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Cloning and Characterization of the *trpC* Gene from an Aflatoxigenic Strain of *Aspergillus parasiticus*†

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The *trpC* gene in the tryptophan biosynthetic pathway was isolated from an aflatoxigenic *Aspergillus parasiticus* by complementation of an *Escherichia coli trpC* mutant lacking phosphoribosylanthranilate isomerase (PRAI) activity. The cloned gene complemented an *E. coli trpC* mutant deficient in indoleglycerol-phosphate synthase (IGPS) activity as well as an *Aspergillus nidulans* mutant strain that was defective in all three enzymatic activities of the *trpC* gene (glutamine amidotransferase, IGPS, and PRAI), thus indicating the presence of a complete and functional *trpC* gene. The location and organization of the *A. parasiticus trpC* gene on the cloned DNA fragment were determined by deletion mapping and by hybridization to heterologous DNA probes that were prepared from cloned *trpC* genes of *A. nidulans* and *Aspergillus niger*. These experiments suggested that the *A. parasiticus trpC* gene encoded a trifunctional polypeptide with a functional domain structure organized identically to those of analogous genes from other filamentous fungi. The *A. parasiticus trpC* gene was expressed constitutively regardless of the nutritional status of the culture medium. This gene should be useful as a selectable marker in developing a DNA-mediated transformation system to analyze the aflatoxin biosynthetic pathway of *A. parasiticus*.

Aspergillus parasiticus is an imperfect fungus which produces aflatoxins. The ubiquitous existence of this mold and the potent toxicity and carcinogenicity of aflatoxins represent a serious threat to food safety and public health. To date, protection against contamination of the food supply with these toxins is limited to preliminary detection, followed by elimination of contaminated items. By investigating expression and regulation of genes involved in alfatoxin biosynthesis it should be possible to design strategies to efficiently control formation of these toxins. It is thus desirable to develop selectable markers for transformation of *A. parasiticus* to provide a mechanism to clone the genes involved in the aflatoxin biosynthetic pathway in this organism. One recently described approach (30) involved the use of the *pyrG* (orotidine 5′-phosphate decarboxylase) gene in the pyrimidine biosynthetic pathway for transformation of *Aspergillus flavus*. An alternative approach is the use of the *trpC* gene, which has been used previously as a selectable marker in several molecular studies.

There is considerable conservation of domain structure of the *trpC* genes throughout the procaryotic and eucaryotic worlds (9, 14). The five biosynthetic steps from chorismate to tryptophan, catalyzed by seven enzymatic activities, appear to be the same in bacteria, yeasts, and filamentous fungi (14). The seven enzymatic functions of *Escherichia coli* are encoded by five genes (*trpA* through *trpE*) in a single operon and are designated domains A through G (14). In the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*, four unlinked genes encode four polypeptides, of which two are monofunctional, one is bifunctional, and one is trifunctional. The activities of the fungal trifunctional gene correspond to the domains G, C, and F of *E. coli*, which encode glutamine amidotransferase (GAT), indoleglycerol-phosphate synthase (IGPS), and phosphoribosylanthranilate isomerase (PRAI), respectively. In *E. coli*, the *trpC* gene product is a bifunctional polypeptide with IGPS and PRAI activities. The genes that encode PRAI activity in different filamentous fungi, including *N. crassa* (26), *A. nidulans* (31), *Aspergillus niger* (16), *Cochliobolus heterostrophus* (28), *Schizophyllum commune* (22), *Penicillium chrysogenum* (25), and *Phycomyces blakesleeanus* (24), have been cloned by complementation of the PRAI deficiency in *E. coli trpC* mutants. We therefore hypothesized that the *A. parasiticus trpC* gene could be selected in suitable *E. coli* mutant strains. In this article we report the isolation of the *trpC* gene from *A. parasiticus* by complementation of the *E. coli trpC* mutation.

MATERIALS AND METHODS

Cultures. The *E. coli* K-12 strain DH5 (F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* $r_k^-m_k^+$) was used for plasmid library construction. DH5 and *E. coli* HB101 [*hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* Sm^r *xyl-5 mtl-1 supE44*] were used to propagate plasmids. *E. coli* JA209 (*trpA36 argH metE xyl recA56* Str^r *glyH*) (8), JA300 (*trpC1117 thr leuB6 thyA thi hsdR hsdM str*) (27), and MC1066 (*trpC9830 Δlac(IPOZYA)X74 galU galK strA hsdR leuB6 pyrF74*::Tn*5* (Km^r)] (4) as well as mutants of strain W3110 (26), each carrying a different mutation in the *trp* operon (Table 1), were used for complementation assays. *E. coli* NM539 (Promega Biotec, Madison, Wis.) was the host for amplification of the plasmid library. The wild-type aflatoxigenic strain *A. parasiticus* NRRL 5862 (SU-1) (1) was the source of DNA for genomic library construction. *A. nidulans* mutant strain FGSC 237 (*pabaA1 yA2 trpC801*, deficient in all trifunctional *trpC* activities) (15) was the host for studies of heterologous gene expression.

