Review Article

Regulation of secreted protein production by filamentous fungi: recent developments and perspectives

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Introduction

Filamentous fungi, typically, are saprophytic organisms which, unlike yeasts, secrete a wide array of enzymes involved in the breakdown and recycling of complex polymers from both plant and animal tissues. This makes them attractive hosts for the production of secreted heterologous proteins (Jeenes *et al.*, 1991; van den Hondel *et al.*, 1991). In only a few examples, however, have the secreted yields of heterologous protein reached the gram per litre levels of many homologous fungal enzymes. In many cases, the problem does not appear to be at the level of transcription but, rather, occurs within the secretory pathway. Although the secretory process has barely been explored in filamentous fungi, we have attempted to identify areas upon which attention should be focused based on current knowledge gained from other systems. We also discuss recent developments in the dissection of transcriptional control in these organisms with particular reference to the interaction of regulatory proteins with fungal promoter regions and to the need for targeting expression cassettes to specific locations in the fungal genome. Understanding the detailed mechanisms of transcriptional control will help in designing modified promoter elements or regulatory factors optimized for a given set of growth conditions. Coupled with the proposed study of the secretory pathway, this should improve the yields of secreted heterologous proteins produced by filamentous fungi.

Transcriptional control

DNA regions controlling expression of a number of fungal genes have been investigated in increasing detail

(Gurr *et al.*, 1987; Unkles, 1992). Various elements constituting a 'core promoter' have been identified (CAAG, CT, TATAAA and CAAT motifs) which are thought to be necessary for basal levels of gene expression in many, but not all, cases. In addition, sequences responsible for gene-specific control of expression have been found where regulatory proteins are known to bind to the DNA. These core promoter and regulatory motifs were initially characterized by sequence homologies using computer analysis and an increasing number of these have now been functionally investigated. Recent studies with *in vivo* titration of regulatory proteins and *in vitro* DNA-protein binding have furthered our understanding of transcriptional control in these organisms.

As with most systems studied, transcription in filamentous fungi is regulated on at least two different levels – one type of control being pathway-specific whereas the other is more global, e.g. carbon or nitrogen regulation. The interaction between these different levels of control is a primary factor in determining the final yield of a particular target protein and understanding the mechanism(s) of this interaction is becoming increasingly important. Accumulating evidence suggests that the binding sites for some regulatory proteins may overlap or be adjacent to each other, indicating that competition for DNA-binding may operate *in vivo*. To date, the majority of transcription factors from filamentous fungi have been identified as activator proteins but a growing number of repressors have also been found. Table 1 lists most of the regulatory proteins identified in filamentous fungi along with their putative target sites where available. A number of other control sequences implicated in transcriptional regulation but for which proteins have yet to be positively identified are listed in Table 2. Some aspects of pathway-specific and wide domain control are now discussed in greater detail, concentrating

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Table 1. Transcriptional regulatory proteins from filamentous fungi

Strain	Regulatory protein	Activator (+) or repressor (-)	Gene(s) under control§	Putative target sequence	References
<i>A. nidulans</i>	ALCR*†	+	<i>alcR, alcA, aldA</i>	CCGCA	Kulmburg <i>et al.</i> (1992a, b)*
	AMDA	+	<i>aciA, amdS</i>	TGAGGGGGANGNG	Saleeba <i>et al.</i> (1992)
	AMDR*	+	<i>amdS, gatA, lamA/B</i>	TCGGCGAAGCCAGCCAATCA	van Heeswijk & Hynes (1991);* Littlejohn & Hynes (1992)
	AnCF*	?	<i>amdS</i>	AGCCAAT	van Heeswijk & Hynes (1991)*
	AnCP1/2*	(+)?	Taa-G2 (<i>amyB</i>) NM, e.g. <i>amdS, niiA/niaD</i>	CCATCCAATTAGAAG	Nagata <i>et al.</i> (1993)*
	AnNP1*	(-)?		Upstream from CCAAT	
	AREA	+		GATA(TATC)	
	BRLA	+	CON, e.g. <i>rodA</i>	AAAGGGG _G	Sophianopoulou <i>et al.</i> (1993); Stankovich <i>et al.</i> (1993)
	CREA*†	-	CC, e.g. <i>alcA, alcR, prnB</i>	GGGGG _{CT}	Chang & Timberlake (1992)
	FACB	+	AC; GLY, e.g. <i>amdS, facA, acuD, acuE</i>	GAGACCCC	Kulmburg <i>et al.</i> (1993);* Sophianopoulou <i>et al.</i> (1993)
	NIRA	+	<i>niiA/niaD</i>	GAGACCCC	Katz & Hynes (1989)
	PACC	+	PH, e.g. <i>pacA</i>	GCAGCTTTCCTTGGC	Burger <i>et al.</i> (1991)
	PALCA	+	PH, e.g. <i>palD</i> ; PEN		Caddick <i>et al.</i> (1986a); Shah <i>et al.</i> (1991)
	PRNA	+	PHO, e.g. <i>pacA, palD</i>		Caddick <i>et al.</i> (1986b)
	QUTA	+	PRO, e.g. <i>prnB</i>		Sharma & Arst (1985)
	QUTH	+	QUIN, e.g. <i>quiB, D, E, G</i> May inactivate QUTA activity directly	GCCAGANCGNTCTNCC	Beri <i>et al.</i> (1987)
	QUTR	-			
<i>A. oryzae</i>	AMDR	+	<i>amdS</i>		Lamb <i>et al.</i> (1992)
<i>N. crassa</i>	CPC1	+	AA, e.g. <i>arg-2, trp-1, his-3</i>	TGACTC	Wang <i>et al.</i> (1992)
	CYS3*	+	SC, e.g. <i>ars-1, cys-3, cys-14</i>	TGACTC	Paluh & Yanofsky (1991)
	NIT2*‡	+	NM, e.g. <i>nit-3</i>	GAGAA	Kanaan & Marzluf (1993)*
	NIT4	+	<i>nit-3, nit-6</i>	TTCGCT	
	NMR	-	NM, e.g. <i>nit-3</i>	TATCTA(TAGATA)	Jarai <i>et al.</i> (1992)*
	NUC1	+	PHO, e.g. <i>pho-2, -3, -4</i>		Yuan & Marzluf (1992)
	NUC2	-	<i>pgov/preg</i>		Young & Marzluf (1991)
	PGOV	(-)	May inactivate NUC1 activity directly		Kang & Metzenberg (1990)
	PREG	(-)			
	SCON2	-	SC, e.g. <i>ars-1, cys-3</i>		Paietta (1990)
	QA1F	+	QUIN, e.g. <i>qa-2, -3</i>	GG _G TAA _{ACAC} TTA _{GTGT} CCC	Geever <i>et al.</i> (1989)
	QA1S	-	May inactivate QA1F activity directly		Anton <i>et al.</i> (1987)

* Studies have included *in vitro* DNA-protein binding assays.

† DNA-binding studies used a glutathione *S*-transferase fusion protein containing the DNA-binding domain of the regulatory protein.

‡ DNA-binding studies used a β -galactosidase fusion protein containing the DNA-binding domain of NIT2.

§ Abbreviations represent genes involved in: AA, amino acid biosynthesis; AC, acetate utilization; CC, carbon catabolite repression; CON, conidiation; GLY, glyoxylate cycle; NM, nitrogen metabolite repression; PEN, penicillin biosynthesis; PH, pH regulation; PHO, phosphate regulation; PRO, proline metabolism; QUIN, quinate utilization; SC, sulphur catabolism.

on systems which have already been used in the production of heterologous proteins.

Pathway-specific regulation

Several pathway-specific activator proteins have now been identified from filamentous fungi (Table 1) but only a few have been characterized in detail. Like other

regulatory proteins they consist of distinct domains that are required for DNA-binding, transcription activation and inducer-/repressor-binding. Some also contain signals involved in nuclear targeting or localization. The most-studied fungal activators are the ALCR and AMDR proteins of *Aspergillus nidulans*, controlling the expression of the ethanol regulon (*alcR/alcA/aldA*) and ω -amino acid inducible genes such as *amdS*, respectively,

Table 2. Control elements in fungal promoters for which regulatory proteins have yet to be identified

