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In situ detection of protein–DNA interactions in filamentous fungi by *in vivo* footprinting

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Abstract

The method described here allows the detection of protein–DNA interactions *in vivo* in filamentous fungi. We outline culture conditions and conditions of *in vivo* methylation that permit uniform modification of all cells in an apically growing, non-uniform organism, and subsequent visualization of protected areas by ligation-mediated PCR.

Filamentous fungi are efficient producers of enzymes, secondary metabolites and organic acids. As such they are important biotechnological organisms. Throughout the last decade, many of the structural and regulatory genes involved in the synthesis of these products have been cloned (1) and their upstream regulatory sequences characterized by *in vitro* gel-shift and footprinting techniques (2). However, these methods give no insight as to the occupancy of functionally important sites under different physiological conditions.

For filamentous fungi, an important technical addition would be the footprinting of the relevant promoter areas *in vivo*. Even if ligation-mediated PCR (LMPCR) *in vivo* footprinting (3) is routinely applied now for *in vivo* studies of cell lines and yeast, major modifications are expected to be necessary to use this technique in filamentous fungi. Fungal mycelium grows apically and therefore contains cells of different ages. As a consequence, the physiology of the culture changes and, in fact, it has been shown that protein secretion is limited to young, apical cells (4) whereas penicillin production, for example, requires cultures that have entered the stationary phase (5). Footprint results may be obscured by nuclei being in different transcriptional states in sections of mycelia at different stages of development. To circumvent this problem we used germinating spores ('germlings') with germ tubes containing <10 nuclei. We assumed that if these young hyphae contain equally active nuclei, a clear footprint would be visible.

To establish the method we chose a well documented metabolic system with sufficient molecular genetic background information, i.e. the nitrate ultilization cluster. Structural and regulatory genes have been cloned and characterized from *Aspergillus nidulans* (6), *Aspergillus niger* (7) and *Penicillium chrysogenum* (8). In *A.nidulans*, nitrate and nitrite induction is mediated by NirA, which binds as a dimer to an asymmetrical, non-repeated consensus sequence defined by a 5′-CTCCGHGG-3′ motif (9,10). Additional control over transcription of the cluster is exerted by *areA*. It encodes a wide domain activator (11,12) which belongs to the so called GATA factor family recognizing a consensus 5′-HGATAR-3′ sequence (13). The positions of NirA and AreA binding sites in the *A.nidulans*, *A.niger* and *P.chrysogenum* intergenic regions (IGR) are shown in Figure 1.

In a preliminary experiment we tested for the response of germlings to induction by nitrate and found no difference between these cultures and cultures containing mature mycelium, in respect to the time and level of induction of the nitrate reductase *(niaD)* gene (data not shown). *Aspergillus nidulans* was pregrown in appropriately supplemented liquid minimal medium (14) containing 5 mM urea as the sole nitrogen source. *Aspergillus niger* IMI 60286 was pregrown in liquid complete medium (CMA) (14) and 5 mM urea. *Penicillium chrysogenum* Q176 was pregrown in modified Vogel's medium (MVM) containing 2% glucose and 5 mM urea as the sole nitrogen source. Control DNA for *in vitro* methylation was obtained from germlings harvested immediately after pregrowth (12 h). For *in vivo* methylation, germlings were collected by filtration and resuspended in fresh medium containing 10 mM (final concentration) sodium nitrate as inducer. Aliquots (18 ml) of induced mycelial suspensions were shaken for 30 min at 37°C and finally used for *in vivo* methylation as follows: 2 ml of methylation buffer (300 mM MES, pH 6.2) containing 40 µl DMS was added to 18 ml mycelial suspension and the incubation was continued for 2 min at 37°C. The methylation was stopped by adding 50 ml of ice cold STOP buffer [10 mM Tris pH 8, 1 mM EDTA, 300 mM LiCl, 2% (v/v) β-mercaptoethanol]. Mycelia were then harvested and ground under liquid nitrogen to a fine powder, resuspended in 0.8 ml 0.1 M Tris–HCl, pH 8.0, containing 1.2 M NaCl, 5 mM EDTA and 2% β-mercaptoethanol and incubated for 20 min at 65°C. The suspensions were extracted once with 1 vol phenol–chloroform–isoamyl alcohol 25:24:1 (by volume) and once with 1 vol chloroform–isoamyl alcohol 24:1 (by volume). Nucleic acids were precipitated and subsequently dissolved in 200 µl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and 100 µg/ml RNAse. Following the addition of 20 µl piperidine the samples were