Vectors. Plasmids pHY101 and pHY201 (32), both containing a complete trifunctional *A. nidulans trpC* gene, as well as plasmid pAB2-1 (16), containing an intact *A. niger trpC* gene, were used as probes in heterologous hybridization study. Plasmid pRK9 (26) was used for the construction of the *A. parasiticus* genomic DNA library. pRK9 is a pBR322

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TABLE 1. Complementation of *E. coli* tryptophan auxotrophs by plasmid pLH23

Recipient strain	Deficient domain(s)[a]	Growth on minimal medium[b]	
		With TRP	Without TRP
MC1066 *trpC9830*	F	+	+
JA300 *trpC1117*	F	+	+
W3110 *trpC9830*	F	+	+
W3110 *trpC9941*	C	+	+
W3110 Δ*trpC10-16*	C, F	+	+
W3110 Δ*trpE5*	E	+	−
W3110 Δ*trpLD102*	E, D	+	−
W3110 *trpB9578*	B	+	−
JA209 *trpA36*	A	+	−

[a] Enzymatic activity encoded by each domain: A, subunit A of tryptophan synthase, which catalyzes the conversion of indole-3-glycerolphosphate to indole; B, subunit B of tryptophan synthase, which catalyzes the conversion of indole to tryptophan; C, IGPS; D, phosphoribosyl transferase; E, anthranilate synthase (with ammonia as the amino group donor); F, PRAI; G, GAT, interacts with the E domain in the glutamine-dependent anthranilate synthase.

[b] Cells of each strain were transformed with pLH23, plated on M9 minimal agar, and incubated overnight at 37°C. Colonies which appeared were transferred to M9 minimal medium with or without tryptophan (TRP).

derivative with a unique *Bam*HI site created by replacing the 380-base-pair (bp) *Eco*RI-*Bam*HI fragment of pBR322 with a 96-bp *Serratia marcescens trp* operon promoter.

General procedures. Plasmid DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, and hybridization analyses were performed by standard procedures (18). DNA probes were radiolabeled with ^{32}P (New England Nuclear Corp., Wilmington, Del.) by the random primer technique (11) to a specific activity of greater than 10^8 cpm/μg of DNA. Filters with DNAs or RNAs were hybridized in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–5× Denhardt solution–40% formamide–0.1% sodium dodecyl sulfate–5 mM EDTA–100 μg of denatured salmon sperm DNA per ml at 65°C for higher stringency and at 37 or 42°C for lower stringency. Filters were washed twice in 0.1% sodium dodecyl sulfate–2× SSC at room temperature for 40 min and then, for a final wash, in 0.1% sodium dodecyl sulfate–0.1% SSC at 65°C for 1 h. Bacteriophage DNA was purified by the procedure of Carlock (3). Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England Nuclear Corp., or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used according to the instructions of the suppliers. Nitrocellulose and Nytran nylon membranes were purchased from Schleicher & Schuell, Inc. (Keene, N.H.); GeneScreen Plus membrane was supplied by New England Nuclear Corp.

Isolation and manipulation of genomic DNA of *A. parasiticus*. High-molecular-weight genomic DNA of *A. parasiticus* was prepared by a procedure modified from that of Cihlar and Sypherd (6). Mycelia of *A. parasiticus* NRRL 5862 were harvested from YES medium (2% yeast extract and 6% sucrose), lyophilized, blended into a powder with a Waring blender, and then ground in a mortar and pestle with glass powder. The resulting mycelial powder was suspended in TSE buffer (100 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 100 mM EDTA) and incubated with 1% sodium dodecyl sulfate at 37°C for 1 h with gentle shaking. The suspension was centrifuged (5,000 × *g*, 10 min) to remove cell debris. The supernatant was extracted twice with TSE-saturated phenol. The nucleic acids were precipitated from the aqueous phase with ethanol, dried under vacuum, and suspended in TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). Contaminating RNA and proteins were removed by treatment with RNase A and proteinase K (37°C, 1 h each). The enzymes were removed by phenol extraction, and the genomic DNA was recovered by ethanol precipitation. When this protocol was used, yields were normally >1 mg of *A. parasiticus* DNA from 10 g of wet mycelia. The majority of the genomic DNA migrated more slowly than phage lambda DNA in agarose gel electrophoresis (larger than 50 kilobases [kb]) and was readily digested with restriction enzymes.

Preparation of RNA from *A. parasiticus*. Total RNA was isolated from *A. parasiticus* NRRL 5862 by the hot-phenol method of Maramatsu (19).

