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A Novel cAMP-response Element, CRE1, Modulates Expression of *nor-1* in *Aspergillus parasiticus**

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The level of aflatoxin accumulation in the filamentous fungus *Aspergillus parasiticus* is modulated by a variety of environmental cues. The presence of glucose (a preferred carbon source) in liquid and solid glucose minimal salts (GMS) growth media strongly stimulated aflatoxin accumulation. Peptone (a non-preferred carbon source) in peptone minimal salts (PMS) media stimulated only low levels of aflatoxin accumulation. Glucose stimulated transcription of the aflatoxin structural genes *ver-1* and *nor-1* to similar intermediate levels in liquid GMS, while on solid media, *ver-1* transcription was stimulated to 20-fold higher levels than *nor-1*. PMS liquid and solid media stimulated very low or non-detectable levels of transcription of both genes. Electrophoretic mobility shift analysis using a *nor-1* promoter fragment (*nor*R) and *A. parasiticus* cell protein extracts revealed specific DNA-protein complexes of different mobility on GMS and PMS solid and liquid media. An imperfect cAMP-response element, CRE1, was identified in *nor*R that mediated formation of the specific DNA-protein complexes. Mutation in CRE1 or AflR1 (AflR *cis*-acting site) caused up to a 3-fold decrease in cAMP-mediated stimulation of *nor-1* promoter activity on GMS agar. South-Western blot analysis identified a 32-kDa protein that specifically bound to *nor*R. p32 could be co-immunoprecipitated by anti-AflR antibody and co-purified with an AflR-maltose-binding protein fusion demonstrating a physical interaction between AflR and p32 *in vitro*. We hypothesize that p32 assists AflR in binding to the *nor-1* promoter, thereby modulating *nor-1* gene expression in response to environmental cues.

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *Aspergillus nomius*, and *Aspergillus parasiticus* on food and feed crops (1). Aflatoxins have a broad range of toxic effects on humans and animals including carcinogenicity, neurotoxicity, and reproductive and developmental toxicity (for a review, see Ref. 2). Therefore, aflatoxin contamination raises serious concerns related to environmental safety, food quality, and human and animal health.

Aflatoxin biosynthesis is a complex process involving up to 20 enzyme-catalyzed reactions (3–5); the genes encoding the aflatoxin enzymes are clustered in the fungal genome (5, 6). The early aflatoxin pathway intermediate, norsolorinic acid, is converted to averantin by Nor-1, a norsolorinic acid reductase (7, 8). Ver-1 participates together with other enzyme activities in converting the middle aflatoxin pathway intermediate, versicolorin A, to demethylsterigmatocystin (9, 10). We have studied *nor-1* and *ver-1* as model aflatoxin genes to understand the molecular mechanisms that regulate aflatoxin synthesis (8, 10–12, 14, 15).[1]

Internal cAMP levels play an important role in induction of aflatoxin gene expression (14, 16). We demonstrated that cAMP at physiological concentrations negatively regulated aflatoxin accumulation through activation of cAMP-dependent protein kinase activity. However, addition of high levels of exogenous cAMP (5 mM) caused strong down-regulation of cAMP-dependent protein kinase activity and resulted in up to a 10-fold increase in aflatoxin accumulation that correlated with up-regulation of *nor-1* and *ver-1* transcription (14). We subsequently observed that wortmannin treatment increases intracellular cAMP, inhibits aflatoxin accumulation, and decreases *nor-1* and *ver-1* promoter activity; wortmannin presumably blocks phosphatidylinositol 3-kinase that in turn prevents activation of phosphodiesterase.[2]

Aflatoxin accumulation is regulated by a number of environmental and nutritional factors and a variety of volatile natural plant compounds (4) including ethylene (15). In fungi, glucose normally inhibits expression of specific fungal genes via carbon catabolite repression (for a review, see Ref. 18; Refs. 19–24). However, glucose stimulates aflatoxin synthesis (25–27), although the molecular mechanisms that mediate this "glucose" response are not known. Buchanan (25–27) demonstrated that cAMP levels are highest during active growth when glucose levels are high; glucose and cAMP then decline prior to induction of aflatoxin gene expression.

Stimulation of aflatoxin biosynthesis by glucose may in part be explained by increased levels of AflR, a positive acting transcription factor (28, 29). AflR binds specifically to consensus AflR binding sites in several aflatoxin promoters, and this interaction is necessary for maximal gene expression (30–33) suggesting that glucose influences aflatoxin synthesis indirectly. Data generated in our laboratory showed that regula-

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[2] J.-W. Lee, L. V. Roze, and J. E. Linz, manuscript in preparation.

tion of *nor-1* via AflR was not the entire story (33). The *nor-1* promoter carries a single AflR binding site (AflR1) that was necessary for maximal expression of *nor-1* in a glucose minimal salts (GMS)[3] liquid medium (33). However, neither recombinant AflR nor protein extracts of *A. parasiticus* grown in a GMS liquid medium formed specific complexes *in vitro* using *nor-1* promoter fragments carrying AflR1. These data suggested that AflR requires additional protein(s) to assist in binding to AflR1.

Miller[1] performed a detailed deletion analysis of the *nor-1* promoter region and identified several conserved *cis*-acting sites including a functional TATA box, AflR binding site (AflR1), and a novel *cis*-acting site called NorL that was also necessary for maximum *nor-1* transcriptional activity.[1] These analyses also suggested that at least one additional unknown transcriptional regulator bound to the *nor-1* promoter at a site located between −117 and +55. The objective of the current study was to identify this *cis*-acting site(s) in the *nor-1* promoter and initiate analysis of its function.

Using *nor-1* promoter fragments in electrophoretic mobility shift assays (EMSAs, *in vitro*) and functional mutation analysis (*in vivo*), we identified the location of a novel *cis*-acting cAMP-response element, CRE1; tentatively identified one protein or protein complex (CRE1bp) that specifically binds this site; and showed that CRE1, at least in part, is required for the cAMP-stimulated (exogenously supplied) up-regulation of *nor-1* transcription. South-Western blot analysis identified a protein, p32, that bound specifically to *nor*R. Based on observed *in vitro* interaction between p32 and AflR, we hypothesize that p32 assists AflR in binding to AflR1 in the *nor-1* promoter.

EXPERIMENTAL PROCEDURES

Strains, Growth Media, and Growth Conditions—Escherichia coli DH5α F′^{e} [F′*/endA1 hsdR17* (r_k^- m^{k-}) *supE44 thi-1 recA1 gyrA* (Nal^r)) *relA1* (*lacZYA argF*) $_{u169}$:(m80)*lacZ M15*) (Invitrogen) was used to amplify plasmid DNA using standard procedures (34). *A. parasiticus* SU-1 (ATCC 56775) is a wild type aflatoxin producer and was the parent for all isogenic strains used in this study. NR-1, a spontaneous mutant (*niaD*) derived from SU-1 (35), was transformed with plasmid pAPGUSNNB to generate strain D8D3 that contains a *nor-1* promoter-GUS reporter fusion (*uidA*; encodes β-D-glucuronidase) integrated into the *nor-1* locus (12). The presence of pAPGUSNNB at the 3′ end of *nor-1* in the *A. parasiticus* SU-1 genome did not affect aflatoxin accumulation, conidiation levels, or *nor-1* expression (transcript or protein accumulation) during growth on YES liquid medium (14). In preliminary studies (data not shown) we demonstrated that SU-1 and D8D3 have similar growth rates and similar levels of conidiation and aflatoxin accumulation on the GMS agar medium used in this study. Growth rate, aflatoxin synthesis, and conidiation in SU-1 and D8D3 were shown to respond similarly to treatment with exogenous DcAMP or cAMP (14). *A. parasiticus* Isolate 4 (I4) contains the *ver-1*-GUS translational fusion integrated into the 3′ region of *ver-1* (10). Another *A. parasiticus* SU-1-derived strain used was strain B62 (*niaD nor-1 br-1*; a norsolorinic acid-accumulating strain) derived from ATCC 24690 (*nor-1 br-1*) (36). Strain TJYP1-22 was derived from B62 by transformation with $fadA^{G42R}$, a dominant activating allele from *Aspergillus nidulans* (37). TJYP1-22 has a fluffy autolytic phenotype, does not produce conidiospores, and does not produce detectable norsolorinic acid. AFS10 (Δ*aflR*) (a gift from Dr. J. Cary) resulted from inactivation of the aflatoxin cluster copy of *aflR* in *A. parasiticus* NR-1 via gene replacement by transformation with a vector containing *aflR* disrupted by *niaD* (38). AFS10 produces no aflatoxin or detectable transcripts from the aflatoxin structural genes *nor-1*, *ver-1*, or *omtA*.

Chemically defined GMS and peptone minimal salts (PMS) media were prepared as described by Buchanan (16) except for the modifications described in Table I. For growth in liquid culture, 100 ml of medium in a 250-ml flask with five 6-mm glass beads were inoculated with 2×10^6 spores and incubated at 29 °C in the dark with shaking at 150 rpm.

TABLE I
Components of liquid media used in this study

Medium[a]	Carbon source	Nitrogen source	Zinc concentration
PMS	Peptone	Ammonium (+peptone)	10 μM
NMS	Glucose	Nitrate	10 μM
GMS	Glucose	Ammonium	10 μM

[a] Base medium was the same for all as described previously (16).

GMS agar medium supplemented with 5 μM Zn^{2+} was used as the base growth medium (16) for experiments conducted on solid culture. 10^4 conidiospores (in 30 μl of H_2O) of D8D3 or I4 were center inoculated onto GMS agar plates (60 × 15 mm) in the presence or absence of 5 mM cAMP or 5 mM DcAMP and grown at 29 °C in the dark. Colonies on GMS did not grow to the edges of dish in the duration (6–7 days) of the experiments.

GUS Activity Assays—Liquid culture GUS activity assays were performed with 1 mg of fresh protein extract as described by Miller (33). Solid culture GUS activity assays were performed essentially as described in Roze *et al.* (14).

RNA and Protein Extraction for Northern and Western Blot Analyses—Liquid cultures of *A. parasiticus* D8D3 and I4 were filtered at the indicated time points through miracloth (Calbiochem). The mycelia were then frozen with liquid nitrogen and stored at −80 °C. For RNA extraction, ~200 mg of frozen mycelia were macerated with a mortar and pestle. TRIzol (Invitrogen) was used to extract total RNA from the ground mycelia following the manufacturer's instructions. For protein extraction, ~200 mg of frozen mycelia were macerated with a mortar and pestle. The ground mycelia were suspended in 0.5 ml of GUS lysis buffer (50 mM NaH_2PO_4, 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 10 mM β-mercaptoethanol, pH 7.0), vortexed for 15 s, and centrifuged for 10 min at 10,000 × *g* at room temperature.

