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2 Conservation of structure and function of the aflatoxin regulatory gene aflR from Aspergillus
3 nidulans and A. flavus.

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22 Running Title: Aspergillus aflatoxin gene control

Abstract

Under limiting growth conditions, Aspergillus nidulans produces a carcinogenic secondary metabolite related to aflatoxin called sterigmatocystin (ST). The genes for ST biosynthesis are coordinately regulated and are all found within a ~60 kilobase cluster of DNA. One of the genes within this region is predicted to encode a CX2CX6CX6CX2CX6CX2 zinc binuclear cluster DNA binding protein that is related to the Aspergillus flavus and Aspergillus parasiticus aflatoxin regulatory gene aflR. Deletion of the A. nidulans aflR homolog resulted in an inability to induce expression of genes within the ST gene cluster and a loss of ST production as predicted if A. nidulans aflR encodes a pathway specific regulator for ST biosynthesis. Because A. nidulans aflR mRNA accumulates specifically under conditions that favor ST production we expect that activation of ST biosynthetic genes is determined by A. nidulans aflR activation. In support of this hypothesis, we demonstrated that induced expression of the A. flavus aflR gene in A. nidulans under conditions that normally suppress ST gene expression resulted in activation of genes in the ST biosynthetic pathway. This result demonstrates that AflR function is conserved between Aspergillus spp. and that aflR expression is sufficient to activate genes in the ST pathway.

Keywords: aflR, aflatoxin, secondary metabolism, zinc binuclear cluster

Introduction

Aflatoxin (AF) and sterigmatocystin (ST) are two well characterized Aspergillus polyketide mycotoxins frequently found contaminating oil seed and grain crops (Diener et al., 1987; Jelinek et al., 1989; Scott et al., 1972). These potent carcinogens form a DNA-adduct, often resulting in mutations through G to T transversions (McConnell and Garner, 1994 and references therein), that have been linked to human liver cancer (Bressac et al., 1991). Because contamination of foods and feeds with AF and ST present health risks to livestock and humans alike (Adamson, 1989; Fujii et al., 1976), international tolerance levels for AF (more frequently found than ST) have been imposed on numerous agricultural products. As many as 20 species of Aspergillus, including Aspergillus nidulans, are reported to produce ST as an end point metabolite (Cole and Cox, 1981), whereas Aspergillus flavus and Aspergillus parasiticus are the two major species commonly reported to produce AF, which is enzymatically derived from ST (Keller et al., 1993; Yu et al., 1993).

The ST/AF biosynthetic pathway has been partially defined through biochemical and genetic studies and is thought to involve at least 10 stable intermediates with ST representing the penultimate precursor of AF (for reviews see Bennett et al., 1994; Bennett and Papa, 1988; Bhatnagar et al., 1992; Dutton, 1988; Herbert, 1989; Keller et al., 1992; Yabe et al., 1989). We have found that several of the A. nidulans genes required for ST biosynthesis are closely related to the A. flavus and A. parasiticus AF biosynthetic genes. Genetic studies in all three organisms have shown that these genes are closely linked and our recent studies in A. nidulans have demonstrated the presence of a ~60 kb genomic region containing at least 25 genes that appear to encode all of the enzymatic activities required for ST biosynthesis (Brown et al., 1995; Keller et al., 1995). Such clustering has been observed for genes involved in the production of other secondary metabolites including the penicillin biosynthetic pathway found in A. nidulans, Penicillium chrysogenum and various actinomycetes (Aharonowitz et al., 1992), and the tricothecene pathway

found in Fusarium spp. (Proctor et al., 1995) suggesting that gene clustering may be a general phenomenon associated with secondary metabolism.