Strain	Gene under control	Control element	Putative function	References
<i>A. nidulans</i>	<i>gpdA</i>	TCCAAATATCGTGCCTCTCTGCTT- -TGCCCGGTGTATGAAACCGGAAAGG	<i>gpd</i> box: enhancer element	Punt <i>et al.</i> (1992)
	<i>pgk</i>	AAGCAAAT	Glycolytic box: carbon source regulation	Streatfield <i>et al.</i> (1992)
<i>A. niger</i>	<i>aldA</i>	TGG (repeated eight times)	Enhancer element	O'Connell & Kelly (1992)
	<i>glaA</i>	GGGGACGGCGAAT CCCCGGG	High expression and starch induction	Fowler <i>et al.</i> (1990); Hata <i>et al.</i> (1992)
	<i>pacA</i>	CTCTCC ^A CTGCC	Phosphate regulation	MacRae <i>et al.</i> (1988)
	<i>niiA/niaD</i>	TA ₆ CT ₆	Nitrate regulation (AREA site?)	Unkles <i>et al.</i> (1992)
<i>A. oryzae</i>	<i>amyB</i>	CCAA ^A T* CACGGG	High expression and starch induction	Tsuchiya <i>et al.</i> (1992); Nagata <i>et al.</i> (1993)*
	<i>glaA</i>	GGGGACGGCGAAT CACGGG	High expression and starch induction	Hata <i>et al.</i> (1992)
<i>N. crassa</i>	<i>acu-3</i>	CREA- and FACB-like sites	Carbon catabolite repression and acetate induction	I. Connerton (pers. comm.)
<i>Penicillium chrysogenum</i>	<i>phoA</i>	CTGCACAA ^A G TTCCAAGGTT	Phosphate regulation	Haas <i>et al.</i> (1992)
<i>Phanerochaete chrysosporium</i>	<i>lip</i>	TGACGTC GGGCTCCC GCACGC	CRE† site AP-2 site XRE site	Ritch & Gold (1992)
<i>T. reesei</i>	<i>cbh1</i>	CREA consensus sequences	Carbon catabolite repression	Ilmén <i>et al.</i> (1992)
	<i>cbh2</i> ‡	ATTGG CAAGTAGAGCCTATTTCTCGCCGGAAAGTC	CCAAT-enhancer binding site CRE† site	Stangl <i>et al.</i> (1993)‡
	<i>pgk</i>	CTCACGTCAC CTTGCTATCGTTGCAGCTT ACGGAATGAACCTGAAGGTTACAT	CRE† site UAS HSE (but non-functional?)	Vanhanen <i>et al.</i> (1991)
<i>T. viride</i>	<i>pgk</i>	TGACGTCA CCCCAGGC ACGGACCCACCT	CRE† sites AP-2 site CREA site (but downstream of ATG)	Goldman <i>et al.</i> (1992)

* Nuclear proteins from *A. nidulans* bind to this site.

† Homologous to mammalian cAMP responsive element, CRE (not to be confused with the CREA-binding site) which operates in conjunction with the cAMP activation protein 2 element, AP-2.

‡ Involved *in vitro* DNA-binding studies with mycelial extracts.

and the QA-1F/QUTA proteins of *Neurospora crassa*/*A. nidulans*, involved in regulating the quinate utilization pathway. DNA-binding in these proteins is mediated by an N-terminal domain comprising a Zn(II)₂Cys₆ zinc-cluster motif characteristic of many eukaryotic transcription factors (Geever *et al.*, 1989; Kulmburg *et al.*, 1991; van Heeswijck & Hynes, 1991). ALCR is slightly unusual in that the DNA-binding domain has a helix-turn-helix structure where the turn is replaced with the binuclear zinc-cluster (Kulmburg *et al.*, 1991). Specific high and low affinity binding sites for this protein have now been identified in the promoter regions of *alcA* (Kulmburg *et al.*, 1992a) and *alcR* (Kulmburg *et al.*, 1992b). Both types of site contain a conserved core sequence (see Table 1) – the high affinity site characterized as a palindromic sequence with dyad symmetry and the low affinity site as a direct repeat. It has been

suggested that *in vivo*, ALCR may dimerize through a putative leucine zipper region C-terminal to the zinc-cluster and make contact with both the high and low affinity sites (Kulmburg *et al.*, 1992a, b) but this has yet to be confirmed experimentally.

Sequence comparisons of AMDR-regulated genes and *in vitro* DNA-protein binding studies have identified a promoter site involved in AMDR binding (van Heeswijck & Hynes, 1991; see Table 1). Interestingly, this region also contains a core CCAAT element to which another, unidentified factor, AnCF, binds *in vitro* (van Heeswijck & Hynes, 1991). Although these two sites possibly overlap with each other, the CCAAT sequence is not essential for AMDR binding. Rather, CCAAT appears to be responsible for high-level basal (or non-induced) expression of the *amdS* gene (Littlejohn & Hynes, 1991). However, a regulatory role, for example in

carbon source control has not been ruled out, perhaps via an interaction between AMDR and AnCF or other transcription factors (van Heeswijk & Hynes, 1991). CCAAT elements, along with several associated binding factors, have been found in many eukaryotic promoters but this is the first example of a proven functional role in filamentous fungi. More recently, other *A. nidulans* nuclear proteins have been identified which bind *in vitro* to a CCAAT element in the *amyB* promoter of *Aspergillus oryzae* (Nagata *et al.*, 1993; see Table 1). In this case, competitive binding of a putative repressor protein isolated from non-induced cultures was described at a site adjacent to the CCAAT sequence. The equivalent proteins from *A. oryzae* have yet to be identified, however. Deletions in the C-terminal half of AMDR have confirmed that some of the amphipathic acidic regions located here are essential for transcription activation (Parsons *et al.*, 1992). In this study, it was proposed that activation was due to conformational changes in promoter-bound AMDR and that the binding of inducer(s) to AMDR modulated this activity by unmasking activation domains. Functionally active hybrid regulatory proteins were created in this study by fusing the DNA-binding domain from one transcription factor (FACB) to the activation domains of another (AMDR). Novel transcription factors created in this way may prove useful in tailoring strains for heterologous protein production.

In other promoters currently used for producing heterologous proteins in filamentous fungi, several regulatory sites for pathway-specific transcription factor binding have been identified (see Table 2). To date, only in the case of the *Trichoderma reesei* *cbh2* promoter have homologous proteins been described that bind to upstream control regions (Stangl *et al.*, 1993). Surprisingly, no regulatory proteins have yet been described for the *glaA* promoters of *Aspergillus niger* and *A. oryzae* even though both share some DNA sequence homologies with the *A. oryzae* *amyB* promoter and all are regulated by similar carbon sources (Hata *et al.*, 1992). However, recent evidence of *in vivo* titration from multicopy *glaA* strains of *A. niger* has indicated that such proteins do exist (Verdoes *et al.*, 1993).

Wide domain controls

The expression of many genes is also regulated by more global or wide domain controls that are mediated by a relatively small number of regulatory proteins. Repression of particular pathways can be controlled by the levels of readily utilizable carbon, nitrogen, phosphorus or sulphur sources and expression is only switched on once one or more of these nutrients becomes limiting. In *A. nidulans*, mutational analysis has identified three

genes (*creA*, *creB* and *creC*) involved in carbon catabolite repression. The negatively acting CREA protein has been shown to contain two zinc finger motifs of the Cys₂His₂ class and an alanine-rich region characteristic of many DNA-binding proteins (Dowzer & Kelly, 1991). The zinc finger domain shows high similarity to the DNA-binding region of the yeast MIG1 repressor, also involved in carbon catabolite repression, and putative binding sites for CREA have been described in several promoters from *A. nidulans* (Sophianopoulou *et al.*, 1993; see Table 1), *N. crassa* (I. Connerton, personal communication), *T. reesei* (Ilmén *et al.*, 1992) and *Trichoderma viride* (Goldman *et al.*, 1992). In the case of the *cbh1* gene from *T. reesei*, deletion or mutation of upstream CREA-like binding sites completely abolished glucose repression of *cbh1* specifically without affecting the regulation of other catabolite-repressed genes (Ilmén *et al.*, 1992). Recently, *in vitro* binding studies with the CREA DNA-binding domain have confirmed the existence of several binding sites in the promoters of both the *alcR* and *alcA* genes (Kulmburg *et al.*, 1993; see Table 1). Interestingly, one of these overlaps the site for ALCR-binding in the *alcR* promoter and another is immediately adjacent to a similar ALCR-binding site in the *alcA* promoter, suggesting that competition between the ALCR activator and CREA repressor may be important in regulating gene expression in the *alc* system.

Nitrogen metabolite repression is mediated by the transcriptional activators AREA in *A. nidulans* and NIT2 in *N. crassa*. Both proteins share a common structure and contain a DNA-binding domain with a single Cys₂His₂ zinc finger which binds to specific upstream sequences (Unkles *et al.*, 1992). AREA function has recently been investigated by truncating the C-terminal region which lies outside the DNA-binding domain (Stankovich *et al.*, 1993). In this study, one particularly large deletion affected the activation of genes under AREA control in markedly different ways, indicating that this region can have different roles depending on the gene under control. A suggested mechanism to explain this unusual behaviour is that AREA interacts at its C-terminus with other transcription factors such as NIRA to effect regulation of gene expression (Stankovich *et al.*, 1993). The presence of overlapping or adjacent sites for AREA, CREA and pathway-specific regulatory proteins, for example, in the *amdS* and *prnB* promoters of *A. nidulans* (see Punt *et al.*, 1992; Sophianopoulou *et al.*, 1993), may well be a feature of the complex transcriptional control of these genes, and will have to be taken into account especially when tailoring promoter elements for heterologous gene expression.