Northern Hybridization Analysis—Northern analysis was performed as described in *Current Protocols in Molecular Biology* (34); total RNA (15 μg) was loaded and resolved by electrophoresis on a 1% agarose gel with 0.4 M formaldehyde. RNA was transferred by capillary action to a Nytran nylon membrane (Schleicher & Schuell) and immobilized by UV cross-linking in a Stratalinker (Stratagene, La Jolla, CA). *nor-1* and *ver-1* DNA probes were generated by PCR using plasmid pQE31 carrying the *nor-1* cDNA (7) and the cosmid *norA* (39), respectively. The primer pairs for each reaction were: *nor-1*, 5′-GCGACACGAACCCAG-3′ and 3′-CGTCCCAAAACGACC-5′; *ver-1*, 5′-AGCGCGGAGCCAAAG-3′ and 3′-CGGGCGACATCCACAG-5′.

The PCR products were gel-purified using the QiaexII gel extraction kit (Qiagen, Santa Clarita, CA). Approximately 120 ng of each DNA fragment were radiolabeled by the random priming method (Roche Applied Science, Indianapolis, IN) using 50 μCi of [^{32}P]dCTP (PerkinElmer Life Sciences). The probes were hybridized to the immobilized RNA for 16 h at 65 °C. The membranes were washed twice at room temperature for 15 min under low stringency (2× SSC, 0.1% SDS) followed by a high stringency wash at 65 °C (0.2× SSC, 0.1% SDS). Signals were detected using an FX phosphorimaging system (Bio-Rad).

Western Blot Analysis—Western blot analyses were performed by standard procedures (34). 30 μg of total protein were resolved by electrophoresis through a 12% acrylamide gel using a Miniprotean II electrophoresis cell (Bio-Rad). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) and probed with anti-Nor-1 (this study) or anti-Ver-1 (10) as primary antibody. Alkaline phosphatase-labeled rabbit anti-IgG (Sigma) was used as a secondary antibody. Colorimetric detection of the enzyme-linked secondary antibody was carried out using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Amresco, Solon, OH). Duplicate gels were stained with Coomassie Brilliant Blue R-250 to evaluate the composition and condition of the protein extracts and to verify equal loading.

nor-1 and ver-1 Promoter Fragments—The 5′ boundary of the *ver-1* promoter region (used in *ver-1*-GUS reported constructs) was delineated by the poly(A) site of the *norA* gene immediately upstream. The 3′ boundary was delineated 25 base pairs downstream of the translational start site. Similarly the 5′ boundary of the *nor-1* promoter region (used in *nor-1*-GUS reporter constructs and EMSA) was delineated by the poly(A) site of the gene immediately upstream, ORF3 (33). The 3′ boundary was delineated 34 base pairs downstream from the transcrip-

[3] The abbreviations used are: GMS, glucose minimal salts; PMS, peptone minimal salts; NMS, nitrate minimal salts; CRE, cAMP-response element; CREB, CRE-binding protein; MBP, maltose-binding protein; EMSA, electrophoretic mobility shift assay; GUS, β-D-glucuronidase; YES, yeast extract sucrose medium; DcAMP, N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; I4, Isolate 4; ATF, activating transcription factor; CREM-1, CRE modulator protein-1; IP, immunoprecipitation; CRE1bp, CRE1-binding protein; ORF, open reading frame.

tional start site. The *nor-1* promoter region was further divided into three subfragments designated *nor*R (right; −117 to +55 relative to the start site of transcription, +1), *nor*M (middle), and *nor*L (left). The 172-bp *nor*R fragment was amplified using pAPGUSNNB as a template (33) and a pair of primers: forward, 5′-TGGCATACCATCAAATGC-3′; and reverse, 5′-ATCGGTCGTGCTAGTTCTCT-3′. The fragment was gel-purified using a QIAEX II gel extraction kit from Qiagen (Valencia, CA) and used for EMSA. *nor*R subfragments R1 and R2 were synthesized using a similar protocol. PCR fragments used as probes in EMSA were cut with BamHI, gel-purified (Qiagen, Santa Clarita, CA), and either labeled using [^{32}P]dCTP (PerkinElmer Life Sciences) in a fill-in reaction (34) or end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using Ready-to-Go Kinase (Amersham Biosciences).

Preparation of Protein Extracts for EMSA—Cellular protein was extracted from *A. parasiticus* cultures using our modifications (31) of the method of Peters and Perez-Esteban (40, 41). Briefly 1-liter flasks containing 10 6-mm glass beads and 500 ml of medium (GMS or PMS) were inoculated with 1×10^8 spores. The cultures were incubated for 48 h at 29 °C in the dark with shaking at 150 rpm. The mycelia were filtered through miracloth (Calbiochem), washed with cold sterile water, frozen with liquid nitrogen, and stored at −80 °C. Frozen mycelia were ground using a mortar and pestle with liquid nitrogen. 5 ml of lysis buffer (25 mM Hepes-KOH, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and 50 μl of protease inhibitor mixture (Sigma) were added per gram of ground mycelia. After stirring on ice for 15 min, saturated ammonium sulfate was slowly introduced to a final concentration of 10%. The suspension was stirred on ice for 15 min, left for 15 min on ice, and then pelleted at 100,000 × *g* for 30 min at 4 °C. Solid ammonium sulfate was added slowly to the supernatant over 1.5 h to raise the concentration to 70% while stirring on ice. The supernatant was then left for 30 min on ice without stirring. The protein was pelleted at 10,000 × *g* for 20 min at 4 °C. The pellet was resuspended in dialysis buffer (15% glycerol, 15 mM Hepes-KOH, pH 7.9, 100 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture, 1 ml/1 liter) and dialyzed twice in 2 liters of dialysis buffer using a 10,000 molecular weight cut-off Slide-A-Lyzer (Pierce). The dialyzed solution was aliquoted and stored at −80 °C. Using liquid culture, the results obtained with the cell protein extracts and nuclear protein extracts were similar. Because preparation of nuclear extracts from mycelia grown in solid culture did not generate satisfactory data we used cellular protein extracts throughout these studies.

EMSA—Double-stranded DNA fragments were 5′ end labeled with [γ-^{32}P]ATP using Ready-To-Go T4 polynucleotide kinase (Amersham Biosciences). EMSA and competition EMSA were performed essentially as described in *Current Protocols in Molecular Biology* (34). 5% acrylamide (80:1 acrylamide:bisacrylamide) non-denaturing gels were used to separate DNA-protein complexes; the gels were dried and exposed to x-ray film. 20 fmol of *nor-1* promoter probes were incubated for 15 min at 30 °C with 2 μg of poly(dI-dC), 7.5 μg bovine serum albumin, and competitor (if desired) with 32 mg of cell protein extract (added last) in a final binding reaction volume of 25 μl.

Supershift EMSA—Two alternative protocols were used. For Protocol 1, fractionated cell protein extract prepared from the FadA mutant TJYP1-22 (37) grown for 43 h on GMS agar was incubated in a DNA binding buffer for 1 h on ice with 2 μl of antibodies against CREB1 (C-21), CREB2 (C-20), ATF2 (N-96), and ATF3 (C-19) (all from Santa Cruz Biotechnology, Santa Cruz, CA). Then the ^{32}P-labeled *nor*R fragment was added, and incubation continued for an additional 15 min at 30 °C. Finally DNA-protein complexes were resolved by electrophoresis in a 4.5% native polyacrylamide gel. The gel was dried and exposed to x-ray film. For Protocol 2, fractionated cell protein extract prepared from FadA mutant TJYP1-22 grown for 43 h on GMS agar was incubated for 15 min at 30 °C with ^{32}P-labeled *nor*R. Next 2 μl of antibodies against CREB1, CREB2, ATF2, and ATF3 were added, and samples were incubated for an additional 15 min at 30 °C. The rest of the procedure was as described for Protocol 1.

EMSA Using CREM-1—The DNA binding reaction contained the same components as described above except that instead of fractionated cell protein extract 20 ng, 100 ng, or 1 μg of CREM-1 (Santa Cruz Biotechnology) was added last.

South-Western Blot Analysis of norR-binding Proteins—The procedure was performed using a protocol previously described (42). Briefly partially purified DNA-binding proteins (used for EMSA) and crude cell protein extracts were separated by electrophoresis on 12% SDS-polyacrylamide gels (43) and electroblotted onto nitrocellulose (0.45 mm, Bio-Rad). The proteins on the membrane were denatured with 6 M guanidine hydrochloride and then renatured by incubation in decreasing concentrations of guanidine hydrochloride. Next the nitrocellulose membrane was exposed to a radiolabeled probe in the absence or presence of non-labeled double-stranded competitor and washed from non-specifically bound DNA followed by autoradiography. The relative molecular size of the proteins binding the probe was determined by comparison with BenchMark™ prestained protein ladder (Invitrogen).

Phosphatase Treatment of Protein Extracts—An aliquot of protein extract used for EMSA was treated in the presence of 0.5 μl of alkaline phosphatase (Sigma) and GMA buffer for 15 min at 37 °C and then placed on ice for 45 min followed by the EMSA protocol. The efficiency of the enzymatic reaction under these conditions was verified by addition of a synthetic substrate for alkaline phosphatase (4-nitrophenyl phosphate) into the reaction mixture. Development of a soluble end product of yellow color was observed almost immediately after 4-nitrophenyl phosphate addition. Heat-inactivated alkaline phosphatase was prepared by incubating the enzyme in 25 mM EDTA at 75 °C for 5 h; this treatment eliminated development of a yellow color after addition of 4-nitrophenyl phosphate.

Immunoprecipitation of p32 with Antibodies against AflR—Immunoprecipitation was performed with the use of Seize X protein A immunoprecipitation kit (Pierce). Crude protein extracts were prepared as described above. 0.5 ml of extract (2400–9400 μg of total protein) was precleared by addition of 50 μl of protein A beads and 100 μl of preimmune serum for 30 min at 4 °C. Simultaneously 1 ml of immune serum was preabsorbed by addition of 50 μl of protein A beads (2 h at room temperature) and washed twice with IP wash buffer. Preabsorbed beads were mixed with precleared protein extract for 1 h at 4 °C. Beads containing immunoprecipitated proteins bound to protein A were washed twice with IP wash buffer containing 0.1% Triton X-100, once with IP wash buffer, and once with IP-quenched buffer (the buffers were provided with the kit). Immune complexes were released by boiling for 4 min in 30 μl of SDS-PAGE sample buffer. Proteins (20 μl/well) were separated via 12% SDS-PAGE followed by South-Western blot analysis described above.