A common feature of fungal gene clusters, including those for secondary metabolites, is the presence of a pathway-specific regulatory gene, e.g. tri6 for tricothecene biosynthesis, prnA regulating proline metabolism, nirA regulating nitrate metabolism, etc. (Proctor et al., 1995; Scazzocchio, 1992). To date, all of these regulatory genes have been found to encode members of the zinc binuclear cluster protein family typified by GAL4, the Saccharomyces cerevisiae transcription factor that regulates galactose metabolism (Giniger et al., 1985). The zinc binuclear cluster binding domain, CX2CX6CX6CX2CX6CX2, is invariably located at the amino terminal end of these proteins and this region confers DNA binding specificity (Scazzocchio, 1992). Recently several laboratories have reported the identification in A. flavus and A. parasiticus, of a putative AF regulatory gene, aflR, (Chang et al., 1993; Payne et al., 1993) that contains a zinc binuclear cluster domain (Woloshuk et al., 1994). Studies in these laboratories have shown that aflR is required for expression of other AF pathway genes (Chang et al., 1993; Payne et al., 1993; Woloshuk et al., 1994) and that the A. parasiticus homolog complements an A. flavus aflR mutant (Chang et al., 1993). Here we present data showing that the sixth gene in the ST cluster encodes the A. nidulans aflR homolog and that it too contains the conserved zinc binuclear cluster binding domain. We demonstrate that (i) A. nidulans aflR is required for transcription of the other ST cluster genes, (ii) A. nidulans aflR deletion mutants cannot produce ST and (iii) controlled expression of A. flavus aflR induces the expression of A. nidulans ST cluster genes, e.g. stcU, *stcS*, etc. This latter result is particularly significant because it shows that aflR expression is sufficient to cause ST gene mRNA accumulation when the genes would not normally be activated. Thus, AflR is a pathway specific activator of ST/AF expression and the regulation of aflR expression determines activation of the pathway. The functional conservation of aflR within ST and AF producing aspergilli should allow a focused approach towards investigating ST/AF regulation in A. nidulans.

Materials and methods

Fungal strains and growth conditions.

Table 1 lists all fungal strains used in this study. JYA1-57 was constructed by transforming FGSC89 with the aflR deletion plasmid pJYDRR using standard techniques (Yelton et al., 1984). TRB5-76 was constructed by transformation of FGSC237 with the niiA(p)::AfaflR fusion plasmid pRB5 (Fig. 1). Transformants were initially selected on medium with ammonia to repress activity of the niiA(p).

Spore inoculum for liquid growth experiments was prepared from A. nidulans strains grown for 4 to 5 days at 37°C in darkness on minimal medium plates with appropriate supplements. Liquid minimal medium (Käfer, 1977) was inoculated with a spore suspension ($1x10^6$ conidia per ml) and incubated at 37°C at 300 rpm. Static liquid cultures for RNA isolation were obtained by inoculating 3 ml of complete medium (Yager et al., 1982) with 2 loops of conidia ($1.0x10^7$ spores) in a 7 ml vial placed at a 45° angle and incubating at 30°C. Mycelia was collected from 3 vials for each time point (cA. 1.0 g each sample). To assay the effect of A. flavus aflR on the transcription of A. nidulans ST pathway genes, strains were grown for 12 h in supplemented liquid minimal media containing NH_4 as the sole nitrogen source. The mycelia were then harvested onto miracloth (Calbiochem), washed with minimal medium, transferred to minimal medium containing NO_3 as a sole nitrogen source and samples were taken at 0, 2, 4, 8, and 12 h after shifting.

Identification of the A. nidulans aflR *gene.*

A single degenerate primer (TG T/C A/T CNAA A/G GA A/G AA A/G CC) was constructed based on sequence similarity between the conserved zinc binuclear cluster domains of A. flavus aflR and the S. cerevisiae transcription factor, GAL4. Plasmid subclones of the cosmid L11C09 were used as template DNA for Taq cycle sequencing with the degenerate primer.

Plasmid constructions.

Plasmids were generated using standard techniques. pJYDRR was used to replace the A. nidulans aflR gene and was generated in two steps. First, pJYR41 was constructed by moving the 1.9 kb XbaI-PstI fragment including the A. nidulans aflR ORF into pBluescript SK. pJYR41 was then digested with BglII, removing a 0.450 kb fragment of A. nidulans aflR including the start codon (ATG), and ligated with a 1.8 kb BamHI fragment from pSalArgB containing the argB gene (Fig. 1A). pRB5 was used to construct an A. nidulans strain in which the A. flavus aflR gene was placed under the control of the niiA promoter and was generated in three steps. First, a 0.9 kb BamHI-XhoI fragment from pniiA2 (Johnstone et al., 1990) containing the A. nidulans nitrite reductase promoter was ligated into pSH96 (Wieser and Adams, 1995) generating pRB1. Next, a 1.9 kb fragment including the A. flavus aflR gene was PCR amplified from cosmid B9 (kindly provided by Dr. G. Payne, North Carolina State Univ.) using primers constructed with KpnI sites. This fragment was digested with KpnI and inserted into the same site in pBluescript SK(-) to give pRB3. Finally, the 1.9 kb KpnI fragment from pRB3 was inserted into pRB1 so that the A. flavus aflR gene was adjacent to the niiA promoter (Fig. 1B).

Nucleic acid isolation and manipulation.