Poly(A)$^+$ mRNAs were separated from total RNA by oligo(dT) cellulose (Boehringer Mannheim) affinity column chromatography, done by using standard procedures (18).

Construction of genomic DNA libraries. For construction of the phage lambda library, high-molecular-weight genomic DNA from *A. parasiticus* was partially digested with restriction endonuclease *Sau*3A1. DNA restriction fragments (15 to 20 kb) were isolated by fractionation on 5 to 25% NaCl gradients and ligated to lambda EMBL3 *Bam*HI arms (Promega Biotec). Recombinant phage DNAs were packaged according to the protocol of the supplier (Promega Biotec). Libraries were amplified by the procedure of Maniatis et al. (18). For construction of the plasmid library, purified genomic DNA fragments with an average size of 5 to 10 kb were generated by partial digestion with *Sau*3A1 and then ligated with *Bam*HI-digested, phosphatase-treated plasmid pRK9 (26). *A. parasiticus* DNA fragments were inserted into the single *Bam*HI site in the *S. marcescens trp* leader region in order to maximize expression of the gene cloned downstream from the promoter. The recombinant plasmids were used to transform competent cells of *E. coli* DH5 (Bethesda Research Laboratories) to ampicillin resistance. The primary plasmid library was amplified as described by Vogeli et al. (29).

Lytic complementation of *E. coli trpC* mutants. Lytic complementation was performed by the procedure of Davis et al. (10). *E. coli trpC* mutants MC1066 and W3110 *trpC9830*, defective in PRAI activity, were infected with 10^7 recombinant phages from the *A. parasiticus* genomic DNA library (multiplicity of infection, <0.001) and plated on M9 medium lacking tryptophan. Plaques appeared after overnight incubation. Phage clones were further purified by reinfecting the susceptible host under selective conditions to isolate single plaques.

Transformation. *E. coli* strains were transformed by the hexamine cobalt chloride method (13). *A. nidulans* was transformed by the procedure of Oakley et al. (23).

RESULTS

Isolation of *A. parasiticus trpC* gene from recombinant phage library. The primary phage library contained 2.1×10^5 recombinant phage clones, 99% of which contained *A. parasiticus* DNA inserts averaging 15 kb. Assuming that the genomic size of *A. parasiticus* is comparable to that of the *A. nidulans* and *N. crassa* genomes, the probability of finding any given sequence in the library was estimated to be greater than 99.9% (7). The primary library was amplified in *E. coli* NM539, which supports only the growth of recombinant phage particles. The amplified library consisted of 6.7×10^9 PFU in total.

E. coli MC1066 and W3110 carrying the *trpC9830* missense mutation were used as recipients for lytic complemen-

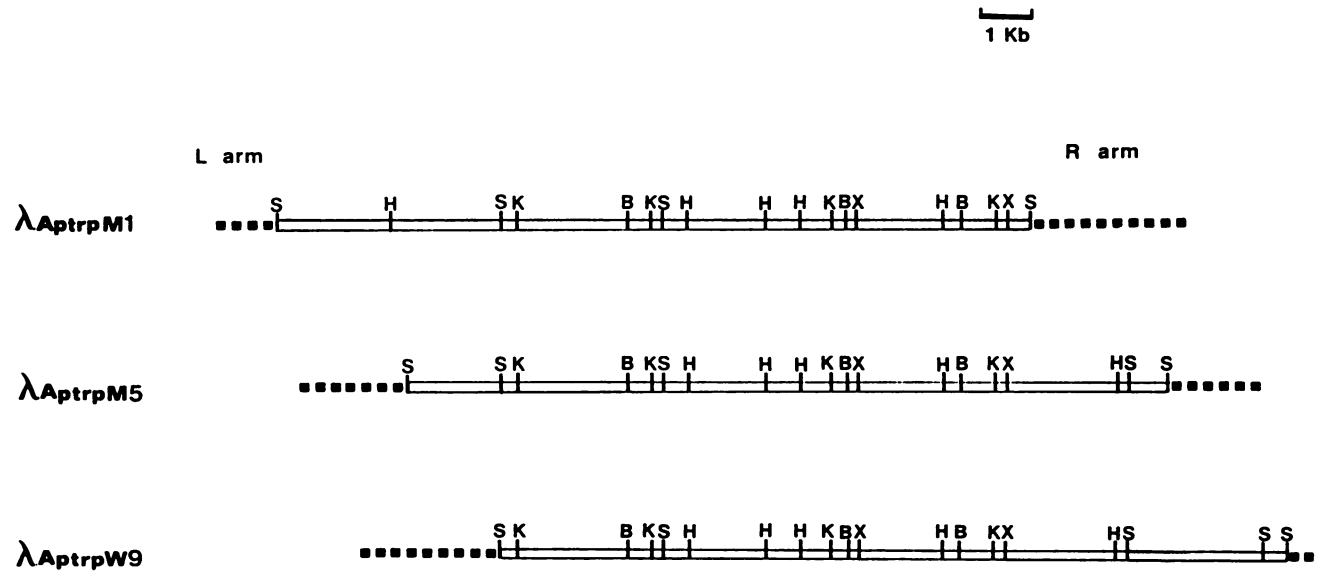

FIG. 1. Restriction endonuclease maps of three *A. parasiticus* DNA inserts (open bars) in lambda EMBL3 which complement the *E. coli trpC9830* mutation. Restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*1; S, *Sal*I; X, *Xba*I.