Co-purification of p32 with AflR-MBP Fusion Protein—Amylose resin (New England Biolabs, Beverly, MA) was washed in column buffer (10 mM sodium phosphate, 0.5 M NaCl, 1 mM sodium azide, 10 mM β-mercaptoethanol, 1 mM EGTA, pH 7.2). 80 μl of washed 50% slurry were mixed with 300 μl of crude extract containing AflR-MBP fusion protein obtained using the pMAL protein fusion and purification kit (New England Biolabs) and 200 μl of column buffer. This mixture was incubated on ice for 20 min. Resin was pelleted by centrifugation for 1 min at 5,000 rpm (4 °C) and washed twice with 1 ml of column buffer and once with 1 ml of wash buffer (0.14 M NaCl, 0.008 M Na_2PO_4, 0.002 M K_2PO_4, 0.01 M KCl, pH 7.4). Precleared crude fungal protein extract (1600 to 3750 μg of total protein) was added to the beads and incubated on ice for 1 h. Beads carrying co-precipitated proteins were washed and analyzed as described above.

Enzyme-linked Immunosorbent Assay—Enzyme-linked immunosorbent assays were conducted by the method of Pestka (44) using polyclonal antibodies against aflatoxin B_1 (Sigma).

Protein Determination—The protein concentration was determined using Bio-Rad protein dye reagent.

RESULTS

Generation of Highly Specific Anti-Nor-1 Polyclonal Antibodies—Purified recombinant Nor-1-maltose-binding protein (7) was used as antigen for polyclonal antibody production in rabbits using procedures published previously for anti-Ver-1 (10) and anti-OmtA (45). In YES liquid shake cultures, the antibody (IgG fraction) detected two major bands at 31 and 28 kDa at 48 and 60 h in *A. parasiticus* SU-1; neither band was detected in a Nor-1-disrupted strain (Δ*nor-1*) (8) at any time point (not shown). Based on the *nor-1* cDNA sequence, the molecular mass of Nor-1 is predicted to be 31 kDa. These data suggest that the smaller band is a cleavage product of the 31-kDa protein and that Nor-1 polyclonal antibody specifically recognizes Nor-1 in fungal extracts.

Carbon and Nitrogen Sources Affect Aflatoxin Accumulation and Aflatoxin Gene Expression—Aflatoxin accumulation was measured in D8D3 (*nor-1*-GUS reporter) and I4 (*ver-1*-GUS) grown for 72 h in liquid shake culture in PMS (peptone, NH_4^+, D8D3 only), NMS (glucose, NO_3^-), or GMS (glucose, NH_4^+). Consistent with previous reports (10, 11, 46), aflatoxin accu-

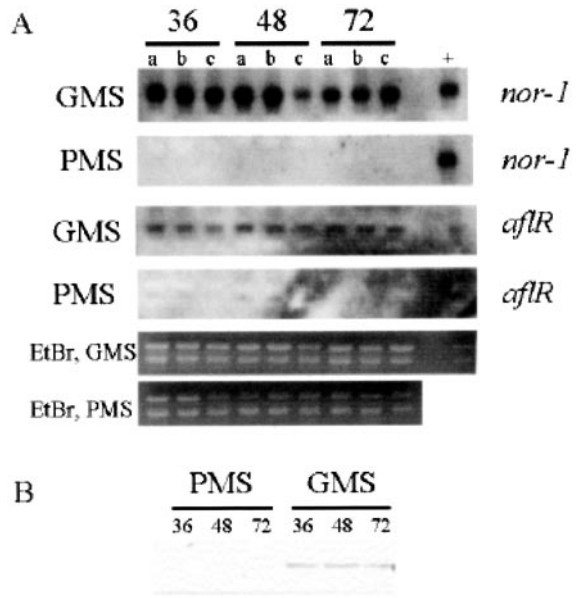

FIG. 1. **Expression of *nor-1* and *aflR* in the reporter strain *A. parasiticus* D8D3.** *A. parasiticus* D8D3 was grown in triplicate in liquid shake culture in GMS or PMS medium. At appropriate time points (36, 48, and 72 h) each replicate culture (*a*, *b*, or *c*) was analyzed for *nor-1* and *aflR* transcripts and Nor-1 protein. *A*, *nor-1* and *aflR* transcript accumulation assessed by Northern hybridization analysis. + *lane*, RNA from a 48-h *A. parasiticus* SU-1 culture in YES medium (positive control). *EtBr*, ethidium bromide staining of triplicate RNA samples. *B*, Nor-1 protein accumulation by Western blot analysis. One flask per time point (36, 48, and 72 h) was analyzed from PMS and GMS cultures.

mulated to intermediate levels (up to 700 μg of aflatoxin/mg of dry weight) in GMS during a transition from active growth to stationary phase (36–48 h in D8D3; 48–60 h in I4). In contrast, D8D3 did not produce detectable aflatoxin in PMS or NMS. I4 produced detectable but greatly reduced (up to 100-fold) aflatoxin levels in NMS (compared with GMS); aflatoxin was not detected in PMS.

Northern and Western blot analyses demonstrated that aflatoxin transcript and protein accumulation paralleled aflatoxin accumulation in D8D3 (Fig. 1) and I4 (data not shown). Native Nor-1 protein and *nor-1* transcript accumulated in GMS cultures at all three time points for both D8D3 and I4 but were not detectable in either PMS (Fig. 1, *A* and *B*) or NMS (not shown); one exception was a barely detectable signal for *nor-1* transcript at 72 h in D8D3. Native Ver-1 protein and *ver-1* transcript levels in I4 followed a similar pattern (not shown).

The transcription factor AflR is required for *nor-1* and *ver-1* gene expression (33). *aflR* transcript accumulated to significant levels at all three time points in D8D3 (Fig. 1*C*) and I4 (data not shown) in GMS cultures but was barely detectable in PMS cultures. *aflR* transcript was not detectable in NMS in D8D3 and I4 (not shown). Transcript steady state levels of *aflR* coincided with *nor-1* and *ver-1* transcript and protein accumulation.

Carbon and Nitrogen Sources Regulate nor-1 and ver-1 in Part at the Level of Transcription—Using *nor-1*-GUS and *ver-1*-GUS reporter constructs carried by strains D8D3 (data not shown) and I4, respectively (Fig. 2), we measured carbon and nitrogen source effects on *nor-1* and *ver-1* expression at the transcriptional level. In liquid shake culture, I4 GMS cultures at 72 h had 60-fold higher *ver-1* promoter activity than I4 NMS cultures (as measured by GUS activity). Results for *nor-1* promoter activity in D8D3 in GMS and NMS liquid shake cultures were similar; neither *ver-1* nor *nor-1* promoter activity was detectable at any time point in PMS liquid shake cultures. On GMS solid (agar) growth medium, glucose stimulated *nor-1* and *ver-1* promoter activity between 48 and 120 h of growth. *ver-1* promoter activity was 20-fold greater on GMS than on PMS agar medium and was ~25-fold lower on agar media than in liquid shake culture. In contrast, *nor-1* promoter activity on PMS agar was stimulated to low, but detectable, levels similar to that on GMS agar. While we cannot eliminate RNA stability as a factor, we can conclude that transcriptional activation of *nor-1* and *ver-1* is in part responsible for changes in aflatoxin gene expression mediated by carbon and nitrogen in both liquid and solid media.

Carbon Source Affects nor-1 Promoter Binding by Cellular Proteins—To determine whether DNA-binding proteins mediate the up-regulation of *nor-1* promoter activity under the influence of carbon source, EMSAs were performed with *A. parasiticus* SU-1 (wild type) and AFS10 (AflR knock-out) cell protein extracts derived from cells grown on GMS and PMS liquid and solid growth media. The DNA probes (*nor*R and derivatives) were generated from the *nor-1* promoter region (for schematics, see Fig. 3, *A–D*).

In liquid media, *nor*R, which contains the functional AflR binding site AflR1 and a TATA box, produced two shifted complexes (P0 and P2) with both SU-1 and AFS10 PMS cell protein extracts (Fig. 4*A*). Complexes of similar mobility (G0 and G2) were also observed with AFS10 GMS cell protein extracts. Two complexes (G4 and G5) with reduced mobilities and increased intensity were observed with SU-1 GMS cell protein extracts (see also Fig. 5*A*). Both *nor*R and *nor*R* (AflR1 changed to non-functional site, TCGgccagCGA to AGTttaaaCAG) (33) were effective competitors for G5 and P2 but were not as effective competitors for G0, G4, or P0 under the growth conditions tested. AFS10 GMS cell protein extracts produced one additional shifted complex (G3) with the *nor*R probe. Neither *nor*R nor *nor*R* competed for G3 suggesting that it, like G0 and G4, is a nonspecific DNA-protein interaction. Based on these data, we conclude the following. 1) P2 and G5 are specific DNA-protein complexes. 2) A functional AflR1 site is not necessary for G5 or P2 formation. 3) Glucose induces G5 complex formation in liquid media. 4) The protein(s) responsible for complex G5 induction in GMS is AflR-dependent. 5) P2 complex formation appears to be AflR-independent (assuming P2 and G5 have similar protein composition).

On solid media, D8D3 GMS cell protein extracts produced a single DNA-protein complex (G1) with lower mobility than complexes seen with D8D3 PMS cell protein extracts (complex P1) or TJYP1-22 (FadA mutant) GMS cell protein extracts (complex F1) (see Fig. 4*B*). Complexes P1 and F1 consisted of closely spaced bands. Formation of complexes G1, P1, and F1 was specific as confirmed by competition EMSA with non-labeled *nor*R as a competitor (not shown). These data suggest that either glucose induces a new complex (G1) consisting of different proteins than those contained in P1 or F1 or glucose induces a new protein that binds to complex P1 but is unable to bind to complex F1 in TJYP1-22 GMS extracts.

Identification of CRE1 Necessary for DNA-Protein Interaction—To further localize the binding site in *nor*R responsible for the specific protein binding observed in EMSA, *nor*R was subdivided into two fragments, *nor*R1 (−52 to −117) and *nor*R2 (+55 to −64) (Fig. 3*C*). These two fragments together with *nor*R and *nor*TATAm (*nor*R with TATA box changed, from 5′-ATATATAG-3′ to 5′-GTTTAAAC-3′) were used as competitors in EMSA with probe *nor*R. Using SU-1, GMS cell protein extracts (Fig. 5*A*), *nor*R1 was the only competitor that failed to compete. These experiments led us to conclude that 1) the TATA box is not necessary for complex G5 formation and 2) the *cis*-acting site for complex G5 is located within *nor*R2.