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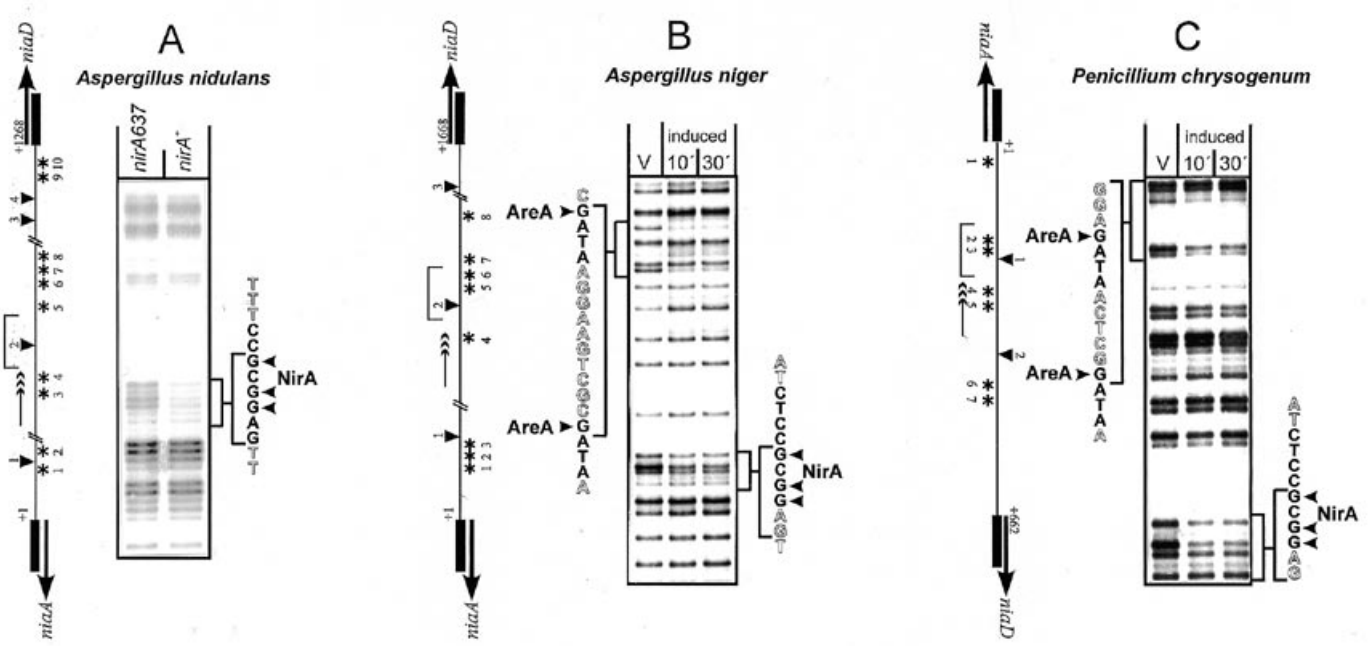