tation and yielded 50 and 340 plaques, respectively, after infection followed by overnight incubation. Twenty phage isolates were purified and replated on both host strains under selective conditions. All 20 phage isolates were able to complement the *E. coli* mutants at a high frequency. Restriction analysis of DNA purified from 10 phage clones revealed three different types of restriction patterns. Three phages, each representing a different restriction pattern, were selected for further analysis. Restriction endonuclease analysis (Fig. 1) of the *A. parasiticus* DNA inserts from these three clones (designated lambda AptrpM1, lambda AptrpM5 and lambda AptrpW9) showed that the inserts were approximately 15 kb and shared a common 10.5-kb region which presumably contained the complementing activity for *trpC9830* mutation. The DNA restriction maps were contiguous, indicating that no identifiable DNA rearrangements occurred during lytic complementation.

Localization and organization of the cloned *A. parasiticus trpC* gene. To identify which portion of the DNA insert contained the *A. parasiticus trpC* gene, DNAs from the three phage clones were digested with restriction enzymes, fractionated by electrophoresis on 1% agarose gels, blotted onto Nytran nylon membranes, and hybridized under low-stringency conditions with DNA probes prepared from the *A. nidulans trpC* gene. The hybridization patterns (Fig. 2, with lambda AptrpW9 as an example) were consistent with the restriction maps of the three phage clones shown in Fig. 1. Both amino- and carboxyl-terminus probes hybridized strongly, suggesting that extensive sequence similarities existed between *trpC* genes of *A. nidulans* and *A. parasiticus*. A similar experiment (data not shown) using DNA probes prepared from the cloned *trpC* gene of *A. niger* (16) was consistent with these findings. Southern hybridization with N- and C-terminus probes also suggested that the *trpC* gene was transcribed in the direction indicated by the arrow in Fig. 2B. The *A. parasiticus trpC* gene, therefore, has the same structural organization as those of *A. nidulans*, *A. niger*, and other filamentous fungi. This result is consistent with previous evidence that enzyme activities encoded by the *trpC* gene are highly conserved in filamentous fungi (9, 14).

Complementation of an *A. nidulans trpC* mutant by the cloned *A. parasiticus trpC* gene. A 3.4-kb *Hin*dIII fragment thought to contain the trifunctional *A. parasiticus trpC* gene was subcloned in opposite orientations into the unique *Hin*dIII site of *E. coli* plasmid pUC19 and *Saccharomyces cerevisiae* vector YIp5 (2). The resulting recombinant plasmids, designated pJH34 and pAP34 (derivatives of pUC19) and YAP43H and YAPH34 (derivatives of YIp5), were used to transform *A. nidulans* FGSC 237, a mutant deficient in all three *trpC* activities. (A detailed restriction map of plasmid pJH34 is shown in Fig. 3.) Each of the plasmids was able to transform this *trpC* mutant to tryptophan prototrophy, indicating that all three *trp* functions were present on the 3.4-kb fragment and were expressed in *A. nidulans* (Table 2). The successful transformation with both types of plasmids containing identical *A. parasiticus* DNA inserts in different orientations suggested that the complementing activity was encoded by the DNA insert, not by the vector sequences.

DNA was purified from five *A. nidulans* FGSC 237 Trp^+ isolates that had been transformed with pJH34 and subjected to Southern hybridization analyses using ^{32}P-labeled pUC19, pJH34, or a 3.4-kb *Hin*dIII restriction fragment excised from pJH34 (Fig. 3) as probe. In all cases, the pUC19 probe hybridized to undigested *A. nidulans* transformant DNA at the position of high-molecular-weight chromosomal DNA (Fig. 4A), indicating that the vector sequences had integrated into the host genome. Hybridization to lower-molecular-weight fragments did not occur, suggesting that unintegrated copies of pJH34 were not detectable in the transformants. However, the data did not rule out the possibility of hybridization of the probe to concatemers of unintegrated transforming plasmids. There was no homology between pUC19 and *A. nidulans* DNA that had not been transformed with pJH34 (Fig. 4A, lane 1). The 3.4-kb *Hin*dIII probe thought to carry the complete *A. parasiticus trpC* gene hybridized with several (including a 3.4-kb) chromosomal-DNA *Hin*dIII fragments from the *A. nidulans* transformants (Fig. 4B), indicating the presence of the 3.4-kb *Hin*dIII fragment of pJH34, i.e., *A. parasiticus trpC* sequences. The *A. parasiticus trpC* gene also hybridized with a 3.4-kb *Hin*dIII fragment of *A. nidulans* FGSC 237. The

