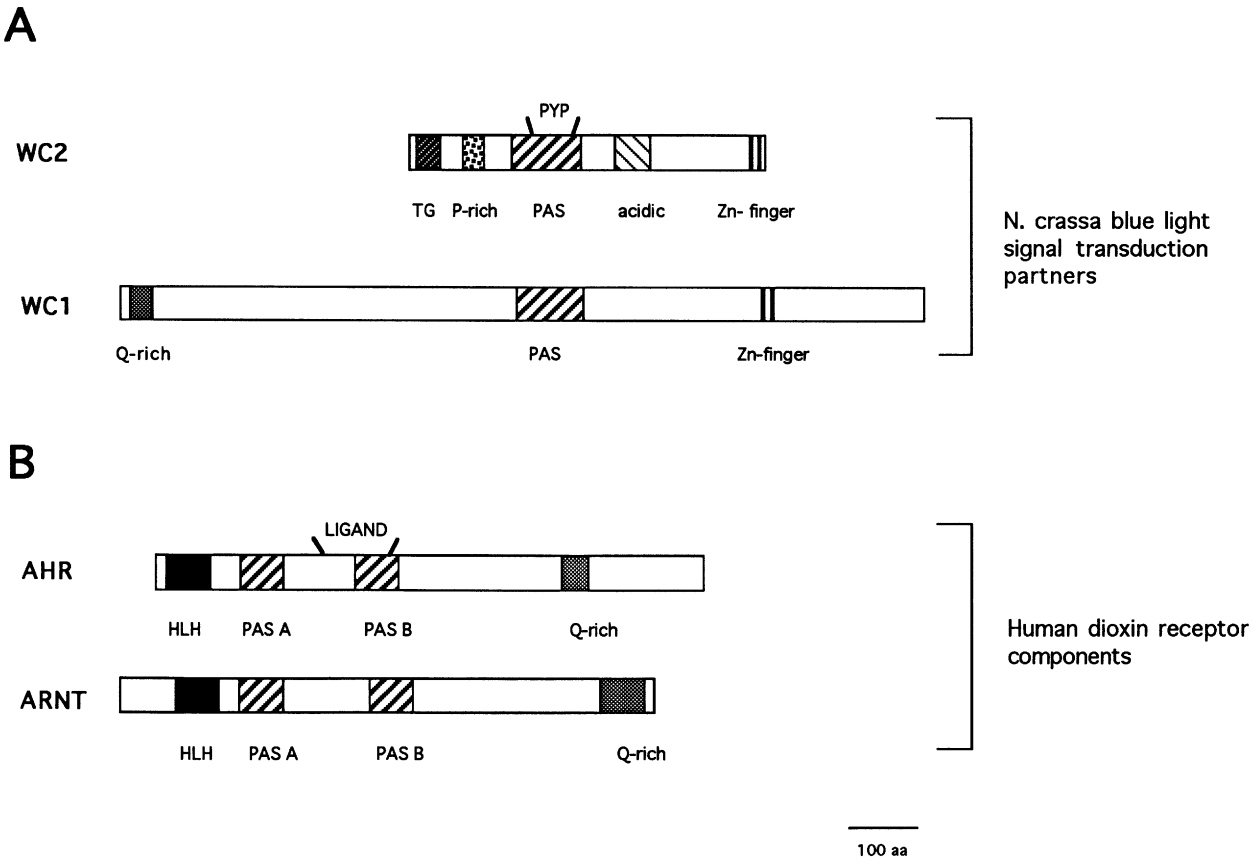

Fig. 7. Domain structure of WC1 and WC2 (**A**) compared with the Ah-receptor (AHR) and ARNT (**B**) (according to Burbach *et al.*, 1992). The position of putative PAS domains in WC1 and WC2 as well as PAS A and PAS B in the dioxin receptor components are indicated by hatched boxes. Other regions indicated include the Thr/Gly, Met/Gly and Ser/Gly repeat region (TG), the proline-rich (P-rich) and acidic domains, and the homology region with the photoactive yellow protein (PYP) in WC2, the putative zinc finger domain in WC1 and WC2, the glutamine-rich (Q-rich) regions and putative helix–loop–helix domains in the AH receptor and ARNT as well as the ligand-binding region of the AH receptor.

all other members of the PAS domain family (Figure 4). The question naturally arises as to which protein(s) interact with WC2 via the putative PAS dimerization domain. Although no definitive answer can be given at present, an intriguing finding was the identification of a putative PAS domain also in WC1 (Figure 4). Both WC1 and WC2 are putative transcription factors containing zinc finger domains involved in light-activated gene expression (Ballario *et al.*, 1996). Therefore, a PAS-mediated protein–protein interaction between WC1 and WC2 could allow or facilitate DNA binding to light-regulated promoters analogous to the basic helix–loop–helix PAS proteins AHR and ARNT (Reyes *et al.*, 1992). Here, we show that a WC2 fusion protein on its own is capable of binding to the *al-3* promoter *in vitro*. However, the putative heterodimerization of WC1 and WC2 could be necessary for binding and/or transcriptional activation *in vivo*.