In *N. crassa*, the positively acting NUC1 protein involved in controlling phosphate acquisition possesses a

DNA-binding domain which comprises an amphipathic helix-loop-helix motif found in several eukaryotic transcription factors (Kang & Metzenberg, 1990). NUC1 activity appears to be controlled by interaction with negative regulatory factors, PREG and PGOV, whose synthesis or activity is in turn controlled by the NUC2 repressor protein (Kang & Metzenberg, 1990). DNA-binding studies to determine the site of action for NUC1 have yet to be carried out. Phosphate regulation in *A. nidulans* is controlled by the wide domain PALCA protein (Caddick *et al.*, 1986b), whose positive effects on gene expression may be analogous to that of NUC1. During sulphur limitation, the CYS3 activator protein of *N. crassa* switches on the expression of several sulphur-controlled genes by binding to specific promoter elements (Kanaan & Marzluf, 1993; see Table 1). DNA-binding of CYS3 is mediated by a bipartite domain, the so-called 'bzip' region comprising a leucine zipper involved in dimerization and an adjacent basic DNA contact region (Kanaan & Marzluf, 1993). Synthesis of CYS3 is in turn regulated by two proteins, SCON1 and SCON2, the latter being a repressor (Paietta, 1990). Similar negatively acting genes have been identified in *A. nidulans* but a CYS3 equivalent still awaits discovery (Natorff *et al.*, 1993).

Finally, another important wide domain control is that exerted by the PACC protein of *A. nidulans*, which is thought to affect gene expression in both a positive and negative manner in response to extracellular pH (Caddick *et al.*, 1986a; Shah *et al.*, 1991). PACC contains a putative DNA-binding domain consisting of two adjacent zinc fingers of the Cys₂His₂ type (Tilburn *et al.*, 1991) and its activity appears to be inhibited by the end-product of the *palA,B,C,E,F* pathway (Caddick *et al.*, 1986a; Shah *et al.*, 1991). Modifying wide domain controls therefore represents another important area of future research in the production of secreted heterologous proteins by filamentous fungi. The use of novel expression cassettes created by deleting or inserting appropriate promoter elements could specifically alter the expression pattern of a particular gene for a given set of growth conditions.

Effect of locus on transcription of integrated genes

It has been known for some time that in higher eukaryotes the genomic site of integration of introduced genes can have profound effects on expression levels (Gridley *et al.*, 1987; Stief *et al.*, 1989). This is also true with filamentous fungi (Miller *et al.*, 1987; Streatfield *et al.*, 1992). Because of copy number and integrating locus effects on expression, several studies on the structure/function relationships within fungal promoters have targeted single expression cassettes to specific loci.

However, this occurred with varying degrees of success depending on the fungal strain used (Unkles *et al.*, 1989; Hata *et al.*, 1992; Streatfield *et al.*, 1992; Tsuchiya *et al.*, 1992). The *cbh1* locus of *T. reesei* appears to be one of high transcriptional activity and has been used successfully to target heterologous gene expression (Harkki *et al.*, 1991; Nyssönen *et al.*, 1993). The high levels of glucoamylase produced by *A. niger* may be a result not only of the strong *glaA* promoter but also a transcriptionally active locus, but this has not been confirmed experimentally. Transforming DNA should therefore be directed to a locus of known high transcriptional activity.

Alternatively, the gene of interest could be introduced using a vector which confers position-independent high transcription. In this context, particular sequences have been identified in higher eukaryotes which can reduce the negative effects of integration site on gene expression. The eukaryotic genome is subdivided into topologically constrained loops that serve to define functional genetic domains (Gasser & Laemmli, 1987). When genes are inactive, the chromatin within the domain is found in a highly compact state, and the DNA is inaccessible to molecular probes such as DNAase I. Transcriptional activation is accompanied by cooperative unravelling of the chromatin including the coding sequences; genomic domains as long as 100 kb which can be preferentially digested with DNAase I were defined in this manner (Stadler *et al.*, 1980). Some of these DNA domains are flanked by regions where a sharp change in chromatin structure takes place, passing from a resistant conformation to a DNAase sensitive state (Phi-Van & Strätling, 1988). Sequences located at these boundaries were found to bind specifically to the internal nuclear skeleton (nuclear matrix/scaffold) and hence termed matrix or scaffold attachment regions (MARs/SARs). MARs of various sizes have been identified in a number of eukaryotes at the boundaries of functional transcription units (Gasser & Laemmli, 1986; Phi-Van & Strätling, 1988) and characterized as being A+T rich (Phi-Van *et al.*, 1990). Those from a number of organisms show only limited sequence homology and do not cross-hybridize to each other. However, MARs have the ability to bind matrices from other species (albeit with differing affinities) demonstrating that they are functionally conserved (Cockerill & Garrard, 1986).

Several studies have shown that MARs flanking a reporter construct can mitigate position-dependent expression in both homologous and heterologous backgrounds (Phi-Van *et al.*, 1990; Breyné *et al.*, 1992; McKnight *et al.*, 1992). Indeed, the MAR from the chicken lysozyme gene domain promoted a 10-fold increase of reporter gene expression, when compared to a construct without MARs (Stief *et al.*, 1989). The use of MARs or related nuclear scaffold elements in filamentous

fungus expression cassettes may therefore obviate the need for locus-specific targeting by insulating against host chromatin effects on gene expression.

The secretory pathway

It is becoming increasingly clear that many of the problems associated with obtaining effective secreted yields of heterologous proteins from expression hosts are a function of the constraints imposed by the secretory process itself. These constraints reflect the functional diversity of a system which must oversee and promote correct protein folding, mediate any required post-translational modifications (such as glycosylation) and sort, process and target proteins to specific cellular sites, all at a rate consistent with the function of the cell as a whole. Molecular dissection of the secretory pathway in a number of organisms has revealed common features and a generalized model has been established. Transport is a vectorial process beginning with entry of proteins into the lumen of the endoplasmic reticulum (ER) and ending with the fusion of secretory vesicles with the plasma membrane. In *A. niger*, the secretion of glucamylase has been shown to occur primarily at growing hyphal tips (Wösten *et al.*, 1991). Retrieval and recycling of proteins from post-ER compartments in the secretory pathway can also occur (see Rothman & Orci, 1992). Although post-ER compartments such as the Golgi complex need not have been observed cytologically in many fungal species, we regard this complex as a likely functional compartment involved in post-translational modification and protein sorting. In addition, specific sorting is likely to operate mainly through a process of selective retention so that, in the absence of specific signals, protein transport occurs by default via a mechanism of 'bulk flow' (Pfeffer & Rothman, 1987). Finally, it is now apparent that many of the components of the secretion machinery are universal so that, for example, some yeast and mammalian homologues are interchangeable and can act as effective replacements for one another (Barinaga, 1993). In this part of the review, we describe some recent developments in understanding the secretory process and highlight areas relevant in formulating strategies for improving the secreted yields of heterologous proteins from fungi. Most data is derived from non-fungal systems and a number of more general reviews detailing the secretion pathway have been published recently (Reid, 1991; Mellman & Simmons, 1992; Rothman & Orci, 1992).

The translocation step

Translocation from cell cytosol to the ER lumen represents the first major obstacle to a protein destined for secretion and, in eukaryotes, mainly occurs co-

translationally. For most proteins, targeting of the nascent polypeptide to the ER membrane is directed by its own signal sequence in conjunction with a multi-component signal recognition particle (SRP), an associated membrane receptor and GTP (see Rapoport, 1992). High affinity binding between the signal sequence and the SRP (High & Dobberstein, 1991) is mediated by a methionine-rich domain of the 54 kDa SRP component which may form a hydrophobic pocket into which the hydrophobic core of the signal sequence can be inserted (Bernstein *et al.*, 1989). *In vivo* and *in vitro* studies with synthetic signal peptides also suggest that residues which allow an extended conformation at the C-terminus are required for efficient processing by the signal peptidase in *Saccharomyces cerevisiae* (Yamamoto *et al.*, 1990). Alternative, SRP-independent, translocation systems also exist which can replace SRP function in SRP-defective mutants (Deshaies *et al.*, 1988; Rapoport, 1992).

The precise nature of the translocation site itself remains unclear although recent evidence supports the existence of a specific-protein conducting channel (Simon & Blobel, 1992). Recent cross-linking studies with *S. cerevisiae* show that Sec61p is a major partner to translocating polypeptides (Müsch *et al.*, 1992) and binds tightly to ribosomes, an event promoted by the presence of nascent chains (Gorlich *et al.*, 1992b). The ability of ribosomes to protect the crosslinking partners from proteolysis suggests that Sec61p comprises part or all of the ribosome receptor site (High *et al.*, 1991). Dissociation of the ribosomal subunits appears to close the translocation channel (Gorlich *et al.*, 1992b), supporting the notion that the nascent chain is fed directly into it from the ribosome. Although homologues of Sec61p have been identified in mammalian cells (Gorlich *et al.*, 1992b), they have yet to be described in filamentous fungi. Interestingly, the transmembrane domains of these proteins contain several conserved hydrophilic residues suggesting a requirement for a hydrophilic environment within the membrane (Rapoport, 1992).