In EMSA using *nor*R* as a probe and a cell protein extract

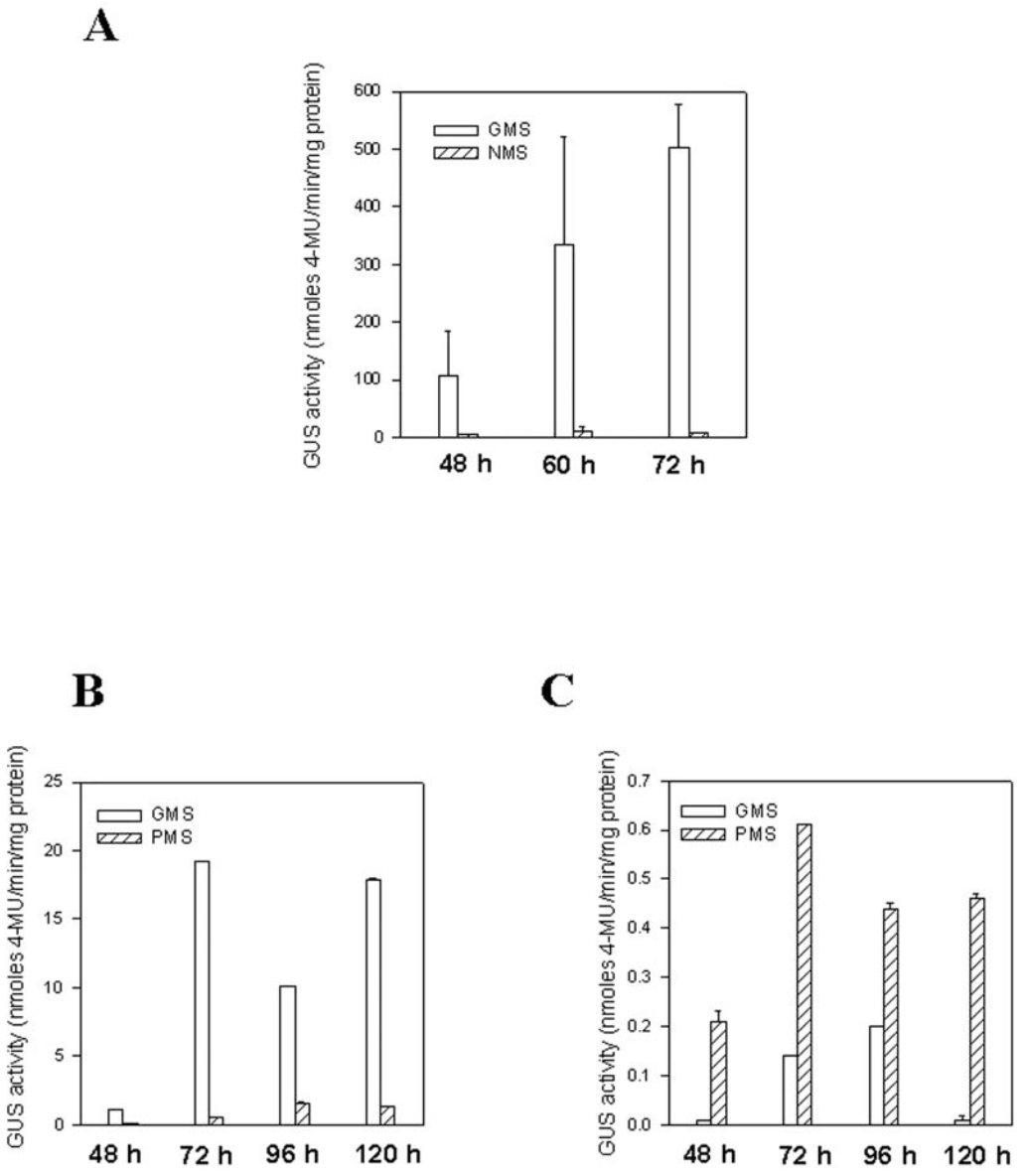

FIG. 2. ***nor-1* and *ver-1* promoter activity in the reporter strains *A. parasiticus* D8D3 and I4.** *A*, strains D8D3 (*nor-1*-GUS; not shown in *A*) and I4 (*ver-1*-GUS) were grown in triplicate in GMS and NMS liquid shake culture for appropriate periods of time (36, 48, 60, or 72 h) and analyzed for GUS activity (see "Experimental Procedures"). These same strains (*B*, I4; *C*, D8D3) were grown in triplicate on PMS or GMS solid medium for 48, 72, 96, or 120 h and analyzed for GUS activity. Values are reported as the mean of triplicates ± S.E. *4-MU*, 4-methylumbelliferone.

from SU-1, we confirmed that the AflR1 binding site is not involved in the *nor*R-protein complex formation (Fig. 5*B*). *nor*R* formed DNA-protein complexes (G4 and G5) of mobility similar to *nor*R. Complex formation was competed by excess *nor*R, *nor*R2, and *nor*R* but not by *nor*R1, by oligonucleotides containing AflR1 or TATA box, or by a strong AflR binding site derived from the *ver-1* promoter.

Because we previously demonstrated that addition of exogenous cAMP (5 mM) induces aflatoxin accumulation and *nor-1* gene expression at the level of transcription (14), we analyzed the *nor-1* promoter for possible cAMP-dependent sites. Nucleotide sequence analysis identified two imperfect cAMP-response elements in the *nor-1*/ORF3 intergenic region that we designated CRE1 and CRE2 (Fig. 3*B*). CRE1 (−14 to −21, TGACATAA) is located in *nor*R2 immediately upstream of the ATG translational start codon. The last nucleotide "A" of CRE1 serves as the first nucleotide in the ATG translational start codon. CRE2 (+247 to +240, TGACATGA) is located in *nor*L. CRE1 and CRE2 sequences each differ by two nucleotides from the consensus CRE, which consists of the inverted octanucleotide repeat TGACGTCA (47). Underlined nucleotides above indicate differences between CRE1, CRE2, and the consensus CRE. There is a single nucleotide difference between CRE1 and CRE2.

To test the hypothesis that CRE1 is necessary for specific DNA-protein interactions in *nor*R and *nor*R2, competition EMSA was conducted with a *nor*R probe and competitors consisting of a 27-bp oligonucleotide containing CRE1 (flanking sequences from *nor*R) or mutant derivatives of the wild type CRE1 sequence (see Fig. 3*D*). The wild type CRE1 oligonucleotide competed effectively for complex G5 (SU-1 liquid GMS cell protein extracts, Fig. 5*A*), complex G1 (D8D3 solid GMS extracts), complex F1 (TJYP1-22 solid GMS extracts), and complex P1 (D8D3 solid PMS extracts) (Fig. 6). A 27-bp oligonucleotide containing a consensus CRE (mammalian) with flanking sequences from *nor*R also competed for G1, F1, and P1 but was a weaker competitor than CRE1 (Fig. 6, *A–C*). Oligonucleotides carrying two, three, or five base changes in CRE1 (CRE1m2, CRE1m3, or CRE1m5; see Fig. 3*D*) either did not compete (m5) or competed much less effectively (m2 and m3)

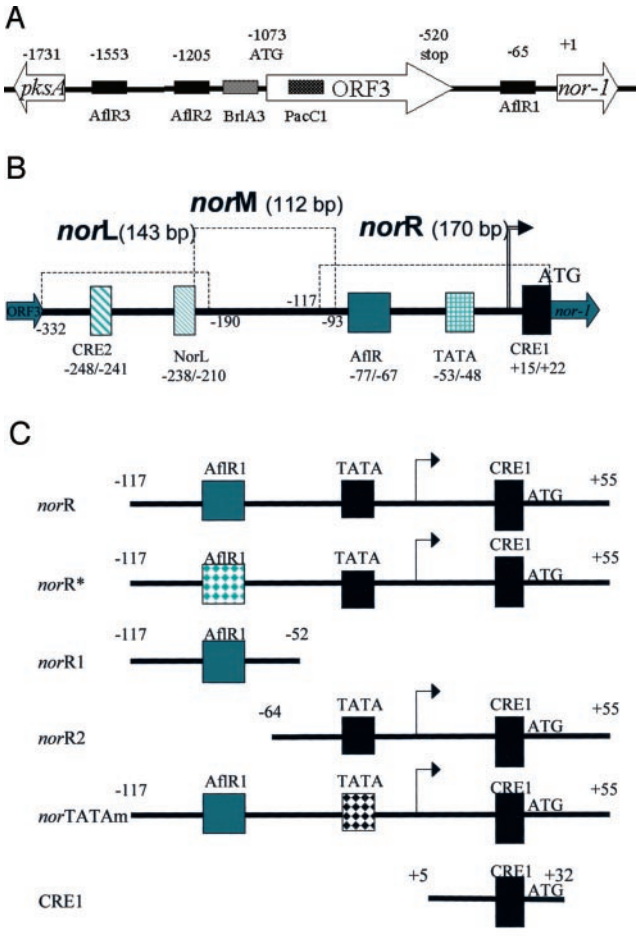

FIG. 3. ***nor-1* promoter and DNA fragments used in EMSA.** *A*, schematic of the *nor-1*/*pksA* intergenic region. In *A*, *B*, and *C* location and size (bp) are shown relative to the *nor-1* transcriptional start site (*bent arrow*). *B*, schematic depicting the intergenic region between *nor-1* and the gene immediately upstream, ORF3. The *nor-1* promoter fragment was subdivided into smaller subfragments designated *nor*R, *nor*M, and *nor*L. *C*, schematic of oligonucleotides used for EMSA; oligonucleotides were based on wild type and mutant sequences in the *nor*R subfragment. *nor*R was further subdivided into *nor*R1 and *nor*R2; location and size (bp) are shown. Three putative *cis*-acting sites are shown including AflR1, TATA, and CRE1 (*filled boxes*). For *nor*R*, AflR1 was changed from wild type (TCGgccagCGA) to AflRm (AGTttaaaCAG, *checkered box*). For *nor*TATAm, the TATA box was changed from wild type (5′-ATATATAG-3′) to TATAm (5′-GTTTAAAC-3′, *checkered box*). *D*, nucleotide sequence of oligonucleotides used in competition EMSA. All oligonucleotides were derived from the *nor-1* promoter region except for *ver*AflR, which was derived from *ver-1* promoter region. *Bold letters* designate the wild type *cis*-acting site. *Bold* and *italicized letters* show positions of mutations. The translation start codon (ATG) in *nor-1* is *underlined*.