Total RNA was isolated as described previously (Adams et al., 1988) and separated by electrophoresis on formaldehyde-agarose gels before transfer to nylon membrane (Hybond-N; Amersham Corp.). Hybridization to ^{32}P-labeled random primed probes was carried out in modified Church buffer (7.0% SDS, 1.0% casein, 1.0 mM EDTA and 0.25 M Na_2HPO_4, pH 7.4)(Church and Gilbert, 1984) at 63°C. A 0.7 kb SmaI fragment of A. nidulans aflR was used as a probe to detect A. nidulans aflR transcript. A 4.2 kb EcoRI-HindIII fragment from pNK10 was used as the probe to detect stcU and stcV transcripts (Keller et al., 1994). The 0.7 kb SmaI-KspI fragment from pRB7 was used as a stcU-specific probe (Keller et al., 1994). A 0.9 kb PstI-HindIII fragment from pNK7 was used as a stcS-specific probe. The 1.9 kb KpnI fragment from pRB3 was used as an A. flavus aflR probe. The control probe was a 12.0 kb EcoRI fragment

located adjacent to the ST cluster that encodes an uncharacterized constitutively expressed gene (approximately a 1.7 kb transcript). A 2.5 kb XbaI fragment from pILJ121 was used as a niaD probe (Johnstone et al., 1990).

Sterigmatocystin production and detection.

Approximately 1.0×10^7 conidia/ml from putative A. nidulans aflR disruptants were used to inoculate 3 ml of liquid complete medium in 7 ml vials. The vials were placed at a 45° angle and incubated at 30°C for 6 days. ST was extracted by adding 1 ml of chloroform to culture vials and was detected by spotting 10 to 20 ml of the chloroform extract from each sample onto precoated thin layer chromatography (TLC) plates (Aldrich). An ST standard (Sigma) was also spotted on the TLC plate. TLC plates were developed with toluene:ethyl acetate:glacial acetic acid (80:10:10, v/v/v) until the solvent front reached 10 cm where the R_f value of ST is 0.65 (Cole and Cox, 1981). After development, the plate was dried in the hood, sprayed with 20% aluminum chloride in 95% ethanol and baked at 80°C for 5 min (Stack and Rodricks, 1971). ST was visualized under long wave UV (320 nm) light.

Results

Identification of the A. nidulans aflR gene.

To test the possibility that an aflR homolog could be found within the A. nidulans ST gene cluster, we synthesized a single degenerate oligonucleotide based on the amino acid sequence conservation observed between the zinc binuclear cluster binding domains of A. flavus AflR and Saccharomyces cerevisiae GAL4. This degenerate oligonucleotide was used as a primer in sequencing reactions with various template plasmids derived from within the ST cluster. One plasmid, pAHK25, a 5.6 kb KpnI subclone of cosmid L11C09, yielded sequence information and additional sequence within this region uncovered a 433 codon open reading frame containing a putative zinc binuclear cluster near the N-terminus as predicted for AflR (Fig. 2). Comparison of this open reading frame

with sequences in various databases showed that it was most closely related to AflR proteins from A. flavus and A. parasiticus. While AflR from A. flavus is almost completely identical to A. parasiticus AflR, the putative A. nidulans AflR is only 33% identical to each of the others. However, the zinc binuclear cluster region is 71% identical and two additional regions near the C-terminus also show high identity between species (Fig. 2).

Functional inactivation of A. nidulans aflR *eliminates ST production and transcription of ST pathway genes.*

To determine whether A. nidulans aflR encoded a regulator of ST gene expression, we deleted the gene by transforming an argB$^-$ strain (FGSC89) with a plasmid construct in which a 450 bp BglII fragment that includes the A. nidulans aflR initiation codon was deleted and replaced with the argB gene (Fig. 1A). Transformants were analyzed to determine if they were able to synthesize ST and if they contained the DNA fragment pattern indicative of an A. nidulans aflR replacement. One transformant, JYA1-57, had the restriction pattern predicted for an A. nidulans aflR gene replacement and failed to produce either ST or any of the ST pathway intermediates while all other transformants tested had the wild-type A. nidulans aflR gene and produced ST. To determine if the requirement for A. nidulans aflR in ST production was for ST gene mRNA accumulation, total RNA from wild-type (FGSC89) and A. nidulans aflR deletion mutant (JYA1-57) strains was isolated from cultures grown for various times. Figure 3 shows that several transcripts from the ST cluster accumulated by 72 h of growth in the wild-type strain but no transcripts were observed in JYA1-57. In contrast, an unregulated transcript from a gene directly adjacent to the ST gene cluster was present in both wild-type and A. nidulans aflR deletion strains. This result supports the hypothesis that A. nidulans aflR is required for the activation of ST cluster pathway genes.