Another important component of the translocation site is the translocation chain associating membrane protein (TRAM), a glycoprotein with multiple membrane-spanning domains which crosslinks specifically to short nascent polypeptides (Krieg *et al.*, 1989). A suggested function is as a signal sequence receptor due to its interaction with the N-terminal residues of the signal peptide (Rapoport, 1992). *In vivo* experiments suggest that TRAM may not be required for transport of all proteins, however (Gorlich *et al.*, 1992a). Other proteins localized to the translocation site include the Sec62p/Sec63p complex, the signal peptidase complex and the oligosaccharyl transferase protein. Mutations in Sec62p or Sec63p impair Sec61p function suggesting a role in

translocation (Sanders *et al.*, 1992). This is supported by recent evidence of a transient physical association between Sec62p and nascent polypeptide chains (Müsch *et al.*, 1992). A striking feature of Sec63p is the homology between its predicted ER luminal domain and DnaJ, the partner of the *E. coli* chaperone DnaK. This has led to the proposal that Sec63p may interact in a similar fashion with the major ER chaperone, BiP, and promote passage of the polypeptide chain through the membrane (Feldheim *et al.*, 1992).

In the context of heterologous gene expression and protein secretion, the complexity of translocation should not act as a deterrent. The translocation mechanism is, by and large, highly conserved amongst both pro- and eukaryotes so that signal peptidases, signal peptides and other components can and do operate in a heterologous context. As well as providing potential targets to further optimize the secretion process, this suggests that the demands of translocation in filamentous fungi, about which little is known, can be met without undue difficulty.

The ER lumen

Perhaps the most critical factor in determining the successful passage of a protein through the secretory pathway is that the target protein is correctly folded, a process largely controlled during transit from ER to Golgi (see Gething & Sambrook, 1992). Partially folded structures can survive in the ER for extended periods before assuming their native conformation, suggesting important rate-limiting steps exist *in vivo*. Folding catalysts such as protein disulphide isomerase (PDI) and peptidyl prolyl isomerase (PPI) isoforms or chaperones such as BiP have been localized to the ER lumen and offer potential targets for manipulating and optimizing the folding process (Lodish & Kong, 1991). The emerging role of more general cellular factors such as Ca^{2+} or ATP levels and the redox state of the ER lumen may also provide useful strategies for controlling protein folding (Edgington, 1992). The part played by glycosylation in this context is dealt with in the following section.

PDI is an abundant dimeric ER enzyme which catalyses thiol/disulphide interchange reactions with broad specificity but with low efficiency and comprises up to 10% of the luminal protein (Lorimer, 1992). Dependent on the redox potential, it promotes the formation, reduction and isomerization of disulphide bonds, probably in a manner similar to thioredoxin (Freedman, 1989). Evidence for its role in the folding of ER proteins comes from several sources. Thus, there is a good correlation between PDI and secreted protein levels in a number of cell types (Freedman, 1984). Addition of PDI also restores co-translational formation of disul-

phide bonds in a rabbit reticulocyte lysate system (Bulleid & Freedman, 1988) and its loss of function is a lethal mutation in *S. cerevisiae* (LaMantia *et al.*, 1991). Recent data showed that the levels of correctly folded Fv and Fab antibody fragments in *E. coli* were unaffected by overexpressing either PDI, PPI or both together (Knap-pick *et al.*, 1993). The validity of such a strategy requires more data, however, as it seems likely that different proteins will show different requirements for folding catalysts and chaperones. In addition, PDI's function as a folding catalyst may have to compete with its other cellular activities since, for example, a PDI subunit is required for activation of the microsomal triglyceride transfer protein (Wetterau *et al.*, 1991). Experiments by M. Kikuchi and colleagues with Cys mutants of human lysozyme produced by *S. cerevisiae* have provided several valuable insights into the relationships both *in vivo* and *in vitro* between disulphide bridge formation, folding, and secretion (Taniyama *et al.*, 1992; Omura *et al.*, 1992). One such mutant was transported more rapidly to the Golgi in both yeast and mammalian cells and showed an eightfold increase in secretion efficiency. Several Cys residues appeared to be dispensable for folding to a compact structure as judged by SDS-PAGE. Formation of these structures correlated well with their secretion efficiency *in vivo*.

Two structurally unrelated families of proteins (cyclophilins and FK506-binding proteins), capable of catalysing the *cis-trans* isomerization of peptidyl-prolyl bonds which is known to limit the rate of folding of some proteins, have been identified (see Lorimer, 1992). The catalytic efficiency of eukaryotic cyclophilins appears to depend significantly on the structural context of the particular prolyl peptide bond in question (Schonbrunner *et al.*, 1991). A recent survey of 154 proteins showed that over 60% contained *cis*-prolyl residues (Stewart *et al.*, 1990), emphasizing the potential importance of this event to secreted proteins. PPIase activity is both abundant and ubiquitous (see Fischer & Schmid, 1990) and is known to promote the refolding of proteins both *in vitro* and *in vivo* (Schonbrunner *et al.*, 1991; Gething & Sambrook, 1992). DNA sequence data from *S. cerevisiae* (Koser *et al.*, 1990), *N. crassa* (Tropschug *et al.*, 1988) and others suggest that PPIases are transported to several cellular compartments, including the ER. The recent detection of a cyclosporin-A-sensitive folding event in the ER of mammalian cells supports this view (Lodish & Kong, 1991). Genetic data indicate that genes encoding PPIase activity are not essential in *S. cerevisiae* (Heitman *et al.*, 1991), possibly due to the presence of other isofunctional proteins. Despite the fact that *in vivo* evidence for a role in protein folding is still poor, recent *in vitro* data suggest that PPIases act synergistically with folding catalysts such as

PDI (Schonbrunner & Schmid, 1992). Additional data show that PPIases bind to (cytosolic) hsp90-class chaperones (Nadea *et al.*, 1993) and interact with hsp70 and hsp90 in maintaining the glucocorticoid receptor monomer in an inactive state (Tai *et al.*, 1992). The ability of this family of proteins to associate with these and other cellular factors has prompted the intriguing speculation that they may have a more general role in modulating protein transport and signal transduction pathways (Kelley & Georgopoulos, 1992).

Binding protein (BiP) is an ATP-dependent hsp70-class chaperone found in the ER of eukaryotic cells (see Gething & Sambrook, 1992). In *S. cerevisiae*, this protein is encoded by the *KAR2* gene (Rose *et al.*, 1989). BiP can form stable associations with misfolded, incorrectly glycosylated or incompletely assembled proteins, an event generally correlated with retention of its bound partner in the ER although not all secretion incompetent proteins associate with BiP. Using anti-sense RNA to decrease BiP levels resulted in up to a threefold increase in secreted levels of a mutant protein from mammalian cells (Dorner *et al.*, 1988). More recently, this group has demonstrated a decrease in levels of secreted proteins in BiP-overexpressing mammalian cells, specific to proteins which normally associate with BiP (Dorner *et al.*, 1992). This effect might be specific to proteins with slow folding kinetics (e.g. some mutant proteins) since it has been suggested that overexpression of BiP could actually increase secreted levels of other proteins (see Edgington, 1992). Whether BiP overexpression increases or decreases the secretion capacity of cells, it is likely to provide a useful tool in optimizing the secretion process, especially in cases where engineered or heterologous products are required.

A number of other factors are now emerging as important elements modulating the *in vivo* folding or chaperoning of secreted proteins. The ER lumen is a major intracellular reservoir of Ca^{2+} and inactivation of a gene encoding a plasma membrane Ca^{2+} -ATPase in *S. cerevisiae* (Rudolph *et al.*, 1989) is known to increase levels of secreted heterologous or mutant yeast proteins. More recently, Suzuki *et al.* (1991) showed that retention of mutant T-cell receptor proteins in the ER of mammalian cells by BiP could be relieved by specifically depleting Ca^{2+} levels in the ER. Release of these proteins from BiP is assumed to result from an increase in BiP's ATPase activity for which Ca^{2+} is a known inhibitor (Kassenbrock & Kelly, 1989). Conversely, depletion of luminal Ca^{2+} can result in BiP-mediated retention of proteins with a Ca^{2+} -dependent folding step such as has been shown recently for a multimeric Ca^{2+} -binding protein in mammalian cells (Lodish *et al.*, 1992). BiP is only one of several ER-resident proteins with Ca^{2+} -binding activity which are associated with the folding

process (others include PDI and GRP94). This therefore raises the real possibility of controlling the release of newly synthesized polypeptides from the folding machinery by manipulating Ca^{2+} levels in the ER.