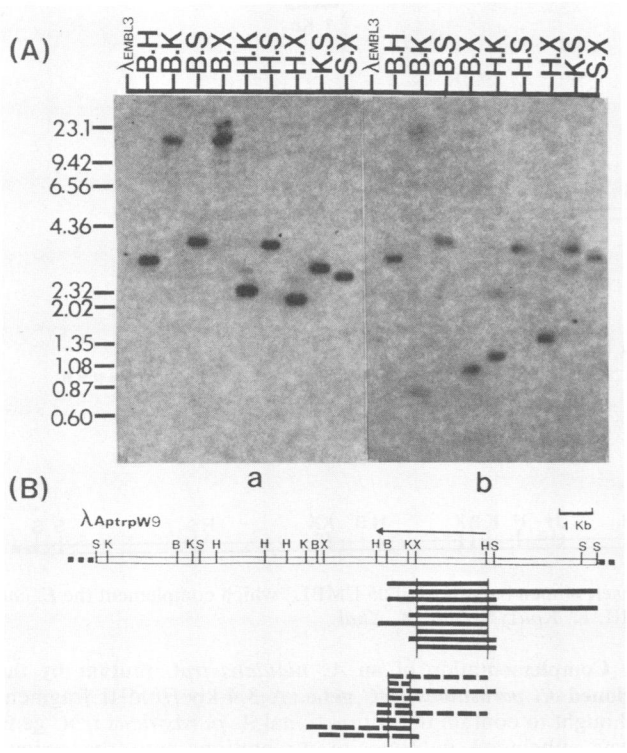

FIG. 2. Southern hybridization of lambda clone AptrpW9 with *A. nidulans trpC* probes. (A) DNA was digested with pairs of restriction enzymes as indicated, blotted to a nylon membrane, and hybridized under conditions of low stringency with the radiolabeled amino-terminal portion (gel a) or carboxyl-terminal portion (gel b) of the *A. nidulans trpC* gene. A 2.5-kb *Eco*RI fragment purified from plasmid pHY201 was used as an N-terminus probe; a 1.8-kb *Eco*RI-*Xho*I fragment purified from plasmid pHY101 was used as a C-terminus probe. Both pHY201 and pHY101 contain complete trifunctional *A. nidulans trpC* genes. Molecular sizes in kilobases are indicated. (B) Diagram of the location and orientation of the *A. parasiticus trpC* gene as determined from hybridization data. Solid bars indicate regions which hybridize with the N-terminus probe; dashed bars indicate regions which hybridize with the C-terminus probe. The minimal overlapping regions are delimited with vertical lines. The arrow shows the predicted direction of transcription of the *A. parasiticus trpC* gene. Restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I.

identity of this 3.4-kb *Hin*dIII fragment in untransformed cells is unclear. Presumably it could be generated from *Hin*dIII sites within the *A. nidulans trpC* gene and its flanking region. When the pUC19 probe was hybridized to the *A. nidulans* DNA cut with *Hin*dIII, one or more chromosomal-DNA fragments in the transformants were detected (Fig. 4C), demonstrating again the presence of vector sequences of pJH34 in the transformants. No hybridization was seen with genomic DNA of the untransformed host. The presence of the *A. parasiticus* 3.4-kb *Hin*dIII fragment concurrent with the Trp^+ phenotype in the transformants suggested that the *A. parasiticus trpC* gene was contained on the 3.4-kb *Hin*dIII fragment and was functionally expressed in *A. nidulans*.

To prove that the cloned DNA insert in pJH34 was indeed derived from the *A. parasiticus* genome, *A. parasiticus* DNA was digested with restriction enzyme *Hin*dIII, and the gel-fractionated digests were probed with ^{32}P-labeled pUC19 or the 3.4-kb *Hin*dIII insert that was excised from pJH34 (data not shown). The 3.4-kb *Hin*dIII DNA probe hybridized to a 3.4-kb fragment from *A. parasiticus* genomic DNA. No hybridization to genomic DNA of either *E. coli trpC9830* or lambda EMBL3 was observed, indicating the absence of a DNA sequence in *E. coli* or phage lambda that would be able to complement the *E. coli trpC9830* mutation. The *A. parasiticus* genome showed no detectable homology to pUC19 under the wash stringency used in this experiment.