Figure 1. Schematic representation of the bidirectional promoter regions (IGR) of *A.nidulans*, *A.niger* and *P.chrysogenum* are shown on the left side of each autoradiograph. In (C) the position of the genes is inverted because primers were set in the opposite direction as compared to (A) and (B). Triangles indicate proven (for *A.nidulans*, 1–4) (9,10) or putative (for *A.niger*, 1–3, and *P.chrysogenum* 1–2) (7,8) NirA binding sites. Asterisks indicate GATA sites. These are proven AreA binding sites for *A.nidulans* (1–10) (12,13) and putative AreA and NRE binding sites for *A.niger* (1–8) and *P.chrysogenum* (1–7) (7,8), respectively. Arrows indicate the positions of the specific primers used in the LMPCR reaction. Brackets indicate, for each IGR, the sequence studied by *in vivo* footprinting. The start codon for *niaA* is indicated by +1, the number at the opposite side of the IGR indicates the number of base pairs from the *niaA* start codon to the start codon of *niaD*, and this is the length of each IGR. Autoradiographs shown are derived from LMPCR products of *A.nidulans* NirA site 2 (A), *A.niger* NirA binding site 2 and AreA binding sites 5 and 6 (B) and *P.chrysogenum* NirA binding site 1 and NRE binding sites 2 and 3 (C). Protected guanines are indicated by arrowheads. (**A**) LMPCR products from induced cultures of *A.nidulans* strains carrying either an intact *nirA* gene (lane *nirA*$^+$) or a mutated *nirA* gene (lane *nirA 637)*. The relevant NirA binding sequence (5′-CTCCGCGG-3′) is shown in bold as the complementary sequence present on the *niaD*-coding strand. (**B**) LMPCR products from cultures of *A.niger* after 10 min (lane 10′) and 30 min (lane 30′) of induction by nitrate. Lane V: LMPCR products from *in vitro* methylated, naked DNA isolated from germinating spores in the absence of inducer. The relevant NirA binding sequence present on the *niaA*-coding strand is shown in bold on the right side of the figure and relevant AreA binding sites (5′-CGATAA-3′) present on the *niaA*-coding strand are shown on the left side. (**C**) LMPCR products from cultures of *P.chrysogenum* treated as described in (B). The putative binding site 1 of the *P.chrysogenum* NirA homologue present on the *niaD*-coding strand is shown in bold letters on the right side of the figure and relevant NRE binding sites (5′-AGATAA-3′) present on the *niaD*-coding strand on the left side. Lane V: *in vitro* methylated DNA of *P.chrysogenum*.

incubated for 30 min at 90°C. After three sequential ethanol precipitations the DNA was resuspended in TE to give a final concentration of 0.4 mg/ml. *In vitro* methylation of genomic DNA was performed as described (3). Methylated and piperidine treated DNA samples were analyzed by LMPCR (3), using Vent polymerase (NEB). [γ-^{32}P]ATP-labelled amplification products were separated on a sequencing gel which was dried and autoradiographed. The following oligonucleotides were used to investigate the *A.nidulans niaA–niaD* IGR: NIR2/1: 5′-TGGCT-AGAGCCGCTTGACGATAATG-3′, NIR2/2: 5′-GAGCCGGCG-ATAAGCATGATGTTGGC-3′ and NIR2/3: 5′-CCGGCGATAA-GCATGATGTTGGCGCTGTC-3′. For investigation of the *A.niger niaA–niaD* IGR, the following oligonucleotides were used: ANIT1: 5′-GGGAAAGACGGATAAGAACTG-3′, ANIT2: 5′-GACGG-ATAAGAACTGGGAATGCGATG-3′ and ANIT3: 5′-CGGAT-AAGAACTGGGAATGCGATGGGTTC-3′. Oligonucleotides used for the *P.chrysogenum niaA–niaD* IGR were the following: PNIT1: 5′-GACCCATGGATGTACCCAAGTATC-3′, PNIT2: 5′-CCTAACCCAACCCAATCGGACCAATC-3′ and PNIT3: 5′-CCCAACCCAATCGGACCAATCACAGCC-3′.