Glycosylation

Glycoproteins show a remarkable diversity of structure, the biological significance of which is only now beginning to be understood in terms of function. Glycosylation can influence solubility, resistance to proteolytic attack, *in vivo* activity and the half-life of a protein (see Hart, 1992). For a given protein, glycosylation patterns are dependent on both cell type and culture conditions. In addition, the stage at which O-glycosylation is mediated in the secretory pathway varies in different organisms, occurring early during translocation into the ER for yeast and some filamentous fungi (see Kubicek *et al.*, 1993). N-linked core oligosaccharide attachment is mediated by a multicomponent oligosaccharyl transferase (OTase) intimately associated with the ER translocation site and is known to be required for correct folding and transport of a number of secreted proteins in mammalian cells (Hoe & Hunt, 1992). Its significance to the expression of non-fungal proteins in filamentous fungal hosts has yet to be assessed. Simultaneous overexpression of two components of the yeast OTase failed to increase the activity of this enzyme, suggesting further components of the system have to be identified (te Heesen *et al.*, 1993). In yeast, mammalian systems, and, it is assumed, filamentous fungi, the Golgi appears to be the site at which major differences in N-linked glycosylation occur. Few data are available (Salovuori *et al.*, 1987) detailing whether the glycosylation machinery of filamentous fungi possesses the enzymes and controlling mechanisms necessary for forming the complex oligosaccharide structures observed in many mammalian glycoproteins and on which *in vivo* activity may depend (Takeuchi *et al.*, 1989). This remains a pressing concern if the secretion potential of filamentous fungi is to be exploited for the production of authentic heterologous proteins intended for therapeutic use.

Gene fusions

Fusions of heterologous genes to those of highly secreted fungal proteins such as glucoamylase or α -amylase, have already proved to be a useful aid in improving the secreted yields of some recombinant proteins (Ward *et al.*, 1990; Korman *et al.*, 1990; Contreras *et al.*, 1991). Similar improvements have also been shown recently for fusions directed by a *Streptoalloteichus hindustanus* protein conferring phleomycin resistance in *Tolypocladium geodes* (Baron *et al.*, 1992). In these cases, the entire coding sequence of the 'carrier' protein was fused

to the target protein. More recently, truncated forms of *A. niger* glucoamylase, GAM (Jeenes *et al.*, 1993; C. van den Hondel, personal communication) or *T. reesei* cellobiohydrolase I, CBHI (Nyyssönen *et al.*, 1993), which exploit the domain structure of these proteins, have been used as carriers for the secretion of a number of recombinant proteins. Fusion of the mature chicken lysozyme (HEWL) sequence to the first 498 residues of *A. niger* glucoamylase provided up to a 20-fold increase in secreted levels of HEWL to 1 g l^{-1} (Jeenes *et al.*, 1993). A KEX2-like endoproteolytic cleavage site similar to that found in many fungal precursor proteins (Calmels *et al.*, 1991) was engineered at the fusion junction and allowed *in vivo* processing and release of the mature product into the medium. All the recombinant HEWL produced was correctly processed and had a native folded structure, suggesting that the *A. niger* KEX2-like activity is capable of dealing with the demands of overproducing strains. A similar strategy has also improved secretion of mature human interleukin-6 by a factor of over 10^4 to 15 mg l^{-1} in *A. niger* (C. van den Hondel, personal communication). In the *T. reesei* system, a 150-fold increase in Fab production to 150 mg l^{-1} was observed as a fusion protein which could later be processed *in vitro* by a *T. reesei* protease that co-purified with the CBHI-Fab fusion (Nyyssönen *et al.*, 1993). The processed Fab showed natural immunological activity despite two additional N-terminal residues. Fusion mRNA showed higher steady-state levels than non-fused controls suggesting an additional benefit of either enhanced transcription or improved mRNA stability (M. Penttilä, personal communication).

It is clear that gene fusion strategies provide substantial benefits in terms of yield, although the precise reasons for this remain unknown. It seems likely that the coding sequences of the GAM and CBHI proteins contain signals optimized for the fungal secretory pathway which facilitate passage of the recombinant protein and may offer protection from proteolytic attack. Fungi produce a wide spectrum of both intracellular and extracellular proteases many of which are active in the secretory pathway presenting major problems to heterologous protein production (Archer *et al.*, 1992). Strains with lowered protease activity have been created by specific gene deletion or by mutagenesis (Mattern *et al.*, 1992) and have been successfully used as improved production hosts (Contreras *et al.*, 1991; Dunn-Coleman *et al.*, 1991).

Future prospects

Improvements in secreted heterologous protein yields from filamentous fungi are likely to develop from increasing heterologous mRNA levels coupled with a

better understanding of the protein secretory pathway. At the transcriptional level, the use of multicopy transformants appears to be a successful approach provided sufficient gene copies are also present for associated regulatory proteins. In addition, modulating the interactions of these proteins with their target sites by creating 'hybrid' transcription factors or promoter elements will be especially useful in designing novel expression cassettes capable of responding to particular growth conditions. The fact that several control sites from filamentous fungi are interchangeable amongst different fungal species increases the feasibility of creating modified promoters with elements derived from various fungal sources. Care must be taken, however, in altering promoter sites since regions involved in pathway-specific and wide domain control appear to overlap in many cases. Increased expression levels may also be achieved by using improved vectors containing matrix attachment regions which can reduce the negative integration site effects sometimes encountered in higher eukaryotes. Understanding and manipulating the secretory pathway in filamentous fungi should significantly increase secreted yields of selected proteins of interest. The use of gene fusions has already proved very successful but other strategies may involve over-producing 'foldases' such as PDI or PPI or chaperones such as BiP. Whilst numerous other proteins are involved in the secretory pathway, the complexity of the system suggests that targeting elements which control factors such as ATP, GTP, Ca^{2+} or redox levels in the ER may be more profitable. Modifying Ca^{2+} levels in the ER by modulating the activity of ER-specific Ca^{2+} -dependent ATPases may prove particularly fruitful. Finally, the use of host strains with reduced protease activity should also improve secreted yields of particularly sensitive proteins. Further research on understanding the complex regulation of proteolytic activity in these organisms will therefore help in developing improved host strains for heterologous protein production.

References

- ANTON, I. A., DUNCAN, K. & COGGINS, J. R. (1987). A eukaryotic repressor protein, the *qa-1S* gene product of *Neurospora crassa* is homologous to part of the AROM multifunctional enzyme. *Journal of Molecular Biology* **197**, 367–371.
- ARCHER, D. B., MACKENZIE, D. A., JEENES, D. J. & ROBERTS, I. N. (1992). Proteolytic degradation of heterologous proteins expressed in *Aspergillus niger*. *Biotechnology Letters* **14**, 357–362.
- BARINAGA, M. (1993). Secrets of secretion revealed. *Science* **260**, 487–489.
- BARON, M., TIRABY, G., CALMELS, T., PARRICHE, M. & DURAND, H. (1992). Efficient secretion of human lysozyme fused to the *Sh ble* phleomycin resistance protein by the fungus *Tolypocladium geodes*. *Journal of Biotechnology* **24**, 253–266.
- BERI, R., WHITTINGTON, H., ROBERTS, C. F. & HAWKINS, A. R. (1987). Isolation and characterization of the positively acting regulatory