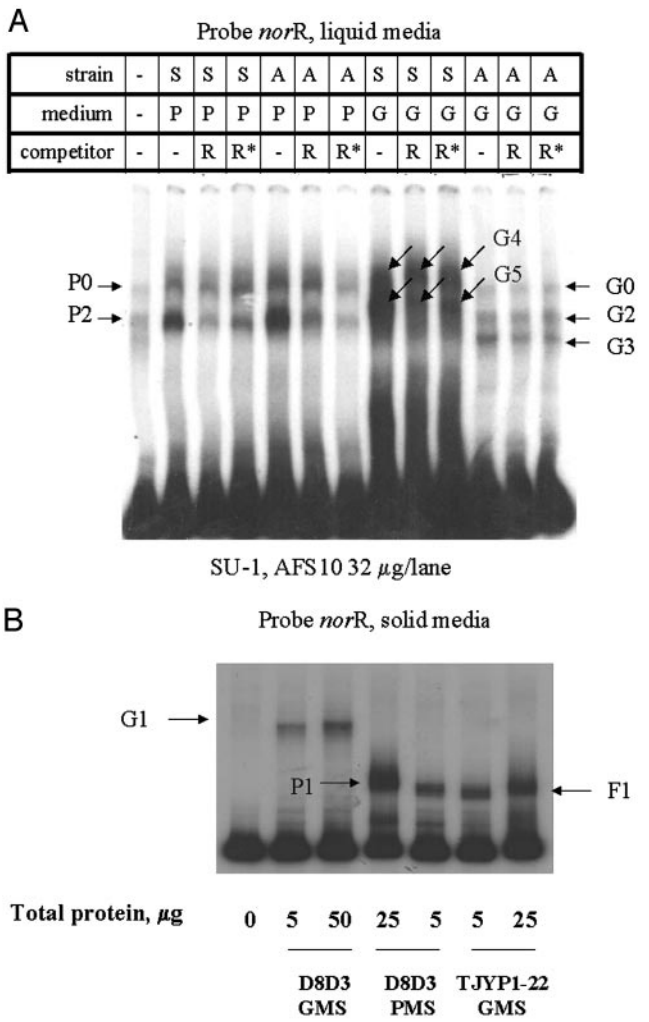

FIG. 4. **EMSA of *nor*R promoter subfragment.** *A*, EMSA of *nor*R, liquid medium. *A. parasiticus* strains SU-1 (*S*) and AFS10 (*A*) were grown for 48 h in liquid shake culture in PMS (*P*) and GMS (*G*). Cell protein extracts were generated from each culture (see "Experimental Procedures"). 32 mg of cell protein were added to the *nor*R probe per lane. Competitors were added at a 250-fold molar excess including *nor*R (*R*) and *nor*R* (*R**). *Arrows* designate DNA-protein complexes formed (P0, P2, G0, G2, G3, G4, and G5) under each set of culture conditions. *B*, EMSA of *nor*R, solid medium. *A. parasiticus* D8D3 was grown for 48 h on GMS or PMS agar medium; *A. parasiticus* TJYP1-22 (FadA) was grown on GMS agar for 43 h. Cell protein extracts were generated from each culture (see "Experimental Procedures"). 0, 5, 25 or 50 μg of each extract were added to *nor*R per lane. *Arrows* designate DNA-protein complexes formed (G1, P1, and F1) under each set of culture conditions.

than CRE1 for complexes G1, F1, or P1 (Fig. 6). Similarly, when a wild type CRE1 oligonucleotide was used as a probe in EMSA (SU-1 liquid GMS extracts), a single, specific DNA-protein complex was formed that effectively could be competed by itself but not by m2, m5, or CRE2 (Fig. 7). This complex was more intense in SU-1 than in AFS10 consistent with EMSA data using a *nor*R probe (Fig. 4*A*).

Mutations in CRE1 or AflR1 Impair cAMP-mediated Induction of nor-1 Transcription in Vivo—Because of its physical similarity to a consensus CRE, we hypothesized that CRE1 was necessary for the cAMP-mediated up-regulation of *nor-1* observed on solid GMS agar media (14). We tested this hypothesis using a reporter strain carrying *nor-1* promoters with either wild type or mutant CRE1 (m2) fused to a GUS reporter. The resulting plasmid constructs were confirmed by nucleotide sequence and restriction enzyme analysis. After transformation, integration of the constructs into the 3′ end of *nor-1* in the

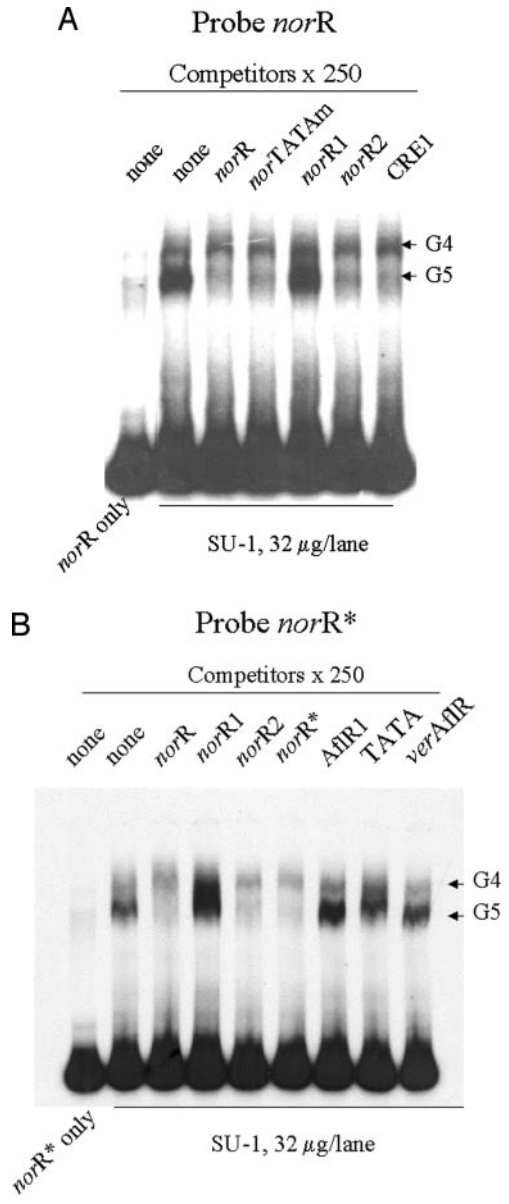

FIG. 5. **Localization of the *cis*-acting site in *nor*R.** *A. parasiticus* SU-1 was grown for 48 h in GMS liquid medium, and cell protein extracts were generated (see "Experimental Procedures"). 32 μg of extract were added to *nor*R per lane in the absence or presence of competitors (250-fold molar excess). *A*, competitors were: *nor*R, *nor*TATAm, *nor*R1, *nor*R2, and CRE1 (oligonucleotide) (see Fig. 3*C*). *B*, competitors were: *nor*R, *nor*R1, *nor*R2, *nor*R*, AflR (oligonucleotide), TATA (oligonucleotide), and *ver*AflR (oligonucleotide). *Arrows* designate DNA-protein complexes formed (G4 and G5) under each set of culture conditions. "*norR only*" and "*norR* only*" *lanes* contain a probe and no protein extract.

genome of *A. parasiticus* NR-1 was confirmed by PCR and Southern hybridization analysis; this location is important for correct transcriptional regulation of *nor-1* (12). To measure *nor-1* promoter activity (GUS activity), *A. parasiticus* reporter strains were grown on GMS agar medium for 72 h. Transformants carrying the m2 mutation in CRE1 did not produce statistically significant differences in *nor-1* promoter activity at 72 h of growth on GMS agar medium (no exogenous cAMP added) as compared with the wild type CRE1 control construct. However, the m2 mutation in CRE1 resulted in only a 1.7-fold induction of *nor-1* promoter activity by addition of exogenous cAMP as compared with a 5-fold induction in the control (Fig. 8).

AflR1 is located nearby to CRE1 in the *nor-1* promoter; this *cis*-acting site has been shown to be necessary for *nor-1* transcriptional activation during growth on YES (aflatoxin-inducing) agar and GMS liquid media (30). However, recombinant AflR-MBP failed to bind *nor*R in EMSA.[1] To determine whether AflR1 plays a role in the cAMP-mediated up-regulation of *nor-1* transcription, a reporter strain containing *nor-1*-GUS with mutations in AflR1 (**AGTTTAAACAG**, AflR1m) was used. The mutations, indicated in bold font and underlined, eliminated *nor-1* transcription in liquid GMS media as detected by reporter activity *in vivo* (33). After 72 h of growth on GMS agar medium, surprisingly a *nor-1* promoter containing mutations in AflR1 (AflR1m) had similar promoter activity without added cAMP as compared with *nor-1* promoter with wild type AflR1 (Fig. 8). However, the up-regulation of *nor-1* expression mediated by cAMP was only 2.1-fold in AflR1m as compared with a 5-fold induction in the control.

EMSA also was performed with a *nor*R probe and cell protein extracts prepared from *A. parasiticus* D8D3 grown on GMS agar medium for 48 h in the presence or absence of DcAMP (5 mM). DcAMP is an analog of cAMP that was previously demonstrated to cause similar effects on aflatoxin accumulation (14). DcAMP decreased the intensity of complex G1 (Fig. 9). In certain experiments, formation of complex G1 was drastically reduced (not shown). However, addition of cAMP or DcAMP directly into the EMSA reaction mixture containing a protein extract of *A. parasiticus* grown in the absence of cAMP did not affect the DNA-protein complex formation (not shown).

CREM-1 Does Not Bind norR—To gain additional information on the nature of the protein(s) binding *nor*R, antibodies against known transcription factors that interact with the consensus CRE were used. Antibodies against CREB-1, CREB-2, ATF2, ATF3, and CREM-1 did not produce supershifts or block DNA-protein complex formation between *nor*R and protein extracts prepared from TJYP1-22 or D8D3 grown on GMS agar (not shown). We performed EMSA with a 34–36-kDa polyhistidine-tagged fusion protein corresponding to a full-length CREM-1 protein of human origin and *nor*R as a probe. The choice of CREM-1 was based on similarity of its molecular mass (34–36 kDa) to the mass of p32 found to bind *nor*R in South-Western analysis (see below). CREM-1 (20 ng–1 μg) did not form DNA-protein complexes as detected by EMSA (not shown).