Using the LMPCR method for genomic footprinting, we investigated the protection pattern in the IGR of the nitrate gene clusters of *A.nidulans*, *A.niger* and *P.chrysogenum*. For the *A.nidulans niaA–niaD* IGR, Figure 1A shows *in vivo* methylated samples of the *nirA637* control strain carrying an in-frame deletion of the DNA-binding domain (M.Muro, J.Strauss, R.Gonzalez, F.Narendja and C.Scazzocchio, submitted) and the *nirA*$^+$ strain under induced conditions. The mutant strain was used as an internal control to check the specificity of the *in vivo* protected DNA sequence. A specific protection is observed only in the *nirA*$^+$ strain and the protected sequence matches well with the *in vitro* determined 5′-CTCCGCGG-3′ binding sequence of site 2 (10).

Figure 1B and C shows protection of guanines in the IGR of *A.niger* and *P.chrysogenum*, respectively. This protection is visible within sequences matching the putative NirA target in *A.niger* (Fig. 1B) and *P.chrysogenum* (Fig. 1C). Furthermore, either two (*A.niger*) or three (*P.chrysogenum*) additional guanines are protected within a 5′-HGATAR-3′ sequence motif. This protection is therefore most likely due to the binding of AreA/NRE (*NRE* = *areA* homologue in *P.chrysogenum*) elements.

The aim of this study was to set up an experimental system for filamentous fungi that allows the identification of sequence specific interaction of DNA binding proteins *in vivo*. We have shown that dimethylsulfate is a suitable footprinting agent which equally penetrates young hyphal cells in submerged cultures. Positive results (i.e. protection) were validated by the use of a loss-of-function mutation in the relevant transcription factor gene.

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REFERENCES

1 Archer,D.B. and Peberdy,J.F. (1997) *Crit. Rev. Biotechnol.*, **17**, 273–306.
2 Jost,J.P. and Saluz,H.P. (1991) *BioMethods*, Vol. 5. Birkhäuser Verlag, Basel.
3 Mueller,P.R. and Wold,B. (1989) *Science*, **246**, 780–786.
4 Wösten,H.A., Moukha,S.M., Sietsma,J.H. and Wessels,J.G. (1991) *J. Gen. Microbiol.*, **137**, 2017–2023.
5 Pérez-Esteban,B., Orejas,M., Gómez-Pardo,E. and Peñalva,M.A. (1993) *Mol. Microbiol.*, **9**, 881–895.
6 Burger,G., Strauss,J., Scazzocchio,C. and Lang,F.B. (1991) *Mol. Cell. Biol.*, **11**, 5746–5755.
7 Unkles,S.E., Campbell,E.I., Punt,P.J., Hawker,K.L., Contreras,R., Hawkins,A.R., Van den Hondel,C.A.M.J.J. and Kinghorn,J.R. (1992) *Gene*, **111**, 149–155.
8 Haas,H. and Marzluf,G.A. (1995) *Curr. Genet.*, **28**, 177–183.
9 Punt,P.J., Strauss,J., Smit, R, Kinghorn,J.R., Van den Hondel,C.A.M.J.J. and Scazzocchio,C. (1995) *Mol. Cell. Biol.*, **15**, 5688–5699.
10 Strauss,J., Muro,M. and Scazzocchio,C. (1998) *Mol. Cell. Biol.*, **18**, 1339–1348.
11 Caddick,M.X, Arst,H.N., Taylor,L.H., Johnson,R.I. and Brownlee,A.G. (1986) *EMBO J.*, **5**, 1087–1090.
12 Kudla,B., Caddick,M.X., Langdon,T., Martinez-Rossi,N.M., Bennett,C.F., Sibley,S., Davies,R.W. and Arst,H.N. (1990) *EMBO J.*, **9**, 1355–1364.
13 Ravagnani,A., Gorfinkiel,L., Langdon,T., Diallinas,G., Adjadj,E., Demais,S., Gorton,D., Arst,H.N. and Scazzocchio,C. (1997) *EMBO J.*, **16**, 3974–3986.
14 Pontecorvo,G., Roper,J.A., Hemmons,L.M., MacDonald,K.D. and Bufton,A.W.J. (1953) *Adv. Genet.*, **5**, 141–238.