Regulation of A. nidulans aflR *mRNA accumulation.*

ST begins to accumulate after cultures have grown for two to three days. We investigated the possibility that as with ST pathway genes, A. nidulans aflR mRNA accumulation was coordinately

regulated with ST biosynthesis. As shown in Figure 4, low levels of A. nidulans aflR mRNA were detectable in wild-type cultures after 24 h of growth but the transcript accumulated to significantly higher levels as growth continued, reaching maximal levels by about 3 days of growth and then decreasing. Accumulation of the transcripts corresponding to stcU, a gene encoding a ketoreductase required for ST biosynthesis, and stcV, a gene encoding a dehydrogenase proposed to be involved in ST production, followed A. nidulans aflR activation. These transcripts were not detectable at 24 h but had accumulated to easily detectable levels by 48 h. Both stcU and stcV mRNA levels decreased at later times and neither was detected after 5 days of growth, a time when A. nidulans aflR mRNA was still detected. A control transcript for an unregulated gene directly adjacent to the ST gene cluster was present at constant levels throughout the experiment.

Aspergillus flavus aflR *activates ST gene expression in* A. nidulans.

To determine if aflR function is conserved amongst the aspergilli and to test the possibility that aflR is directly involved in activating ST cluster genes, we constructed a strain (TRB5-76) containing a copy of the A. flavus aflR gene fused to the A. nidulans niiA promoter (Fig. 1B). niiA transcription is nutritionally regulated such that relatively high levels of expression are observed when cells are grown in medium containing nitrate as a sole nitrogen source, but this expression is repressed in the presence of ammonia (Cove, 1979; Johnstone et al., 1990). Fig. 5 shows that A. flavus aflR transcript accumulated to easily detectable levels by 2 h after shifting from ammonia to nitrate containing medium. This controlled activation of A. flavus aflR was paralleled by activation of A. nidulans stcU (Fig. 5A) as well as numerous other transcripts from within the ST gene cluster (Fig. 5B). Neither A. flavus aflR nor stcU message was detected at these early growth points in the A. nidulans wild-type strain.

Discussion

Contamination of food and feeds with the carcinogenic Aspergillus secondary metabolites, AF and ST, has long been an issue of worldwide concern. Repeated failures in controlling contamination through traditional agronomic practices has shifted efforts towards understanding the molecular mechanism of AF/ST regulation (Bennett et al., 1994; Trail et al., 1995) with the goal of eventually designing rational control strategies. Because progress in research generally proceeds most rapidly using established model systems, we have selected A. nidulans to examine the regulation of ST/AF production in the aspergilli. A. nidulans has long been used to study basic biological questions and, fortuitously, produces high amounts of ST. Our original contention, that ST biosynthesis in A. nidulans and AF biosynthesis in A. flavus and A. parasiticus would be near-identical has proven to be correct. Studies in the past few years have convincingly shown that ST and AF biosynthetic genes are assembled in large, conserved gene clusters in all three Aspergillus species (Brown et al., 1995; Keller et al., 1995; Keller et al., 1994; Skory et al., 1992; Trail et al., 1995; Yu et al., 1995). These clusters each contain a regulatory gene, aflR, that is predicted to encode a zinc binuclear cluster DNA binding protein that is required for AF (Chang et al., 1993; Payne et al., 1993; Woloshuk et al., 1994) and ST biosynthesis (this paper). Most significantly, by demonstrating that A. flavus aflR functions in A. nidulans, we have shown that AflR function is conserved between ST- and AF-producing fungi.

It is interesting that the AflR homologs in A. flavus and A. parasiticus are nearly completely identical (>95% over 373 amino acids) but the A. nidulans AflR is only about 31% identical to either of these. This contrast reflects the current taxonomical grouping of aspergilli into Subgenera and Sections. Six Subgenera containing 1-6 Sections each have been erected based primarily on morphological features (e.g. conidiophore morphology, spore texture, sexual stage, color, etc.) and growth habits (Klich and Pitt, 1988), although secondary metabolite production has also been considered as a criterion (Samson, 1992). The most commonly cited ST/AF

producing fungi are found in Subgenus Nidulantes, Sections Nidulantes (A. nidulans) and Versicolores (A. versicolor, ST) and Subgenus Circumdati, Section Flavi (A. flavus and A. parasiticus) (Klich and Pitt, 1988). Although atoxigenic, other members of Section Flavi, A. oryzae and A. sojae, retain several AF cluster genes which are very similar to the A. flavus and A. parasiticus homologs, again in contrast to those of A. nidulans (Chang et al., 1995). Presumably, the ancestral ST/AF-producing Aspergillus spp. have diverged considerably through the evolutionary history of this genus. It would be interesting to determine if ST genes in A. *versicolor* are closer in homology to those of A. nidulans than to A. flavus and A. parasiticus as predicted by the current taxonomic divisions. In any case, it is likely that a functioning aflR homolog is present in all ST/AF producing spp. including other genera such as Chaetomium (Barnes et al., 1994) and Bipolaris (Aucamp and Holzapfel, 1970).