The PAS domains identified in other regulatory proteins seem to reflect not only a dimerization domain of DNA-binding proteins but have also been shown to be involved in more sophisticated regulatory functions. In PER for example (which has no DNA-binding region), PAS has been shown to be the site of interaction with its partner TIM in the formation of the heterodimeric complex which seems to play a role in the circadian clock (Zeng *et al.*, 1996). In the AHR, heterodimerization with its partner ARNT, nuclear transport and subsequent transcriptional activation were shown to be dependent on ligand binding (Burbach *et al.*, 1992). Interestingly, the dioxin-binding site of AHR is part of the PAS dimerization domain (Figure 7B) and it was concluded, therefore, that PAS-mediated protein–protein interactions may be connected to ligand binding. Unknown ligands were proposed to participate in the regulation of the other PAS proteins (Huang *et al.*, 1993). Is it possible that the proposed binding of WC1 and WC2 involves specific ligands? An intriguing homology between the WC2 PAS domain and the photoactive yellow protein PYP was found. PYP is a member of the protein family of xanthopsins and is proposed to encode a blue-light photoreceptor involved in negative phototaxis (Baca *et al.*, 1994; Kort *et al.*, 1996). Although the chromophore binding site of PYP is not conserved in WC2, the region of homology covers 50% of the entire PYP polypeptide. Therefore, analogously to the AHR–ARNT model, it is tempting to speculate that WC1 and WC2 heterodimerize and activate transcription as a consequence of co-factor binding. This co-factor may participate either in blue-light perception or in signal transduction. Further research will be necessary in order to support this model.

To summarize, only two *wc* mutants have been isolated so far (*wc-1* and *wc-2*), despite extensive screening both in our laboratory and in others (Degli-Innocenti and Russo, 1984; Linden *et al.*, 1997). The corresponding proteins,

WC1 and WC2, represent the first two putative GATA transcription factors that have been characterized in any organism which seem to be dedicated to light-activated gene regulation rather than involved in general transcriptional regulation. Although WC1 and WC2 share homology in some proposed functional regions, no overall homology between them was identified. Ballario *et al.* (1996) proposed a two component signal transduction system with the participation of only WC1 and WC2. Here, we present a model in which the two putative transcription factors WC1 and WC2 form a complex via their putative PAS domains present in both proteins. We propose that this putative heterodimerization might be influenced by light and/or co-factor binding. Given the evolutionary conservation of the PAS domain, it is possible that such a domain is present in light signal transduction components in higher plants, although none has been identified as yet. It will be interesting to determine whether PAS-type proteins also participate in blue-light signal transduction in plants.

Materials and methods

Neurospora crassa strains, growth conditions and transformation

The *N.crassa* wild-type strains 74OR23-1*A* (FGSC 987) and 74OR23-1*a* (FGSC 988) and the mutants *wc-1*, allele ER53 (FGSC 4397) and *wc-2*, alleles ER33 (FGSC 4407) and 234w (FGSC 3817) were obtained from the Fungal Genetic Stock Center (Kansas City, KS). The construction of strain 13-1 (*al-3p::mtr*$^{+}$, *trp-2, mtr, cot-1, ylo-1, A*) was described previously (Carattoli *et al.*, 1995). The general growth medium was comprised of Vogel's minimal medium supplemented with 2% sucrose on agar plates; sexual crossings and maintenance of stock cultures were performed as described by Davis and deSerres (1970). For growth under selection, the plates were supplemented with hygromycin (300 μg/ml), anthranilic acid (50 μg/ml), the amino acid analogue FPA (50 μg/ml) and arginine (1 mg/ml) according to Linden *et al.* (1997) and were then incubated for 2 days at 29°C in an incubator (model G25, New Brunswick Scientific Co. Inc., Edison, NJ) equipped with seven fluorescent lamps (18 W, Philipps) and a programmable timer. A dark–light cycle of 3 h dark and 1 h light was applied. Transformation of *Neurospora* was carried out according to Vollmer and Yanofsky (1986).

Plasmids and libraries

Plasmid pES200 (Staben *et al.*, 1989) was used for the insertional mutagenesis. The subcloning of restriction fragments from genomic library clones was performed in plasmid pCB1004 (Caroll *et al.*, 1994). The *E.coli* expression vector pGEX-2T (Smith and Johnson, 1988) was used for expression of a GST–WC2 fusion protein. The genomic *wc-2* gene was isolated from an ordered cosmid library of *N.crassa* (Cabibbo *et al.*, 1991). The cDNA clones were isolated from a λ ZAP library which was purchased from the Fungal Genetic Stock Center (Orbach *et al.*, 1990).

Integrational mutagenesis and mutant selection

After transformation of *Neurospora* strain 13-1 with plasmid pES200, the transformation samples (~2×10^4 transformants/sample) were incubated in darkness in 250 ml flasks (20 flasks per transformation sample) containing 30 ml of solid Vogel's medium supplemented with hygromycin (300 μg/ml). After 1 week, the conidia were harvested and frozen in pools. About 10^5 conidia from each pool (corresponding to ~2×10^4 different transformants) were grown under the described selection conditions. Surviving colonies were inoculated in a second selection step under the same conditions and purified in several rounds of platings in order to obtain homokaryotic mutants which were then examined phenotypically.