To define more precisely the region in the cloned 3.4-kb *Hin*dIII insert that was essential for the trifunctional activities, a 930-bp *Hin*dIII-*Xho*I fragment at the beginning of the insert in pJH34 (Fig. 3) was deleted. The remaining *trpC* DNA fragment was purified and subcloned into pUC19. The resulting plasmid (pIV238) was used to transform an *A. nidulans trpC* mutant. This plasmid failed to complement the *trpC* mutation (Table 2), indicating that the deleted 930-bp *Hin*dIII-*Xho*I fragment was necessary for complete *trpC* function.

Isolation, complementation analysis, and deletion mapping of plasmid pLH23. *E. coli trpC* mutants were transformed with DNAs pooled from the *A. parasiticus* plasmid library. Trp^+ isolates were obtained and screened by using radiolabeled pJH34 as a probe. A plasmid designated pLH23 (Fig. 3), which contained a 4.9-kb *A. parasiticus* DNA insert, was recovered. On the basis of a comparison by restriction enzyme analysis of this insert and the *trpC* gene isolated from the lambda library, the insert was thought to encode an incomplete *A. parasiticus trpC* gene with a short deletion in the 5′ end. Consistent with this, pLH23 was unable to transform *A. nidulans trpC* mutants to Trp^+ phenotype (Table 2). However, pLH23 complemented all *E. coli* strains with a deficiency in PRAI, IGPS, or both activities (Table 1), whereas pJH34 and pAP34 complemented only strains with PRAI deficiency but not strains with IGPS deficiency (data not shown). These results indicated that the regions within the *A. parasiticus trpC* gene allowed for expression of the PRAI gene sequences but not of the IGPS gene sequences in *E. coli* and that *S. marcescens trp* promoter from pRK9 in pLH23 allowed for transcription of the *A. parasiticus trpC* (IGPS) sequence in *E. coli*. Plasmid pLH23 did not complement *E. coli* strains with deficiencies in domain A, B, D, or E (Table 1).

To confirm the functional organization of the *A. parasiticus trpC* gene as revealed by heterologous hybridization, we determined the relative locations of the IGPS and PRAI domains on pLH23 by deletion mapping. Plasmids were constructed with deletions in the *trpC* gene and analyzed by restriction endonuclease digestion to confirm the desired deletions. Deletion plasmids were then tested for Trp function in various Trp^- mutants of *E. coli* (Fig. 5). The deduced domain organization of the *A. parasiticus trpC* polypeptide (Fig. 5, bottom) was fully consistent with that obtained from heterologous hybridizations and was similar to that described for other filamentous fungi. These data also showed that a 280-bp *Hin*dIII-*Bam*HI fragment located at the 3′ end of the insert was necessary for PRAI activity. This result, together with the inferences that both a 930-bp *Hin*dIII-*Xho*I fragment on pJH34 (see previous section) and a minimum of 2.3 kb are necessary for the complete *trpC* function in filamentous fungi (5, 17, 21, 24–26), suggested that the *A. parasiticus trpC* transcript was initiated near the 3′ end of the 930-bp *Hin*dIII-*Xho*I fragment and terminated within the 280-bp *Hin*dIII-*Bam*HI fragment (Fig. 3, longer arrow on plasmid pJH34).

Analysis of *A. parasiticus trpC* transcript. To determine the transcriptional pattern and direction of the cloned *trpC* gene,