The putative zinc binuclear cluster region is the most highly conserved domain amongst the Aspergillus AflR homologs having 71% identity over a 42 amino acid region. This domain has been shown to determine DNA binding specificity for numerous zinc binuclear cluster proteins so the high degree of similarity likely reflects conserved binding specificity. In keeping with this idea, we have shown that the A. flavus aflR can activate ST gene expression in A. nidulans. This is particularly significant in that it was recently proposed that the A. parasiticus and A. flavus AflR proteins bind to the palindromic sequence TTAGGCCTAA (Chang et al., 1995). This sequence is completely absent in the A. nidulans ST gene cluster indicating that the true AflR binding site differs or there is some degeneracy in binding specificity. This discrepancy should be resolved as the AflR binding sites are more precisely elucidated in various fungi. Some additional short stretches of high identity were observed between AflR proteins in the carboxy terminus but it is not yet clear if these regions contribute to AflR function.

The finding that aflR expression is sufficient to activate expression of other genes within the ST/AF gene cluster has important implications regarding the mechanism regulating ST/AF

biosynthesis. Although a great deal of effort has gone into trying to determine what regulates secondary metabolism - mostly from the viewpoint of increasing production of useful secondary metabolites like penicillin - much is speculated but little is known. The literature is rich with descriptions of the confounding effects of dozens of different parameters on fungal secondary metabolism including growth phase (secondary metabolism is typically confined to idiophase), nitrogen source, carbon source, and pH. All of these factors have been implicated in control of AF/ST metabolism but little is known about how different environmental conditions influence AF/ST gene expression. The demonstration here that inducing aflR transcription is sufficient to activate expression of genes required for ST biosynthesis supports the hypothesis that the transcriptional regulation of aflR is an important determinant to ST pathway induction. Thus, understanding the mechanisms through which various environmental and physiological factors influence aflR transcription is an essential step in designing rational approaches to blocking undesirable ST and AF production.

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Table 1. Aspergillus nidulans strains used in this study.

Fungal strains	Genotype	Source
FGSC237	pabaA1 yA2; trpC801 veA1	FGSC[a]
FGSC26	biA1; veA1	FGSC
FGSC89	biA1; argB2; veA1	FGSC
TRB5-76	pabaA1 yA2; veA1 niiA(p)::AfaflR	This study
JYA1-57	biA1	This study

[a] Fungal Genetics Stock Center

1 **Figure legends**

2

3 Fig. 1.

4 A. A map of the portion of the A. nidulans gene cluster containing the aflR, stcD and stcE genes.
5 A 1.8 kb BamHI fragment containing argB-gene was used to replace the 0.450 kb BglII fragment
6 from A. nidulans aflR to construct a deletion mutant. B. The integration of the A. flavus aflR
7 (AfaflR) niiA(p):::AfaflR construct at the *trpC* locus in A. nidulans. Restriction sites are
8 abbreviated: B, BamHI; Bg, BglII ; Ev, EcoRV; K, KpnI; P, PstI; Xb, XbaI; Xh, XhoI.

9

10 Fig. 2

11 Comparison of the A. nidulans AflR sequence with the A. parasiticus and A. flavus AflR.
12 Asterisks indicate identical amino acids. The conserved zinc binuclear cluster is shown by the gray
13 box. The six conserved cysteines are shown in bold.

14

15 Fig. 3

16 aflR is required for expression of other genes in the ST cluster. Total RNA from an A. nidulans
17 wild-type (FGSC89, Lane 1) and A. nidulans aflR disruption strain (JYA1-57, Lane 2) was
18 probed with pNK10 to detect mRNAs corresponding to stcW, stcV, stcU, and stcT. A fragment
19 of A. nidulans DNA falling outside of the 60 kb gene cluster, hybridizing with a 1.7 kb
20 uncharacterized transcript, was used as a control.