Detection of p32 via South-Western Analysis—To further characterize the protein(s) that bind *nor*R, South-Western blot analysis was performed. *A. parasiticus* protein extracts used in EMSA were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Two proteins, designated p32 and p55 (molecular masses of 32 and 55 kDa, respectively) were detected after exposure to the labeled *nor*R probe (Fig. 10*A*). p32 was detected in fungal extracts prepared under aflatoxin-inducing and non-inducing conditions. However, p55 was not detectable in TJYP1-22/GMS or D8D3/PMS extracts. Binding of *nor*R to p32 but not p55 could be competed with an excess of unlabeled *nor*R (100- and 1000-fold). Unlabeled CRE1 (3000-fold) either did not compete or competed much less effectively (Fig. 10*B*).

p32 Associates with AflR in Vitro—To determine whether AflR interacts with p32, protein extracts used for EMSA were incubated with AflR antibodies produced in our laboratory. Immune complexes were absorbed on protein A-Sepharose beads, released in SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, and probed with ^{32}P-labeled *nor*R. The immunoprecipitate isolated from D8D3 GMS extracts (solid medium) contained a faint band corresponding to p32 based on its electrophoretic mobility in SDS-PAGE (Fig. 11*A*). Association of AflR with p32 was also supported by pu-

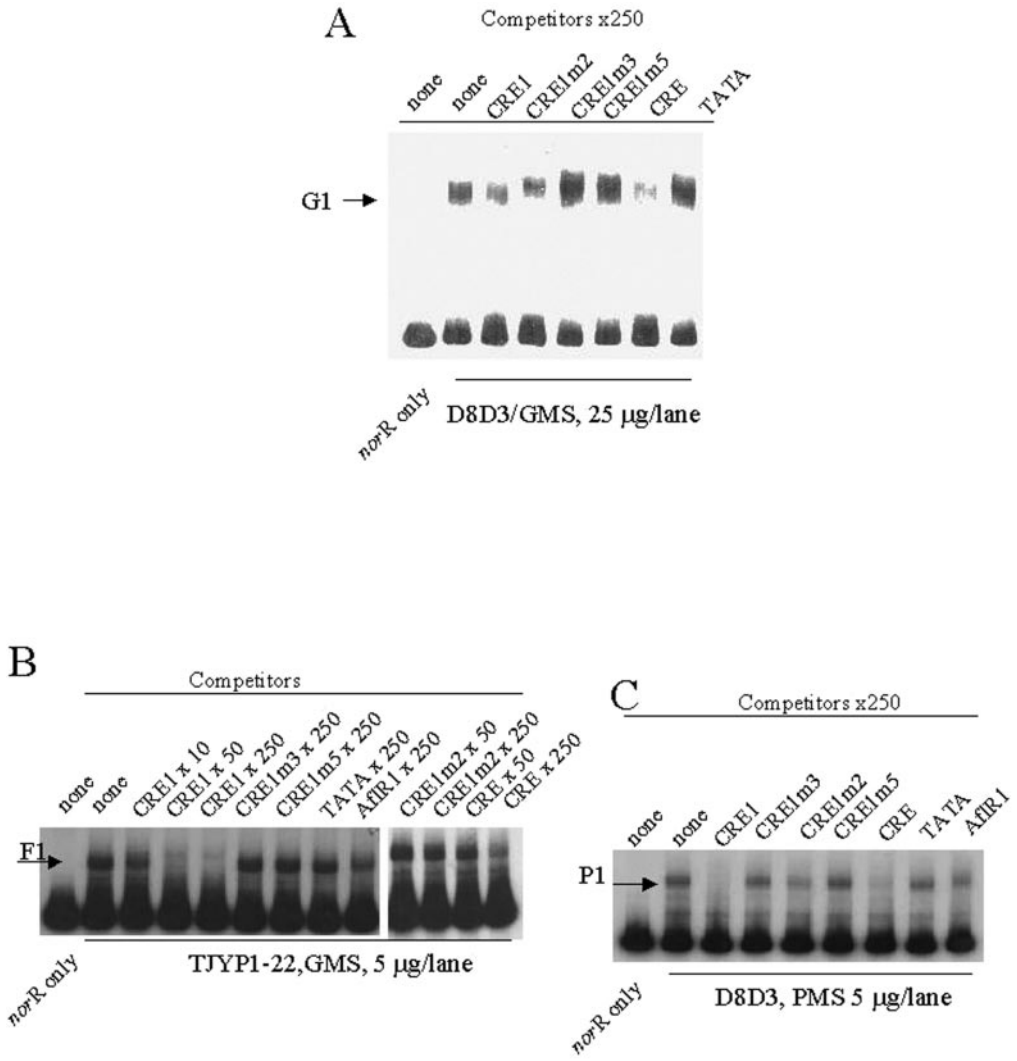

FIG. 6. **CRE1 mediates *nor*R-protein binding *in vitro*.** *A. parasiticus* strains D8D3 (*A* and *C*) and TJYP1-22 (*B*) were grown for 48 h on GMS (*A* and *B*) or PMS (*C*) solid medium, and cell extracts were generated (see "Experimental Procedures"). Competition EMSA was conducted using 25 (*A*) or 5 μg (*B* and *C*) of cell protein extract using a *nor*R probe in the absence (*none*) or presence of either a 250-fold molar excess of competitors (*A* and *C*) or increasing quantities of competitor (*B*; 10-, 50-, or 250-fold molar excess). Sequences for oligonucleotide competitors (CRE1, CRE1m2, CRE1m3, CRE1m5, CRE, TATA, and AflR1) are shown in Fig. 3*D*. *Arrows* designate DNA-protein complexes formed (G1, P1, and F1) under each set of culture conditions. "*norR only*" *lane* contains a probe and no protein extract.

rification of p32 from fungal cell extracts (SU-1 YES) by AflR-MBP fusion followed by detection of p32 in South-Western analysis with ^{32}P-labeled *nor*R probe (Fig. 11*B*).

Based on these experiments, we hypothesized that *nor*R-protein complexes observed in EMSA may contain AflR. However, antibody against AflR did not supershift or block DNA-protein complex formation in EMSA (not shown).

Aflatoxin Gene Promoters Possess CRE-like Motifs—The promoter region of *A. parasiticus aflR* (560 bp upstream of ATG codon) and intergenic regions (between ATG codons) of *A. parasiticus norA/ver1* (837 bp), *A. parasiticus nor1/pksA* (1687 bp), *A. parasiticus omtA/ordA* (1320 bp), *A. parasiticus fas1/fas2* (701 bp), *A. nidulans stcJ/stcK* (494 bp), and *A. nidulans fasA/fasB* (1606 bp) were searched for CRE-like motifs containing the first four 5′ residues of the consensus CRE sequence (TGAC) (48). Analysis of the promoters of fatty-acid synthase genes from *A. parasiticus* and *A. nidulans* was of a particular interest because the *fas1* and *fas2* promoter regions had previously been shown not to possess an AflR binding motif (28). Rangan *et al.* (49) showed that in hepatocytes cAMP affects expression of fatty-acid synthase through an inverted CCAAT box in the gene promoter; therefore we analyzed whether a CCAAT box motif is also present in the aflatoxin gene promoters.

All promoter regions analyzed contained CRE-like motifs (Table II). The *aflR* promoter region contained five CRE-like motifs; two of these carried five of eight conserved residues (the first five 5′ nucleotides), and one carried six of eight conserved residues (two mismatched bases in the 3′ half). The *nor-1/pksA* intergenic region contained 15 CRE-like motifs; three of these carried five of eight conserved residues (the first five 5′ nucleotides), and four of these carried six of eight conserved residues (two mismatched bases in the 3′ half). Interestingly two of the three motifs with five of eight conserved residues were identical (**TGACG**GGT). There were four CRE-like sites in the *norA/ver-1* intergenic region; two of these carried five of eight conserved residues (the first five 5′ residues). Of nine CRE-like motifs found in the *omtA/ordA* intergenic region, five carried five of eight conserved residues (the first five 5′ nucleotides), four contained six of eight conserved residues, and one motif contained seven of eight conserved residues as compared with the consensus CRE.

Genes encoding α- and β-subunits of fatty-acid synthase present in *A. parasiticus* and *A. nidulans* are divergently transcribed. Only one of three CRE-like motifs located in the *fas1/fas2* intergenic region carried five of eight conserved residues (the first five 5′ nucleotides). *A. nidulans stcJ* and *stcK* are

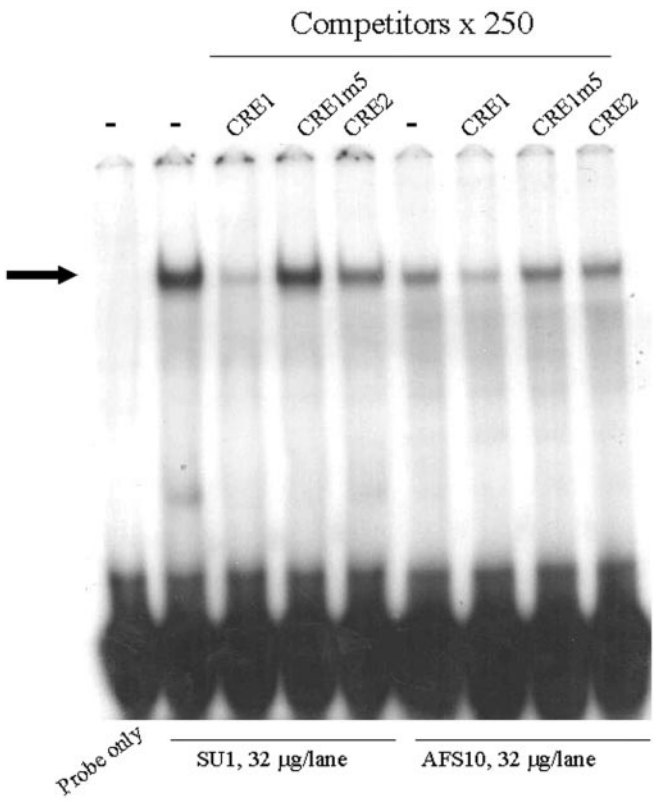

FIG. 7. **DNA-protein complexes in EMSA using a CRE1 oligonucleotide probe.** *A. parasiticus* SU-1 or AFS10 was grown for 48 h in GMS liquid shake culture, and cell protein extracts were generated (see "Experimental Procedures"). Competition EMSA was conducted with 32 μg of cell protein extract and a CRE1 oligonucleotide probe (see Fig. 3*D*) in the absence (−) or presence of oligonucleotide competitors. Sequences of oligonucleotide competitors (CRE1, CRE1m5, and CRE2) are shown in Fig. 3*D*. *Arrow* indicates a DNA-protein complex that consists of CRE1 oligonucleotide probe and CRE1-binding protein(s). "*Probe only*" *lane* contains a probe and no protein extract.