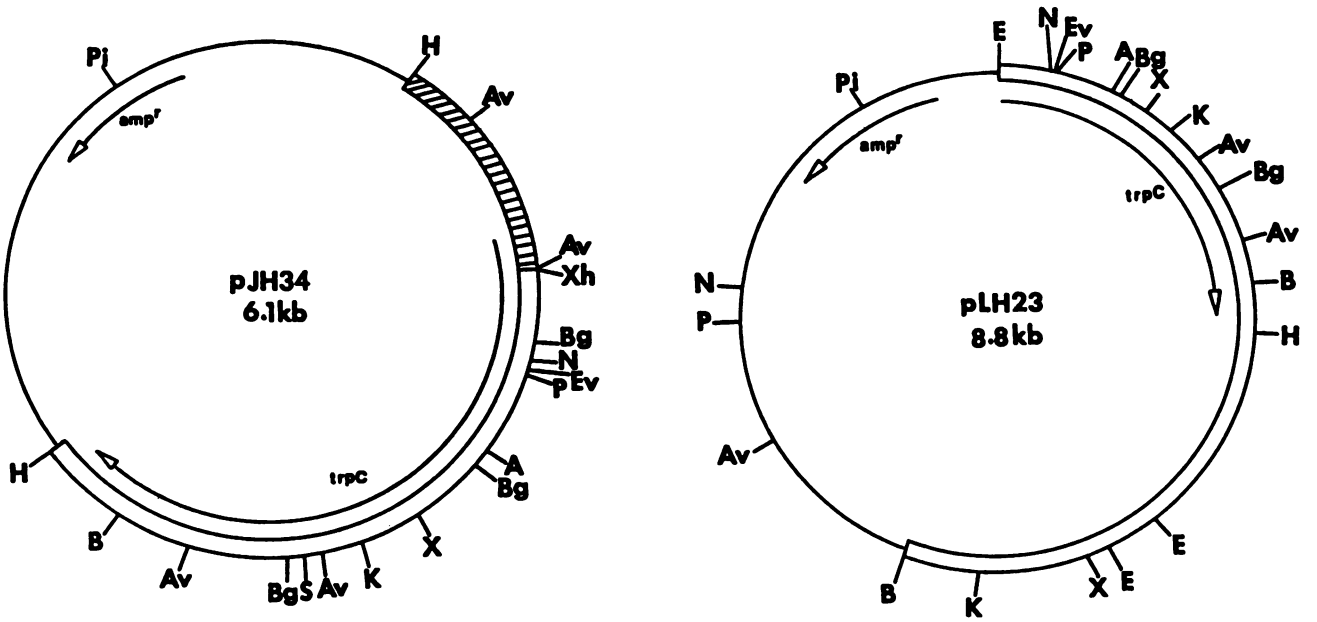

FIG. 3. Restriction endonuclease maps of plasmids containing the *A. parasiticus trpC* gene. The single lines represent sequences derived from vector pRK9 (plasmid pLH23) or pUC19 (plasmid pJH34). The double lines indicate *A. parasiticus* DNA inserts. The longer arrows show the coding regions and directions of transcription. The striped region in pJH34 was deleted to create plasmid pIV238. Only selected restriction sites are shown for pLH23 outside of the *trpC* coding region. Plasmids were not drawn to scale. Abbreviations: A, *Acc*I; Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; Pi, *Pvu*I; P, *Pvu*II; S, *Sac*I; X, *Xba*I; Xh, *Xho*I. The following restriction enzymes have no cutting sites on the 3.4-kb *Hin*dIII insert in plasmid pJH34: *Bgl*I, *Cla*I, *Eco*RI, *Nco*I, *Nru*I, *Pst*I, *Pvu*I, *Sal*I, *Sma*I, *Sph*I, and *Sst*I.

filters with poly(A)$^+$ RNAs purified from *A. parasiticus* mycelia grown in minimal and rich medium were hybridized to ^{32}P-labeled RNA probes generated from *trpC* restriction fragments by using an SP6 transcription system (Riboprobe System II; Promega Biotec). Probe 1 but not probe 2 hybridized to a single species of poly(A)$^+$ RNA, 2.7 kb in length, which was approximately the same size as *trpC* transcripts from other filamentous fungi (Fig. 6) (5, 17, 21, 24–26). The direction of transcription of the *A. parasiticus trpC* gene (Fig. 3, longer arrow) must therefore have been the same as that of the template which gave rise to probe 2. These data were fully consistent with those obtained by heterologous hybridization analysis of phage clones and by

TABLE 2. Transformation of *A. nidulans trpC* mutant with the *A. parasiticus trpC* gene cloned in different plasmids

Plasmid	Transformation frequency[a] (transformants/μg of DNA)
No DNA	0
pUC19	0
pRK9	0
YIp5	0
pLH23	0
pIV238	0
pJH34	15
pAP34	12
YAPH34	10
YAP43H	13
pHY201	26

[a] Protoplasts of *A. nidulans* FGSC 237 were transformed with 10 μg of each of the plasmid DNAs and plated onto minimal medium. Stable Trp$^+$ transformants were scored after incubation at 37°C for 4 days.

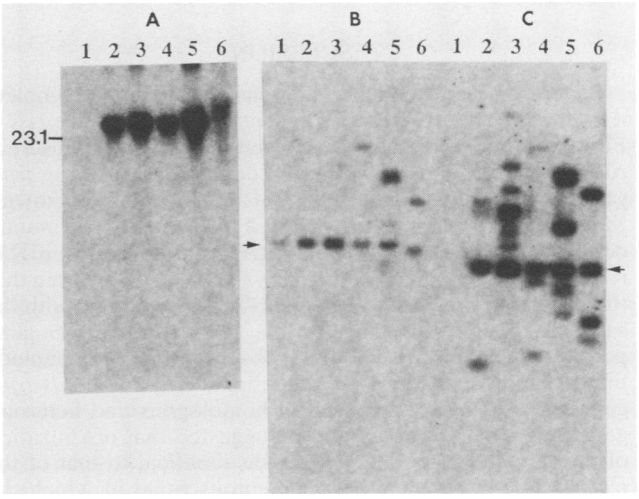

FIG. 4. Southern hybridization analysis of five *A. nidulans* Trp$^+$ transformants. (A) Undigested genomic DNAs were electrophoresed in a 0.7% agarose gel, blotted onto a nitrocellulose membrane, and hybridized with radiolabeled pUC19. The band at the top of lane 5 was caused by DNA molecules that did not migrate into the gel. (B and C) DNAs were digested with *Hin*dIII, blotted onto a nitrocellulose membrane, and hybridized with a radiolabeled 3.4-kb *Hin*dIII fragment excised from plasmid pJH34 (B) or pUC19 (C). Blots were hybridized and washed under high-stringency conditions. Fragments indicated by arrows are 3.4 kb (B) and 2.7 kb (C), respectively. Lanes 1, *A. nidulans* FGSC 237; lanes 2 through 6, *A. nidulans* FGSC 237 transformed with pJH34.