21

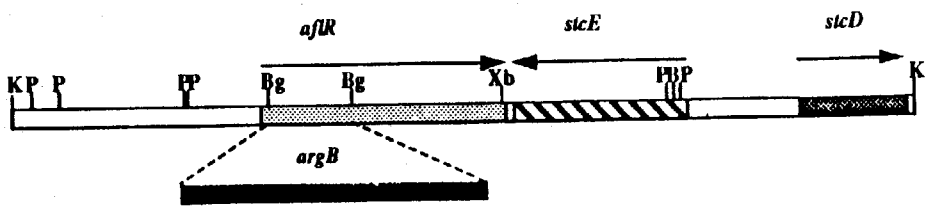
22 Fig. 4

23 Regulation of A. nidulans aflR mRNA accumulation. Total RNA was extracted from stationary
24 cultures at the time points indicated and fractionated on a formaldehyde-agarose gel. Probes for
25 stcU, stcV, and A. nidulans aflR are described in Experimental Procedures. The bottom panel was
26 probed with the control transcript (see Figure 3).

1 Fig. 5.

2 A. A. flavus aflR (AfaflR) and stcU transcript accumulation from strain TRB5-76 at various time
3 points after induction of the niiA promoter. B. Induction of AfaflR leads to the accumulation of
4 transcripts of specific ST pathway genes. Total RNA from samples of TRB5-76 and FGSC26
5 taken 8h after shifting to niiA(p) inducing media was probed with labeled fragments corresponding
6 to AfaflR, stcU, stcN, stcP, stcM, stcO, and niaD as described in the Experimental Procedures.
7

A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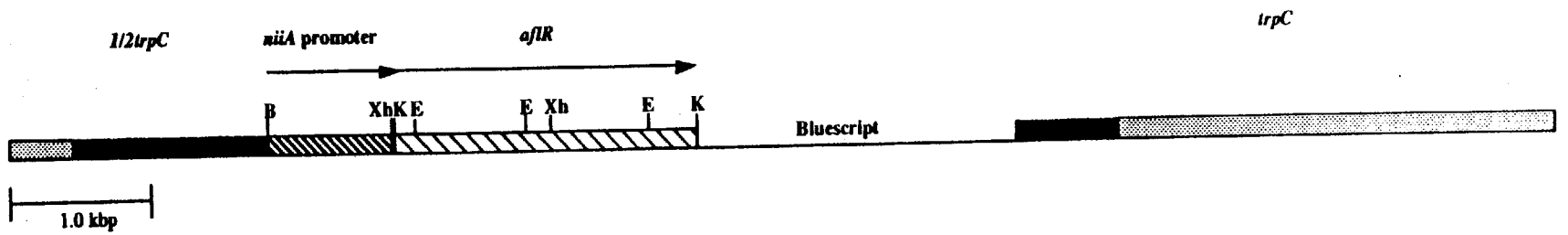
stcE
stcD
K P P
PP
Bg
Bg
Xb
PB P
K
argB