- gene *qutA* from *Aspergillus nidulans*. *Nucleic Acids Research* **19**, 7991–8001.
- BERNSTEIN, H. D., PORITZ, M. A., STRUB, K., HOBEN, P. J., BRENNER, S. & WALTER, P. (1989). Model for signal sequence recognition from amino acid sequence of 54K subunit of signal recognition particle. *Nature, London* **340**, 482–486.
- BREYNE, P., VAN MONTAGU, M., DEPICKER, A. & GHEYSEN, G. (1992). Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco. *Plant Cell* **4**, 463–471.
- BULLEID, N. J. & FREEDMAN, R. B. (1988). Defective co-translational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. *Nature, London* **335**, 649–651.
- BURGER, G., TILBURN, J. & SCAZZOCCHIO, C. (1991). Molecular cloning and functional characterization of the pathway-specific regulatory gene *nirA*, which controls nitrate assimilation in *Aspergillus nidulans*. *Molecular and Cellular Biology* **11**, 795–802.
- CADDICK, M. X., BROWNLEE, A. G. & ARST, H. N., JR (1986a). Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. *Molecular and General Genetics* **203**, 346–353.
- CADDICK, M. X., BROWNLEE, A. G. & ARST, H. N., JR (1986b). Phosphate regulation in *Aspergillus nidulans*: responses to nutritional starvation. *Genetical Research* **47**, 93–102.
- CALMELS, T. P. G., MARTIN, F., DURAND, H. & TIRABY, G. (1991). Proteolytic events in the processing of secreted proteins in fungi. *Journal of Biotechnology* **17**, 51–66.
- CHANG, Y. C. & TIMBERLAKE, W. E. (1992). Identification of *Aspergillus brlA* response elements (BREs) by genetic selection in yeast. *Genetics* **133**, 29–38.
- COCKERILL, P. N. & GARRARD, W. T. (1986). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**, 273–282.
- CONTRERAS, R., CARREZ, D., KINGHORN, J. R., VAN DEN HONDEL, C. A. M. J. J. & FIEERS, W. (1991). Efficient KEX2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus nidulans* and secretion of mature interleukin-6. *Bio/Technology* **9**, 378–381.
- DESHAIES, R. J., KOCH, B. D., WERNER-WASHBURNE, M., CRAIG, E. A. & SCHEKMAN, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature, London* **332**, 800–805.
- DORNER, A. J., KRANE, M. G. & KAUFMAN, R. J. (1988). Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. *Molecular and Cellular Biology* **8**, 4063–4070.
- DORNER, A. J., WASLEY, L. C. & KAUFMAN, R. J. (1992). Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *EMBO Journal* **11**, 1563–1571.
- DOWZER, C. E. A. & KELLY, J. M. (1991). Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Molecular and Cellular Biology* **11**, 5701–5709.
- DUNN-COLEMAN, N. S., BLOEBaum, P., BERKA, R. M., BODIE, E., ROBINSON, N., ARMSTRONG, G., WARD, M., PRZETAK, M., CARTER, G. L., LACOST, R., WILSON, L. J., KODAMA, K. H., BALIU, E. F., BOWER, B., LAMSA, M. & HEINSOHN, H. (1991). Commercial levels of chymosin production by *Aspergillus*. *Bio/Technology* **9**, 976–982.
- EDGINGTON, S. M. (1992). Rites of passage: moving biotech proteins through the ER. *Bio/Technology* **10**, 1413–1420.
- FELDHEIM, D., ROTHBLATT, J. & SCHEKMAN, R. (1992). Topology and functional domains of sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Molecular and Cellular Biology* **12**, 3288–3296.
- FISCHER, G. & SCHMID, F. X. (1990). The mechanism of protein folding. Implications of *in vitro* refolding models for *de novo* protein folding and translocation in the cell. *Biochemistry* **29**, 2205–2212.
- FOWLER, T., BERKA, R. M. & WARD, M. (1990). Regulation of the *glcA* gene of *Aspergillus niger*. *Current Genetics* **18**, 537–545.
- FREEDMAN, R. B. (1984). Native disulphide bond formation in protein biosynthesis: evidence for the role of protein disulphide isomerase. *Trends in Biochemical Sciences* **9**, 438–441.
- FREEDMAN, R. B. (1989). Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell* **57**, 1069–1072.
- GASSER, S. M. & LAEMMLI, U. K. (1986). The organization of chromatin loops: characterization of a scaffold attachment site. *EMBO Journal* **5**, 511–518.
- GASSER, S. M. & LAEMMLI, U. K. (1987). A glimpse at chromosomal order. *Trends in Genetics* **3**, 16–22.
- GEEVER, R. F., HUIET, L., BAUM, J. A., TYLER, B. M., PATEL, V. B., RUTLEDGE, B. J., CASE, M. E. & GILES, N. H. (1989). DNA sequence, organization and regulation of the *qa* gene cluster of *Neurospora crassa*. *Journal of Molecular Biology* **207**, 15–34.
- GETTING, M.-J. & SAMBROOK, J. (1992). Protein folding in the cell. *Nature, London* **355**, 33–44.
- GOLDMAN, G. H., GEREMIA, R. A., CAPLAN, A. B., VILA, S. B., VILLARROEL, R., VAN MONTAGU, M. & HERRERA-ESTRELLA, A. (1992). Molecular characterization and regulation of the phosphoglycerate kinase gene from *Trichoderma viride*. *Molecular Microbiology* **6**, 1231–1242.
- GORLICH, D., HARTMANN, E., PREHN, S. & RAPOPORT, T. A. (1992a). A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature, London* **357**, 47–52.
- GORLICH, D., PREHN, S., HARTMANN, E., KALIES, K.-U. & RAPOPORT, T. A. (1992b). A mammalian homolog of sec61p and secYp is associated with ribosomes and nascent polypeptides during translocation. *Cell* **71**, 489–503.
- GRIDLEY, T., SORIANO, P. & JAENISCH, R. (1987). Insertional mutagenesis in mice. *Trends in Genetics* **3**, 162–166.
- GURR, S. J., UNKLES, S. E. & KINGHORN, J. R. (1987). The structure and organization of nuclear genes of filamentous fungi. In *Gene Structure in Eukaryotic Microbes, Special Publication of the Society for General Microbiology vol. 22*, pp. 93–139. Edited by J. R. Kinghorn. Oxford: IRL Press.
- HAAS, H., REDL, B., FRIEDLIN, E. & STÖFFER, G. (1992). Isolation and analysis of the *Penicillium chrysogenum phoA* gene encoding a secreted phosphate-repressible acid phosphatase. *Gene* **113**, 129–133.
- HARKKI, A., MÄNTYLÄ, A., PENTTILÄ, M., MUTTILAINEN, S., BÜHLER, R., SUOMINEN, P., KNOWLES, J. & NEVALAINEN, H. (1991). Genetic engineering of *Trichoderma* to produce strains with novel cellulase profiles. *Enzyme and Microbiol Technology* **13**, 227–233.
- HART, G. W. (1992). Glycosylation. *Current Opinion in Cell Biology* **4**, 1017–1023.
- HATA, Y., KITAMOTO, K., GOMI, K., KUMAGAI, C. & TAMURA, G. (1992). Functional elements of the promoter region of the *Aspergillus oryzae glaA* gene encoding glucoamylase. *Current Genetics* **22**, 85–91.
- HAWKINS, A. R., LAMB, H. K. & ROBERTS, C. F. (1992). Structure of the *Aspergillus nidulans qut* repressor-encoding gene: implications for the regulation of transcription initiation. *Gene* **110**, 109–114.
- TE HEESSEN, S., KNAUER, R., LEHLE, L. & AEBI, M. (1993). Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity. *EMBO Journal* **12**, 279–284.
- VAN HEESWICK, R. & HYNES, M. J. (1991). The *amdR* product and a CCAAT-binding factor bind to adjacent, possibly overlapping DNA sequences in the promoter region of the *Aspergillus nidulans amdS* gene. *Nucleic Acids Research* **19**, 2655–2660.
- HEITMAN, J., MOVVA, N. R., HESTAND, P. C. & HALL, M. N. (1991). FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 1948–1952.
- HIGH, S. & DOBBERSTEIN, B. (1991). The signal sequence interacts with the methionine-rich domain of the 54 kD protein of signal recognition particle. *Journal of Cell Biology* **113**, 229–233.
- HIGH, S., GORLICH, D., WIEDMANN, M., RAPOPORT, T. A. & DOBBERSTEIN, B. (1991). The identification of proteins in the proximity of signal anchor sequences during their targeting to and insertion into the membrane. *Journal of Cell Biology* **113**, 35–44.
- HOE, M. H. & HUNT, R. C. (1992). Loss of one asparagine-linked oligosaccharide from human transferrin receptors results in specific cleavage and association with the endoplasmic reticulum. *Journal of Biological Chemistry* **267**, 4916–4923.
- VAN DEN HONDEL, C. A. M. J. J., PUNT, P. J. & VAN GORCOM, R. F. M. (1991). Heterologous gene expression in filamentous fungi. In *More Gene Manipulations in Fungi*, pp. 396–428. Edited by J. W. Bennett & L. L. Lasure. London: Academic Press.