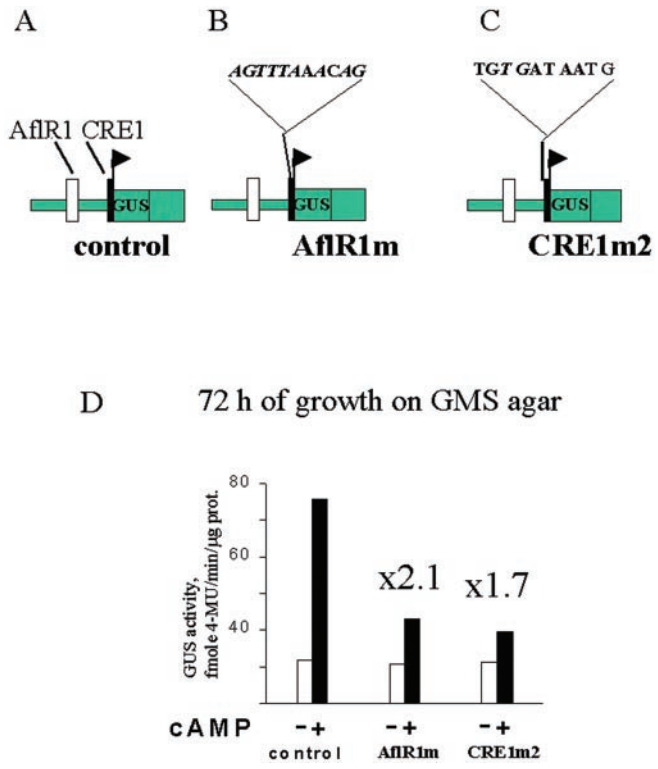

FIG. 8. **Mutations in AflR1 and CRE1 affect cAMP-mediated regulation of *nor-1* transcription.** *A. parasiticus* strains carrying *nor-1*-GUS reporter constructs with wild type promoter (*A*) or mutations in either AflR1 (*B*) or CRE1 (*C*) were grown for 72 h on GMS agar medium containing cAMP (5 mM). Nucleotide residues replaced in mutant sequences are designated in *italics* (*B* and *C*). *D*, GUS activity was assessed in the fungal colonies using a fluorescence-based assay (see "Experimental Procedures"). In total, two experiments were performed using independent transformants carrying wild type and mutant promoters; these experiments showed similar results. The result of one experiment is presented. *4-MU*, 4-methylumbelliferone.

homologous to *A. parasiticus fas2* and *fas1*. The *stcJ*/*stcK* intergenic region contains four CRE-like motifs; two have five of eight conserved residues (the first five 5′ nucleotides), and one carries six of eight conserved residues (two mismatches in the 3′ end). *A. nidulans fasA* and *fasB* are involved in primary fatty acid metabolism in contrast to the *fas1*, *fas2*, *stcJ*, and *stcK* fatty-acid synthases that carry out the initial steps in aflatoxin biosynthesis. Eight CRE-like motifs are present in the *fasA*/*fasB* intergenic region. None of them carry the characteristic five of eight conserved residues, and only one motif contains six of eight conserved residues (two mismatches in the 3′ half). A search for the CCAAT box in the same promoter set revealed that only *aflR* and *norA*/*ver1* do not possess the CCAAT box motif.

DISCUSSION

Data presented here and in previous studies suggest that aflatoxin accumulation and aflatoxin gene expression are strongly influenced by nutrients (carbon and nitrogen sources) and media composition (liquid shake or solid culture) (25–27). Several distinct levels of aflatoxin gene activation are observed depending on growth conditions. In liquid shake culture, growth in PMS or NMS media results in non-detectable or barely detectable levels of aflatoxin accumulation and gene expression. In liquid GMS containing a preferred carbon and nitrogen source, aflatoxin accumulation and aflatoxin gene expression are detected at intermediate levels. In a rich liquid growth medium (YES), aflatoxin accumulation and aflatoxin gene expression are detected at high levels (11). On solid media, GMS stimulates intermediate levels of aflatoxin accumulation and *ver-1* promoter activity but low levels of *nor-1* promoter activity; PMS agar stimulates very low levels of aflatoxin accumulation and *nor-1* and *ver-1* promoter activity. Furthermore addition of cAMP to GMS agar medium increases afla-

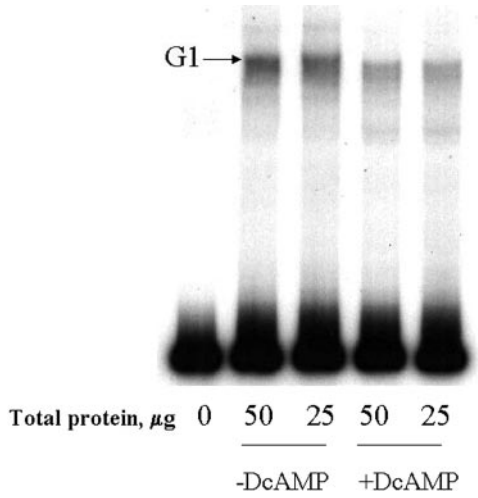

FIG. 9. **Effect of DcAMP on binding of *nor*R to proteins *in vitro*.** *A. parasiticus* D8D3 was grown for 48 h on GMS agar medium in the absence (−*DcAMP*) or presence (+*DcAMP*) of DcAMP (5 mM). Cell protein extracts were generated (see "Experimental Procedures"), and EMSA was conducted with these extracts (0, 25, or 50 μg) and a *nor*R probe. The *arrow* designates DNA-proteins complexes formed under each set of culture conditions.

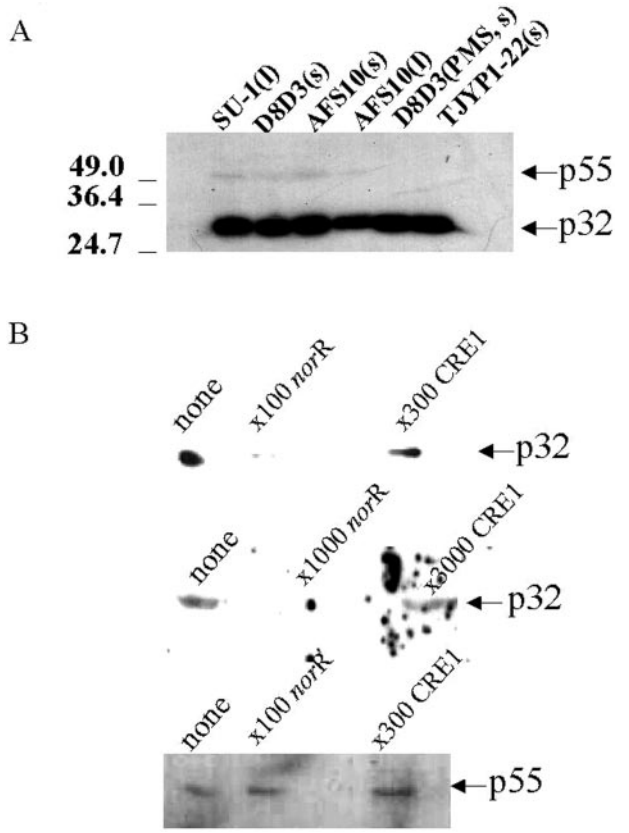

FIG. 10. **South-Western blot analysis of *nor*R- and CRE1-binding proteins in *A. parasiticus* protein extracts.** Cell protein extracts were generated and resolved by electrophoresis (12% SDS-PAGE). The proteins were transferred to nitrocellulose membrane and incubated with ^{32}P-labeled probe, and the resulting blot was exposed to film (see "Experimental Procedures"). *A*, *A. parasiticus* strains SU-1, D8D3, AFS10, and TJYP1-22 were grown in liquid shake culture (*l*) or on solid culture (*s*) in GMS or PMS for 48 h. The position of molecular mass markers resolved on the same gel is shown to the *left* of the photograph. *Arrows* (p55 and p32) designate the *nor*R-binding proteins. *B*, South-Western blot analysis using protein extract from *A. parasiticus* D8D3 grown on solid GMS for 48 h, ^{32}P-labeled *nor*R as a probe, and non-labeled *nor*R and CRE1 as competitors. The competitor and its quantity are shown *above* the lanes. "*none*," no competitor was added. *Arrows* (p55 and p32) designate the *nor*R-binding proteins.

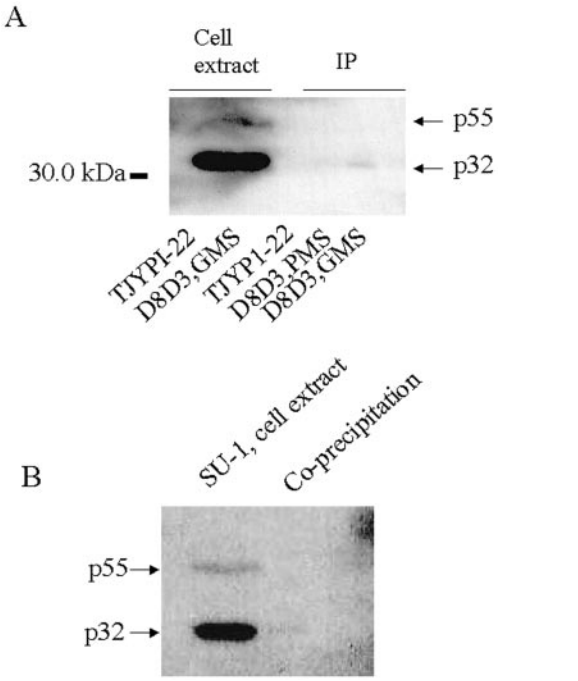

FIG. 11. **Interaction of AflR and p32 *in vitro*.** *A*, immunoprecipitation by anti-AflR antibody and South-Western blot analysis using a *nor*R probe. Precleared cell protein extracts (see "Experimental Procedures") from *A. parasiticus* strains D8D3 and TJYP1-22 grown on GMS or PMS agar were mixed with polyclonal antibodies to *A. parasiticus* AflR, and the resulting immune complexes were captured using 50 μl of preabsorbed protein A-Sepharose beads. Immune complexes were released in 30 μl of SDS-PAGE sample buffer, and 20 μl of this sample were resolved by electrophoresis (12% PAGE) and transferred to nitrocellulose (*IP*). Cell protein extracts (TJYP1-22, GMS agar; D8D3, GMS agar) without immunoprecipitation (*Cell extract*) were resolved on the same gel (90 μg/lane). The resulting blot was probed with radiolabeled *nor*R. The *arrow* designates the DNA-protein complexes formed under each set of experimental conditions. *B*, co-precipitation with AflR-MBP fusion followed by South-Western blot analysis using *nor*R. Precleared cell protein extracts from SU-1 (GMS, liquid) were mixed with amylose resin carrying an MBP-AflR fusion protein. The resin was washed, and bound protein complexes (*Co-precipitation*) were released in 40 μl of SDS sample buffer. The resulting samples were resolved by electrophoresis (12% SDS-PAGE), transferred to nitrocellulose, and probed with radiolabeled *nor*R as above. As a control, cell extracts without co-precipitation (*SU-1, cell extract*) were resolved by electrophoresis and probed in the same manner.

toxin accumulation (5–10-fold) and *nor-1* promoter activity (greater than 5-fold). In the current study, we demonstrate that interaction of CRE1bp, AflR/AflR1, and cAMP all play a role in the molecular switch that modulates *nor-1* expression.