B


1/2trpC
niiA promoter
aflR
trpC
B
XhK E
E Xh
E
K
Bluescript
1.0 kbp

22

```
           1                                                  50
AfaflR     MVDHISPRAS PGPIRSSQTR RARKLRDSCT SCASSKVRCT KEKPACARCI
ApaflR     MVDHISPRAS PGPIRSSQTR RARKLRDSCT SCASSKVRCT KEKPACARCI
AnaflR      MEPPAISQQ STPTAPGGTQ GTRKLRESCI SCSRSKVKCN KEKPTCSRCV
Consensus                *     *    **** **  **  *** *  **** * **

           51                                                100
           ERGLACQYMV SKRMGR.... NPRAPSPLDS TRRPSESLPS ARSEQGLPAH
           ERGLACQYMV SKRMGR.... NPRAPSPLDS TRRPSESLPS AGSEQGLPAH
           RRGLPCEYMV SRRTGRTRVI GVEQPKTAPS PTTPTNTTAA TTATKAGPPV
            *** * *** * * **          *    *    *               *

           101                                               150
           NTYSTPHAHT QAHTHAHSHP QPHPQSHPQS NQPPHALPTP NGSSS.....
           NTYSTPHAHT QAHTHAHSHP QPHPQSHPQS NQPPHALPTP NGSSS.....
           TTDSA..VHT PVITTAPSPK PVQIQSPPAE PDLWGAILSP NTSTSTDLSS
             * *   **    * * *       ** *        *   * * * *

           151                                               200
           .......VSA IFSHQSPPPP VETQGLGGDL AGQEQSTLSS LTVDSEFGGS
           .......VSA IFSHQSPPPL VETQGLGGDL AGQAQSTLSS LTVDSEFGGS
           LLSVNTNFSQ LFASLSPSLL EGMDGMDAEM HAPE...LGA LSVADPSSSM
                    *   *   **        *              *   * *

           201                                               250
           LQSMEHGNHV DFLAESTGSL FDAFLEVGTP MIDPFLESAP LPPFQARYCC
           LQSMEHGNHA DFLAESTGSL FDAFLEVGTP MIDPFLESAP LPPFQARYCC
           MQGLEAPNAA QPPSSNTTS. .......... .......... ......HSYC
            *  *  *         * *                                 *

           251                                               300