- ILMÉN, M., ONNELA, M.-L., SALOHEIMO, A. & PENTTILÄ, M. (1992). Regulation of expression of cellobiohydrolase I gene in *Trichoderma reesei*. In *Proceedings of the First European Conference on Fungal Genetics*, Nottingham, 1992, abstract P2/65.
- JARAI, G., TRUONG, H.-N., DANIEL-VEDELE, F. & MARZLUF, G. A. (1992). NIT2, the nitrogen regulatory protein of *Neurospora crassa*, binds upstream of *nia*, the tomato nitrate reductase gene, *in vitro*. *Current Genetics* **21**, 37–41.
- JEENES, D. J., MACKENZIE, D. A., ROBERTS, I. N. & ARCHER, D. B. (1991). Heterologous protein production by filamentous fungi. *Biotechnology and Genetic Engineering Reviews* **9**, 327–367.
- JEENES, D. J., MARCZINKE, B., MACKENZIE, D. A. & ARCHER, D. B. (1993). A truncated glucoamylase gene fusion for heterologous protein secretion from *Aspergillus niger*. *FEMS Microbiology Letters* **107**, 267–272.
- KANAAN, M. N. & MARZLUF, G. A. (1993). The positive-acting sulfur regulatory protein CYS3 of *Neurospora crassa*: nuclear localization, autogenous control, and regions required for transcriptional activation. *Molecular and General Genetics* **239**, 334–344.
- KANG, S. & METZENBERG, R. L. (1990). Molecular analysis of *nuc-1⁺*, a gene controlling phosphorus acquisition in *Neurospora crassa*. *Molecular and Cellular Biology* **10**, 5839–5848.
- KASSENBRÖCK, C. K. & KELLY, R. B. (1989). Interaction of heavy chain binding protein (BiP/GRP78) with adenine nucleotides. *EMBO Journal* **8**, 1461–1467.
- KATZ, M. E. & HYNES, M. J. (1989). Isolation and analysis of the acetate regulatory gene, *facB*, from *Aspergillus nidulans*. *Molecular and Cellular Biology* **9**, 5696–5701.
- KELLEY, W. L. & GEORGIOPOULOS, C. (1992). Chaperones and protein folding. *Current Opinion in Cell Biology* **4**, 984–991.
- KNAPPICK, A., KREBBER, C. & PLÜCKTHUN, A. (1993). The effect of folding catalysts on the *in vivo* folding process of different antibody fragments expressed in *Escherichia coli*. *Bio/Technology* **11**, 77–83.
- KORMAN, D. R., BAYLISS, F. T., BARNETT, C. C., CARMONA, C. L., KODAMA, K. H., ROYER, T. J., THOMPSON, S. A., WARD, M., WILSON, L. J. & BERKA, R. M. (1990). Cloning, characterization and expression of two α -amylase genes from *Aspergillus niger* var. *awamori*. *Current Genetics* **17**, 203–212.
- KOSER, P. L., SYLVESTER, D., LIVI, G. P. & BERGSMAN, D. J. (1990). A second cyclophilin-related gene in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **18**, 1643.
- KRIEG, U. C., JOHNSON, A. E. & WALTER, P. (1989). Protein translocation across the endoplasmic reticulum membrane: identification by photocross-linking of a 39 kD integral membrane glycoprotein as part of a putative translocation tunnel. *Journal of Cell Biology* **109**, 2033–2043.
- KUBICEK, C. P., MESSNER, R., GRUBER, F., MACH, R. L. & KUBICEK-PRANZ, E. M. (1993). The *Trichoderma* cellulase regulatory puzzle: from the interior life of a secretory fungus. *Enzyme and Microbial Technology* **15**, 90–99.
- KULMBURG, P., PRANGÉ, T., MATHIEU, M., SEQUEVAL, D., SCAZZOCHIO, C. & FELENBOK, B. (1991). Correct intron splicing generates a new type of a putative zinc-binding domain in a transcriptional activator of *Aspergillus nidulans*. *FEBS Letters* **280**, 11–16.
- KULMBURG, P., JUDEWICZ, N., MATHIEU, M., LENOUEVE, F., SEQUEVAL, D. & FELENBOK, B. (1992a). Specific binding sites for the activator protein, ALCR, in the *alcA* promoter of the ethanol regulon of *Aspergillus nidulans*. *Journal of Biological Chemistry* **267**, 21146–21153.
- KULMBURG, P., SEQUEVAL, D., LENOUEVE, F., MATHIEU, M. & FELENBOK, B. (1992b). Identification of the promoter region involved in the autoregulation of the transcriptional activator ALCR in *Aspergillus nidulans*. *Molecular and Cellular Biology* **12**, 1932–1939.
- KULMBURG, P., MATHIEU, M., DOWZER, C., KELLY, J. & FELENBOK, B. (1993). Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Molecular Microbiology* **7**, 847–857.
- LAMANTIA, M. L., MIURA, T., TACHIKAWA, H., KAPLAN, H. A., LENNARZ, W. J. & MIZUNAGA, T. (1991). Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 4453–4457.
- LAMB, H. K., ROBERTS, C. F. & HAWKINS, A. R. (1992). A second gene (*qutH*) within the *Aspergillus nidulans*-quinic-acid utilization gene cluster encodes a protein with a putative zinc-cluster motif. *Gene* **112**, 219–224.
- LITTLEJOHN, T. G. & HYNES, M. J. (1992). Analysis of the site of action of the *amdR* product for regulation of the *amdS* gene of *Aspergillus nidulans*. *Molecular and General Genetics* **235**, 81–88.
- LODISH, H. F. & KONG, N. (1991). Cyclosporin A inhibits an initial step in folding of transferrin within the endoplasmic reticulum. *Journal of Biological Chemistry* **266**, 14835–14838.
- LODISH, H. F., KONG, N. & WIKSTRÖM, L. (1992). Calcium is required for folding of newly made subunits of the asialoglycoprotein receptor within the endoplasmic reticulum. *Journal of Biological Chemistry* **267**, 12753–12760.
- LORIMER, G. H. (1992). Role of accessory proteins in protein folding. *Current Opinion in Structural Biology* **2**, 26–34.
- MCKNIGHT, R. A., SHAMAY, A., SANKARAN, L., WALL, R. J. & HENNIGHAUSEN, L. (1992). Matrix attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6943–6947.
- MACRAE, W. D., BUXTON, F. P., SIBLEY, S., GARVEN, S., GWYNNE, D. I., DAVIES, R. W. & ARST, H. N., JR (1988). A phosphate-repressible acid phosphatase gene from *Aspergillus niger*: its cloning, sequencing and transcriptional analysis. *Gene* **71**, 339–348.
- MATTERN, I. E., VAN NOORT, J. M., VAN DEN BERG, P., ARCHER, D. B., ROBERTS, I. N. & VAN DEN HONDEL, C. A. M. J. J. (1992). Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Molecular and General Genetics* **234**, 332–336.
- MELLMAN, I. & SIMMONS, K. (1992). The Golgi complex: *in vitro* veritas. *Cell* **68**, 829–840.
- MILLER, B. L., MILLER, K. Y., ROBERTI, K. A. & TIMBERLAKE, W. E. (1987). Position-dependent and -independent mechanisms regulate cell-specific expression of the *SpoC1* gene cluster of *Aspergillus nidulans*. *Molecular and Cellular Biology* **7**, 427–434.
- MÜSCH, A., WIEDMANN, M. & RAPOPORT, T. A. (1992). Yeast sec proteins interact with polypeptides traversing the endoplasmic reticulum membrane. *Cell* **69**, 343–352.
- NADEA, K., DAS, A. & WALSH, C. T. (1993). Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *Journal of Biological Chemistry* **268**, 1479–1487.
- NAGATA, O., TAKASHIMA, T., TANAKA, M. & TSUKAGOSHI, N. (1993). *Aspergillus nidulans* nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene. *Molecular and General Genetics* **237**, 251–260.
- NATORFF, R., BALIŃSKA, M. & PASZEWSKI, A. (1993). At least four regulatory genes control sulphur metabolite repression in *Aspergillus nidulans*. *Molecular and General Genetics* **238**, 185–192.
- NYSSÖNEN, E., PENTTILÄ, M., HARKKI, A., SALOHEIMO, A., KNOWLES, J. K. C. & KERÄNEN, S. (1993). Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Bio/Technology* **11**, 591–595.
- O'CONNELL, M. J. & KELLY, J. M. (1992). *Cis*-acting control elements 5' to *aldA*, the aldehyde dehydrogenase-encoding gene of *Aspergillus niger*. *Gene* **117**, 151–156.
- OMURA, F., OTSU, M. & KIKUCHI, M. (1992). Accelerated secretion of human lysozyme with a disulfide bond mutation. *European Journal of Biochemistry* **205**, 551–559.
- PAIETTA, J. V. (1990). Molecular cloning and analysis of the *scon-2* negative regulatory gene of *Neurospora crassa*. *Molecular and Cellular Biology* **10**, 5207–5214.
- PALUH, J. L. & YANOFSKY, C. (1991). Characterization of *Neurospora* CPC1, a bZIP DNA-binding protein that does not require aligned heptad leucines for dimerization. *Molecular and Cellular Biology* **11**, 935–944.
- PARSONS, L. M., DAVIS, M. A. & HYNES, M. J. (1992). Identification of functional regions of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. *Molecular Microbiology* **6**, 2999–3007.