Recombinant DNA procedures

Standard recombinant DNA techniques were carried out according to Sambrook *et al.* (1989). For routinely performed PCR, the Ampli Taq polymerase (Perkin Elmer) was used; PCR for DNA sequencing and cloning of fragments was done by use of the ULTma DNA polymerase (Perkin Elmer).

For the inverse PCR and cloning of the *Neurospora* DNA region flanking the integrated plasmid, the site of integration in the plasmid was determined. This was carried out by Southern analysis of T13 DNA digested with different restriction enzymes. For PCR, 5 μg of T13 genomic DNA was then digested with *Sal*I. This digestion produced fragments containing vector sequences together with adjacent *Neurospora* DNA sequences. Restriction fragments were ligated in a reaction volume of 1 ml in order to favour the formation of monomeric circles. The DNA was precipitated and the flanking region was amplified by inverse PCR. Two oligonucleotide primers complementary to the plasmid sequence close to the integration site were used. These primers were oriented such that extension proceeded outward from the integrated plasmid region. The amplified DNA fragment was digested with *Sal*I and *Cla*I, and the 0.6 kb fragment containing only *Neuropora* DNA was cloned.

For the synthesis of first strand cDNA in the RT–PCR experiments, the Supercript II reverse transcriptase (Gibco-BRL) was used. *Neurospora* DNA was prepared as described by Sherman *et al.* (1978).

Northern blot analysis

Neurospora RNA was isolated according to the miniprep RNA extraction procedure described by Sokolowsky *et al.* (1990). After 2 days growth in darkness, the mycelia were collected by filtration either directly or after a 20 min light induction (constant saturating light, 10 W/m^2) and frozen in liquid nitrogen. The mycelia were then powdered under liquid nitrogen and RNA was extracted. For Northern blot analysis, total RNA (10 μg) was denatured in formaldehyde, electrophoresed on a 1.5% agarose gel containing 6% formaldehyde, transferred to Hybond N membranes (Amersham) and hybridized in the presence of 50% formamide after addition of 1.5×10^6 c.p.m./ml of ^{32}P-labelled probes. The following *N.crassa* gene probes were used: the light-regulated carotenoid biosynthesis genes *al-1* (Schmidhauser *et al.*, 1990) and *al-3* (Carattoli *et al.*, 1991), the conidiation-specific gene *con-10* (Roberts *et al.*, 1988), the *wc-1* gene (Ballario *et al.*, 1996) and, for normalization, the non-photoinducible superoxide dismutase gene *sod-1* (Chary *et al.*, 1990).

DNA sequencing and analysis

The nucleotide sequence of *wc-2* was determined for both strands using TAQ FS DNA polymerase and fluorescent dideoxy terminators in a cycle sequencing method, and the resulting DNA fragments were electrophoresed and analysed using an automated Applied Biosystems 373A Stretch DNA sequencer. For sequencing of cDNAs and mutant *wc-2* genes, the Sequenase system, USB, was used. The nucleotide and derived amino acid sequences were analysed using the MacMolly Tetra program (Soft Gene GmBH). Protein comparisons and alignments were carried out by Blitz Database Searches (SwissProt protein sequence database, gap penalty 8). Minor changes for the multiple alignments were introduced subsequently.

Electrophoretic mobility shift assay (EMSA)

For the expression of a GST–WC2 fusion protein, a PCR fragment amplified from the *wc-2* cDNA clone (corresponding to amino acids 178–530, as indicated in Figure 3) was cloned in-frame in plasmid pGEX-2T. Protein extracts were prepared according to Sessa *et al.* (1993). Binding reactions were performed in a buffer containing 20 mM HEPES, pH 7.4, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT) and glycerol (25%). The bacterial extracts were mixed in 20 μl reactions including 1 μg of poly(dI–dC) (Boehringer), bovine serum albumin (0.1 mg/ml) and the radioactive labelled probe, and incubated for 15 min on ice. Electrophoresis was performed on a 5% polyacrylamide gel in 0.5× TBE. A 78 bp *Hin*dIII–*Cla*I fragment from the *al-3* promoter was used as labelled probe, and labelling was carried out by filling in the recessed ends using the Sequenase™ (USB). The specific radioactivity of the probe was ~5×10^4 c.p.m./ng DNA. For the competition experiments, the following unlabelled oligonucleotides were used: *al-3* oligo (wild-type), GCGGTATCGTCATAGCGTGCGGGTATCGAATATTGCC; *al-3* mutant oligo, GCGGTATGAGAGTCATAGCGTGCGGGAGGAGAATATTGCCC; control oligo, unrelated, CCGAGCACAAACTTCCGTTAGC.

Nucleotide sequence accession number

The DDBJ/EMBL/GenBank accession number for the *wc-2* sequence reported in this paper is Y09119.

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