           FSLALQTLTH LFPHAPLGCQ LRLTDGEDSS CNLMTTDMVI SGNKRATDAV
           FSLALQTLTC LFPHAPLGCQ LRLTDGEDSS CNLMTTDMVI SGNKKATDAV
           LSICLDTLMR LFPNAGANCE ..RPGHESNP GKLFTIESVI EDNKQILDTA
            *  * **   *** *   *        *      * *   **   **   *

           301                                               350
           RKILGCSCAQ DGYLLSMVVL IVLKVLAWYA AAAGTQCTST AAGGETNSGS
           RKILGCSCAQ DGYLLSMVVL IVLKVLGWYA AAAGTQCTST AAGGETNSGS
           QTILACRCAE DEYVVTLVSL IVFKVLGWYV AAARDRSSDP GREEDFNWST
             ** * **  * *    * * ** *** **  ***               *

           351                                               400
           CSNSPATVSS GCLTEERVLH LPSMMGEDCV DEEDQPRVAA QLVLSELHRV
           CSNSPATVSS GCLTEERVLH HPSMVGEDCV DEEDQPRVRD SLF.......
           AQDSR..RGS VSSFEEQVLH LPTVVGTYCI DGHHQSRMAA QLVLSELYRV
              *     *     ** ***  *   *  *  *   * *     *

           401                                               450
           QSLVNLLAKR LQ........ ..EGGDDAAG IPAHHPASPF SLLGFSGLEA

           QRLVTQVSRR LESIRRRSSS SSSSASSNTT DSDGGMSTPL SSTTLVHLED
           * **     * *                              *  *      **

           451                                   487
           NLRHRLAPCP PTLLITCIEN EEKPHRQEQM TGRFPLH

           DLRKRLRAVS SETISILRHA ......... .......
             * **
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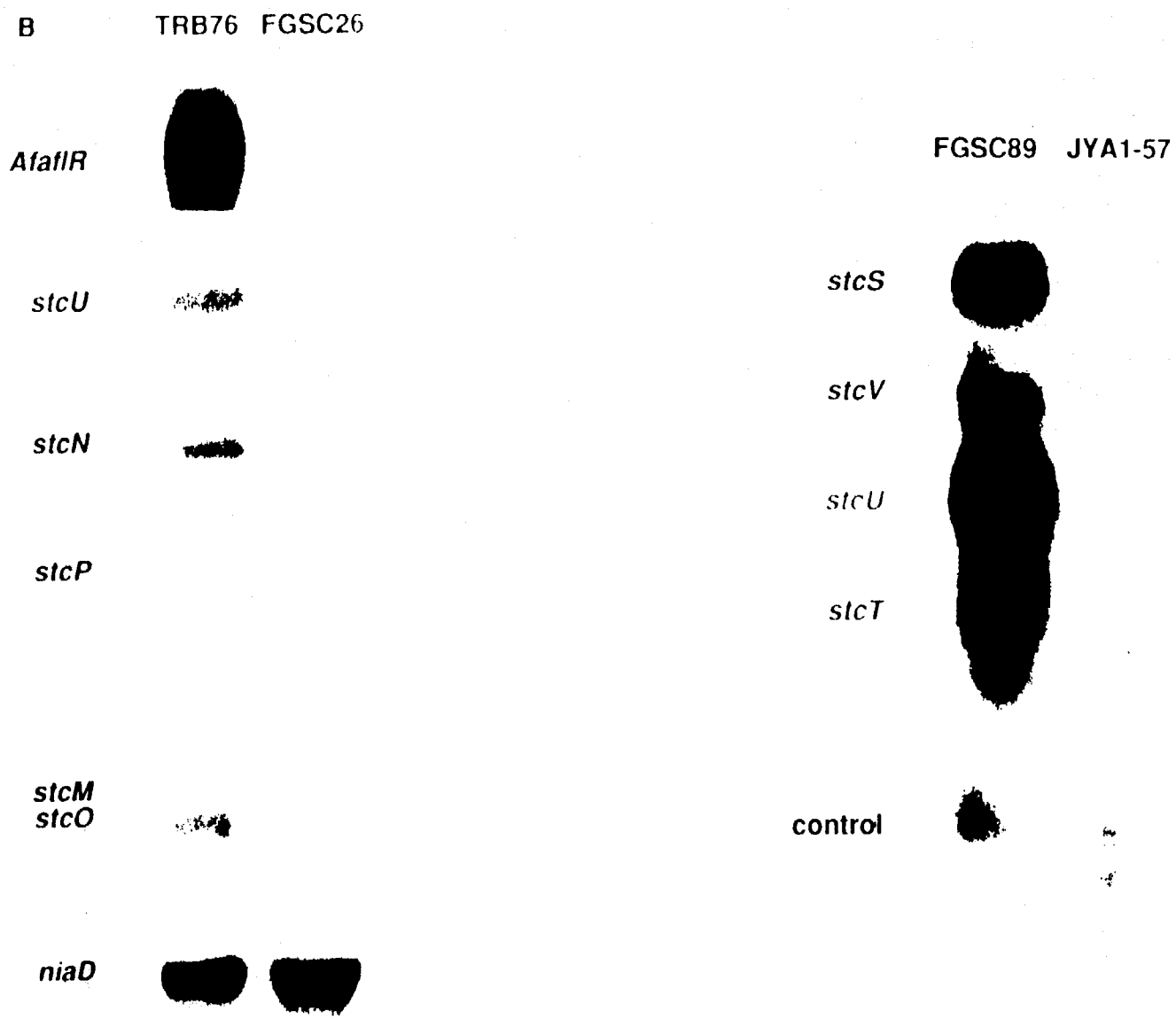
-23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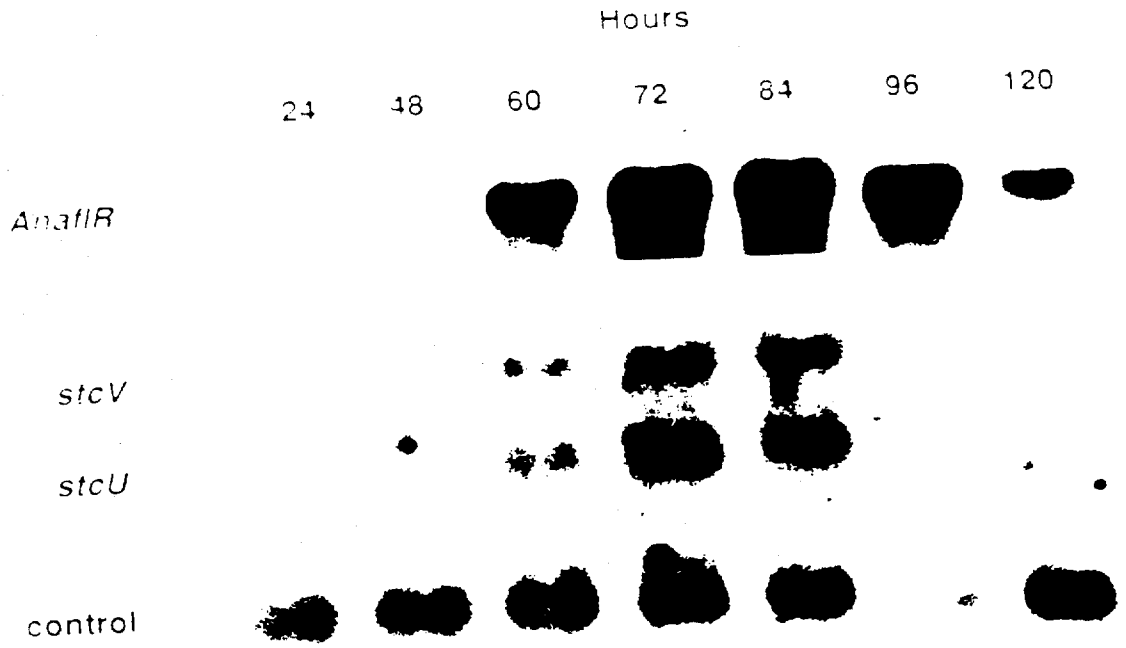
Hours
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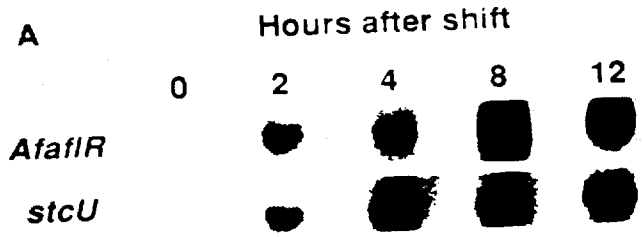
60
72
84
96
120
AnafIR
stcV
stcU
control


A
Hours after shift
0
2
4
8
12
AfafIR
stcU