- PFEFFER, S. R. & ROTHMAN, J. E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annual Reviews of Biochemistry* **56**, 829–852.
- PHI-VAN, L. & STRÄTLING, W. H. (1988). The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO Journal* **7**, 655–664.
- PHI-VAN, L., VON KRIES, J. P., OSTERTAG, W. & STRÄTLING, W. H. (1990). The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Molecular and Cellular Biology* **10**, 2302–2307.
- PUNT, P. J., KRAMER, C., KUYVENHOVEN, A., POWELS, P. H. & VAN DEN HONDEL, C. A. M. J. J. (1992). An upstream activating sequence from the *Aspergillus nidulans* *gpdA* gene. *Gene* **120**, 67–73.
- RAPOPORT, T. A. (1992). Transport of proteins across the endoplasmic reticulum membrane. *Science* **258**, 931–936.
- REID, G. A. (1991). Protein targeting in yeast. *Journal of General Microbiology* **137**, 1765–1773.
- RITCH, T. G., JR & GOLD, M. H. (1992). Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chrysosporium*. *Gene* **118**, 73–80.
- ROSE, M. D., MISRA, L. M. & VOGEL, J. P. (1989). *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78. *Cell* **57**, 1211–1221.
- ROTHMAN, J. E. & ORCI, L. (1992). Molecular dissection of the secretory pathway. *Nature, London* **355**, 409–415.
- RUDOLPH, H. K., ANTEBI, A., FINK, G. R., BUCKLEY, C. M., DORMAN, T. E., LEVITRE, J., DAVIDOW, L. S., MAO, J. & MOIR, D. T. (1989). The yeast secretory pathway is perturbed by mutations in *PMR1*, a member of a Ca^{2+} ATPase family. *Cell* **58**, 133–145.
- SALIEBA, J. A., COBBETT, C. S. & HYNES, M. J. (1992). Characterization of the *amdA*-regulated *aciA* gene of *Aspergillus nidulans*. *Molecular and General Genetics* **235**, 349–358.
- SALOVUORI, I., MAKAROW, M., RAUVALA, H., KNOWLES, J. & KÄÄRIÄINEN, L. (1987). Low molecular weight high-mannose type glycans in a secreted protein of the filamentous fungus *Trichoderma reesei*. *Bio/Technology* **5**, 152–156.
- SANDERS, S. L., WHITFIELD, K. M., VOGEL, J. P., ROSE, M. D. & SCHEKMAN, R. (1992). Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell* **69**, 353–365.
- SCHONBRUNNER, E. R., MAYER, S., TROPSCHUG, M., FISCHER, G., TAKAHASHI, N. & SCHMID, F. X. (1991). Catalysis of protein folding by cyclophilins from different species. *Journal of Biological Chemistry* **266**, 3630–3635.
- SCHONBRUNNER, E. R. & SCHMID, F. X. (1992). Peptidyl-prolyl *cis-trans* isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 4510–4513.
- SHAH, A. J., TILBURN, J., ADLARD, M. W. & ARST, H. N., JR (1991). pH regulation of penicillin production in *Aspergillus nidulans*. *FEMS Microbiological Letters* **61**, 209–212.
- SHARMA, K. K. & ARST, H. N., JR (1985). The product of the regulatory gene of the proline catabolism gene cluster in *Aspergillus nidulans* is a positive acting protein. *Current Genetics* **9**, 299–304.
- SIMON, S. M. & BLOBEL, G. (1992). Signal peptides open protein-conducting channels in *E. coli*. *Cell* **69**, 677–684.
- SOPHIANOPOULOU, V., SUÁREZ, T., DIALLINAS, G. & SCAZZOCCHIO, C. (1993). Operator derepressed mutations in the proline utilization gene cluster of *Aspergillus nidulans*. *Molecular and General Genetics* **236**, 209–213.
- STADLER, J., LARSEN, A., ENGEL, J. D., DOLAN, M., GROUDINE, M. & WEINTRAUB, H. (1980). Tissue specific DNA cleavages in the globin chromatin domain introduced by DNase. *Cell* **20**, 451–460.
- STANGL, H., GRUBER, F. & KUBICEK, C. P. (1993). Characterization of the *Trichoderma reesei* *cbh2* promoter. *Current Genetics* **23**, 115–122.
- STANKOVICH, M., PLATT, A., CADDICK, M. X., LANGDON, T., SHAFFER, P. M. & ARST, H. N., JR (1993). C-terminal truncation of the transcriptional activator encoded by *areA* in *Aspergillus nidulans* results in both loss-of-function and gain-of-function phenotypes. *Molecular Microbiology* **7**, 81–87.
- STEWART, D. E., SARKAR, A. & WAMPLER, J. E. (1990). Occurrence and role of *cis* peptide bonds in protein structures. *Journal of Molecular Biology* **214**, 253–260.
- STIEF, A., WINTER, D. M., STRÄTLING, W. H. & SIPPEL, A. E. (1989). A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature, London* **341**, 343–345.
- STREATFIELD, S. J., TOEWS, S. & ROBERTS, C. F. (1992). Functional analysis of the expression of the 3'-phosphoglycerate kinase *pgk* gene in *Aspergillus nidulans*. *Molecular and General Genetics* **233**, 231–240.
- SUZUKI, C. K., BONIFACINO, J. S., LIN, A. Y., DAVIS, M. M. & KLAUSNER, R. D. (1991). Regulating the retention of T-cell receptor α chain variants within the endoplasmic reticulum: Ca^{2+} -dependent association with BiP. *Journal of Cell Biology* **114**, 189–205.
- TAI, P.-K. K., ALBERS, M. W., CHANG, H., FABER, L. E. & SCHREIBER, S. L. (1992). Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* **256**, 1315–1318.
- TAKEUCHI, M., INOUE, N., STRICKLAND, T. W., KUBOTA, M., WADA, M., SHIMIZU, R., HOSHI, S., KOZUTSUMI, H., TAKASAKI, S. & KOBATA, A. (1989). Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7819–7822.
- TANIYAMA, Y., OGASAHARA, K., YUTANI, K. & KIKUCHI, M. (1992). Folding mechanism of mutant human lysozyme C77/95A with increased secretion efficiency in yeast. *Journal of Biological Chemistry* **267**, 4619–4624.
- TILBURN, J., MUNGRÖO, J., SARKER, S. & ARST, H. N., JR (1991). The *Aspergillus nidulans* pH regulatory gene *pacC*. In *Proceedings of the EMBO Workshop on the Molecular Biology of Filamentous Fungi*, Berlin, 1991, abstract B47.
- TROPSCHUG, M., NICHOLSON, D. W., HARTL, F. U., KOHLER, H., PFANNER, N., WACHTER, E. & NEUPERT, W. (1988). Cyclosporin A-binding protein (cyclophilin) of *Neurospora crassa*. One gene codes for both the cytosolic and mitochondrial forms. *Journal of Biological Chemistry* **263**, 14433–14440.
- TSUCHIYA, K., TADA, S., GOMI, K., KITAMOTO, K., KUMAGAI, C. & TAMURA, G. (1992). Deletion analysis of the Taka-amylase A gene promoter using a homologous transformation system in *Aspergillus oryzae*. *Bioscience, Biotechnology, and Biochemistry* **56**, 1849–1853.
- UNKLES, S. E. (1992). Gene organization in industrial filamentous fungi. In *Applied Molecular Genetics of Filamentous Fungi*, pp. 28–53. Edited by J. R. Kinghorn & G. Turner. Glasgow: Blackie Academic & Professional.
- UNKLES, S. E., CAMPBELL, E. I., DE RUITER-JACOBS, Y. M. J. T., BROEKHUIJSEN, M. P., MACRO, J. A., CARREZ, D., CONTRERAS, R., VAN DEN HONDEL, C. A. M. J. J. & KINGHORN, J. R. (1989). The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation. *Molecular and General Genetics* **218**, 99–104.
- 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. & KINGHORN, J. R. (1992). The *Aspergillus niger* *niaD* gene encoding nitrate reductase: upstream nucleotide and amino acid sequence comparisons. *Gene* **111**, 149–155.
- VANHANEN, S., SALOHEIMO, A., ILMÉN, M., KNOWLES, J. K. C. & PENTTILÄ, M. (1991). Promoter structure and expression of the 3-phosphoglycerate kinase-encoding gene (*pgk1*) of *Trichoderma reesei*. *Gene* **106**, 129–133.
- VERDOES, J. C., PUNT, P. J., SCHRICKX, J. M., VAN VERSEVELD, H. W., STOUTHAMER, A. H. & VAN DEN HONDEL, C. A. M. J. J. (1993). Glucoamylase overexpression in *Aspergillus niger*: molecular genetic analysis of strains containing multiple copies of the *glaA* gene. *Transgenic Research* **2**, 84–92.
- WANG, X. W., HYNES, M. J. & DAVIS, M. A. (1992). Structural and functional analysis of the *amdR* regulatory gene of *Aspergillus oryzae*. *Gene* **122**, 147–154.
- WARD, M., WILSON, L. J., KODAMA, K. H., REY, M. W. & BERKA, R. M. (1990). Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Bio/Technology* **8**, 435–440.
- WETTERAU, J. R., COMBS, K., MCLEAN, R., SPINNER, S. N. & AGGERBECK, L. P. (1991). Protein disulfide isomerase appears

- necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. *Biochemistry* **30**, 9728–9735.
- WÖSTEN, H. A. B., MOUKHA, S. M., SIETSMA, J. H. & WESSELS, J. G. H. (1991). Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology* **137**, 2017–2023.
- YAMAMOTO, Y., TADAYASU, O., KOHARA, A., TANAKA, T., TANAKA, T. & KIKUCHI, M. (1990). Conformational requirement of signal sequences functioning in yeast: circular dichroism and ^1H nuclear magnetic resonance studies of synthetic peptides. *Biochemistry* **29**, 8998–9006.
- YOUNG, J. L. & MARZLUF, G. A. (1991). Molecular comparison of the negative-acting nitrogen control gene, *nmr*, in *Neurospora crassa* and other *Neurospora* and fungal species. *Biochemical Genetics* **29**, 447–459.
- YUAN, G.-F. & MARZLUF, G. A. (1992). Transformants of *Neurospora crassa* with the *nit-4* nitrogen regulatory gene: copy number, growth rate and enzyme activity. *Current Genetics* **22**, 205–211.