EMSA identified a *cis*-acting site in *nor*R (170 bp) designated CRE1 (TGACATAA) that mediated specific CRE1-protein complex formation in liquid and solid growth media under toxin-inducing (GMS) and non-inducing (PMS) conditions. A short oligonucleotide (27 bp) containing CRE1 also effectively and specifically formed a DNA-protein complex under EMSA conditions. This protein or protein complex was designated CRE1bp.

Utilizing South-Western blot analysis, an ~32-kDa protein (p32) was found to specifically bind *nor*R, and a protein of the same size interacted with AflR *in vitro*. Preliminary data also suggest that p32 binds to CRE1, although the signal is much less intense than the signal observed using a labeled *nor*R probe. These data were consistent with the observation that *nor*R was an effective competitor of the p32-*nor*R complex in South-Western blots, whereas the CRE1 oligonucleotide was a much less effective competitor. One possible explanation for the differences between EMSA and South-Western blot data could be related to the different conditions under which DNA-protein complexes are formed. In EMSA, DNA-protein complexes form between molecules freely distributed in solution. In contrast, during South-Western analysis, to form a complex, DNA binds a protein that first is separated by SDS-PAGE, then immobilized on a membrane, and finally renatured. These manipulations may alter protein conformation, which affects DNA binding. Although we propose that p32 is one component of CRE1bp, it is necessary to confirm this in future studies.

GMS liquid medium stimulated a shift from non-detectable to intermediate levels of aflatoxin synthesis, an increase to intermediate levels of *nor-1* and *ver-1* promoter activity, and the formation of a more intense and slower mobility specific complex (G5) as compared with PMS (P2). These data suggest that a new or modified protein takes part in complex G5 formation that is strongly associated with up-regulation of *nor-1* promoter activity in liquid media. Complex G5 formation (this study) and *nor-1* expression (33) are AflR-dependent, and AflR transcript accumulation is up-regulated in GMS liquid media. These data provide a strong link between AflR activity and glucose stimulation of *nor-1* and *ver-1* expression and suggest that regulation of these two promoters is similar in a liquid GMS medium. We propose that p32 acts as a positive regulator

TABLE II
CRE-like and CCAAT sites in aflatoxin gene promoters

Intergenic regions (between ATGs) of *A. parasiticus* aflatoxin biosynthetic genes, *A. nidulans* fatty-acid synthases for primary and secondary metabolism, and 560 bp region upstream ATG of *A. parasiticus aflR* were searched for the presence of TGAC (first four conservative bases of consensus cAMP-response element) and for CCAAT box using Gene Runner (Hastings Software, Inc.). The number indicates quantities of sites detected. +, forward direction; −, reverse direction.

	CRE-like sites				CCAAT
	All CRE-like sites containing TGAC	Five first conserved as in CRE	One mismatch base in 3′ half	Two mismatch bases in 3′ half	
aflR 560 bp upstream ATG	5	2	None	1	None
nor1/*pksA* (1687 bp)	15	3	None	4	2 (−)
norA/*ver1* (837 bp)	4	2	None	2	None
ordA/*vbs* (511 bp)	None	None	None	None	1 (+)
omtA/*ordA* (1320 bp)	9	5	1	4	1 (+), 3 (−)
fas1/*fas2* (701 bp)	3	1	None	None	1 (+)
fasA/*fasB A. nidulans* (1606 bp)	8	None	None	1	1 (−)
stcJ/*stcK A. nidulans* (494 bp)	4	2	None	1	2 (+)

of *nor-1* expression in a GMS liquid medium by assisting in AflR binding to AflR1.

On GMS agar, addition of exogenous cAMP decreased or completely eliminated complex G1 formation (depending on the experiment) and strongly stimulated aflatoxin and *nor-1* and *ver-1* promoter activity. However, *nor-1* promoter activity on GMS agar is up to 20-fold lower than *ver-1* promoter activity suggesting that the two genes are regulated differently on GMS agar. We propose that p32 can also act as a negative regulator of *nor-1* expression on solid media. p32 limits *nor-1* expression on solid GMS; its removal from CRE1 is stimulated by cAMP resulting in a shift from low to intermediate levels of *nor-1* promoter activity. In preliminary supershift EMSA experiments using anti-phosphoserine and anti-phosphothreonine antibodies, we demonstrated that G1 complex formation is associated with phosphorylation of DNA-binding proteins. Similarly phosphatase treatment of cell protein extracts blocked G1 complex formation in ~50% of the experiments conducted. These data are consistent with a scenario in which exogenous cAMP down-regulates cAMP-dependent protein kinase activity (see below) resulting in a decrease in phosphorylation of DNA-binding protein (likely p32) and disappearance of the complex.

We demonstrated a functional role for CRE1 and AflR1 in the cAMP-mediated up-regulation of *nor-1* on GMS agar. Interestingly neither CRE1 nor AflR1 was required for the low level *nor-1* expression observed on GMS agar (without cAMP). A second CRE-like motif (TGACATGA, designated CRE2) identified in *nor*R differed from the consensus CRE at two residues (underlined). CRE2 did not compete effectively for CRE1-protein complex formation suggesting that CRE1 is the only functional *cis*-acting site in *nor*R; these data also suggest that the first four 5′ residues plus an as yet undetermined number of the four 3′ residues in CRE1 are important for protein binding. It was demonstrated previously that the four 3′ residues in the consensus CRE are less conserved than the 5′ residues (48).

p32 was detected in cell protein extracts from several different strains prepared under aflatoxin-inducing and non-inducing conditions. Interestingly almost all CRE-binding transcription factors, with the exception of inducible cAMP early repressor, are housekeeping genes and constitutively expressed (47). These data are consistent with the observed formation of specific complexes in both PMS (P1 and P2) and GMS (G1 and G5) media. The reason that a specific complex is not detected in TJYP1-22 cell protein extracts is not clear. These data are also consistent with the observation that complex P2 and G2 formation is AflR-independent. p32 is apparently expressed even in the AflR knock-out strain (AFS10) so it is available to form specific complexes. However, the generation of lower mobility complexes G1 (solid medium) and G5 (liquid medium) is AflR-dependent, again supporting the idea that an additional or modified protein mediates the shift in mobility.

We conclude that p32 is not AflR or AflJ, each of which has a molecular mass of ~48 kDa. In support of this conclusion, antibody to AflR developed in our laboratory detected an ~48-kDa protein in D8D3 cell protein extracts (YES liquid medium for 68 h), a recombinant AflR-MBP fusion (not shown), a non-functional AflR expressed from the second copy of *aflR* in AFS10 (not shown), but not a 32-kDa protein. In addition, Chang *et al.* (50) recently demonstrated that a His-tagged recombinant AflR containing the DNA-binding domain does not interact with an oligonucleotide containing a consensus CRE.

Consensus CREs mediate transcriptional regulation of a number of eukaryotic genes under the influence of internal cAMP (47). The regulation of gene transcription by cAMP, however, is indirect. The regulatory subunit of a specific protein kinase (cAMP-dependent protein kinase) binds cAMP and releases the active catalytic subunit. After translocation into the nucleus, cAMP-dependent protein kinase phosphorylates and thus activates a number of transcription factors that dimerize and bind to cAMP-response elements in the promoters of many genes and influence their transcription. The levels of intracellular cAMP are dependent on the balance of activity of adenylate cyclase and phosphodiesterase, which are modulated by numerous extra- and intracellular stimuli. In *A. parasiticus*, for example, constitutive activation of a heterotrimeric G protein α subunit resulted in a physiological increase in intracellular cAMP, a significant increase in basal cAMP-dependent protein kinase activity (14), and the suppression of aflatoxin biosynthesis and aflatoxin gene expression. In keeping with this signaling scheme, we provide direct evidence that p32 binds *nor*R and that formation of the complex is responsive to cAMP on solid media.

Sequence analysis of several intergenic regions in the aflatoxin and sterigmatocystin gene clusters (*aflR*, *nor1*/*pksA*, *norA*/*ver1*, *omtA*/*ordA*, *ordA*/*vbs*, *fas1*/*fas2*, *stcK*/*stcJ*, and *fasA*/*fasB*) revealed that all except *ordA*/*vbs* contain CRE-like motifs with five or more conserved 5′ residues. Interestingly the CRE1 motif shows 75% identity with the consensus CRE (four of four conserved 5′ residues and two of four conserved 3′ residues. The fact that a consensus CRE effectively competed for CRE1-protein complex formation suggests that at least one of the conserved 3′ residues contributes to DNA binding. None of the CRE-like motifs in the aflatoxin and sterigmatocystin clusters were identical to CRE1, CRE2, or each other, which may indicate that a high degree of variability can exist in a functional CRE-like motif.

Rangan *et al.* (49) reported an inverted CCAAT motif in the

fatty-acid synthase promoter of hepatocytes that mediates transcriptional regulation of the gene by cAMP. In *A. nidulans*, the sequence CCAAT was found to be necessary for the DNase I hypersensitivity in the 5′ region of the *amdS* (51). The CCAAT box is found in several *A. nidulans* gene promoters involved in penicillin biosynthesis (13, 17, 52). The search for analogous motifs in intergenic regions in the aflatoxin gene cluster revealed that all but *aflR* and *norA*/*ver1* possess this motif. The presence of CRE-like motifs and CCAAT box motifs in intergenic regions of *fas1*/*fas2*, *stcK*/*stcJ*, and *fasA*/*fasB* prompts the intriguing hypothesis that cAMP coordinates a growth phase-dependent shift in transcription of fatty-acid synthases involved in primary and secondary metabolism. One focus of future work is to determine the functionality of the CRE and CCAAT sites.

In summary, this study describes a novel *cis*-acting element, CRE1, in the *nor-1* promoter. CRE1-protein complex formation is involved in modulation of *nor-1* promoter activity in liquid and solid GMS media in response to environmental cues related to nutrient levels and media composition. Nucleotide sequence analysis provided support for the hypothesis that cAMP mediates transcription of several other aflatoxin genes in the cluster.

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