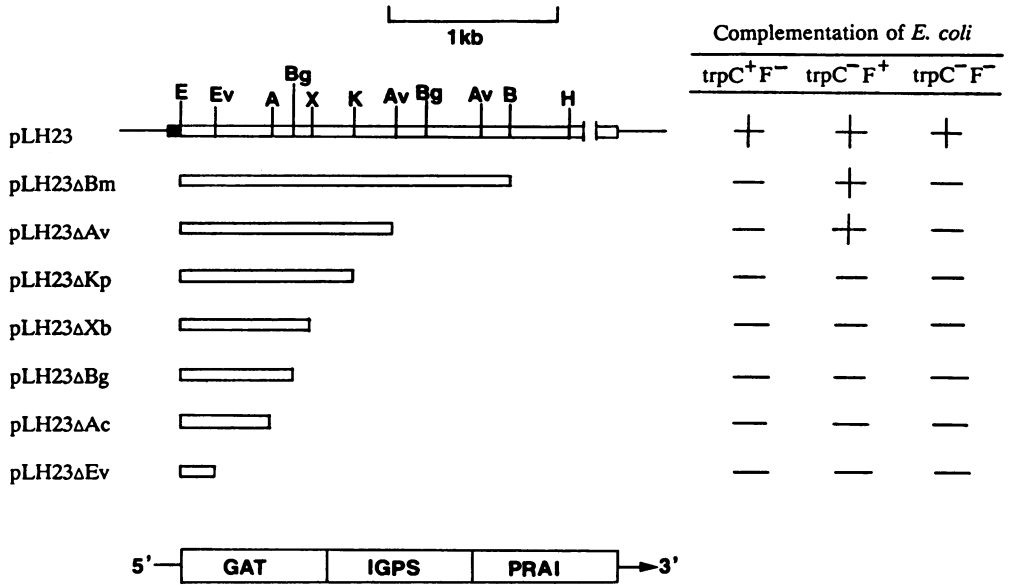

FIG. 5. Localization of the Trp functions in pLH23 by deletion mapping. Deletions were constructed by digesting pLH23 with suitable restriction enzymes, recovering the remaining fragment containing the *Serratia trp* promoter, and recircularizing by ligation. Open blocks represent the *A. parasiticus trpC* DNA sequences which remained in the final plasmid constructs; single lines are vector pRK9 sequences. The solid block indicates the *Serratia trp* promoter region. The ability of each of these plasmids to complement the three *E. coli* tryptophan auxotrophs is given on the right-hand side. The deduced domain organization of the trifunctional *A. parasiticus trpC* gene product is depicted at the bottom. Restriction sites are shown only for regions containing the Trp functions. For abbreviations see the legend to Fig. 3. IGPS was encoded by *trpC*, and PRAI was encoded by *trpF*.

deletion mapping of pLH23. Transcriptional analysis did not show significant differences in the level of the *trpC* mRNA in cells grown in minimal medium or in rich medium, indicating a substantial constitutive expression of the *A. parasiticus trpC* gene.

DISCUSSION

Several lines of evidence demonstrate that the complete *A. parasiticus trpC* gene is present on the plasmid pJH34. First, Southern blot analysis showed that the 3.4-kb insert of pJH34 was homologous with all coding regions of the *trpC* genes of *A. nidulans* (Fig. 2) and *A. niger* (data not shown). Moreover, transformation of an *A. nidulans trpC* mutant deficient in GAT, IGPS, and PRAI activities with pJH34 resulted in tryptophan prototrophy (Table 2), indicating that the *A. parasiticus trpC* gene codes for a polypeptide with the same enzymatic activities as the *A. nidulans trpC* gene product. On the basis of these observations, we conclude that plasmid pJH34 carries a functional *A. parasiticus trpC* gene which can be expressed in homologous and heterologous fungal cells. The data also suggested that organization of the *A. parasiticus trpC* gene was identical to that of the *trpC* (*trp-1*) genes of other filamentous fungi in which the gene product is a trifunctional polypeptide harboring *trpG* (GAT), *trpC* (IGPS), and *trpF* (PRAI) activities arranged in the order NH_2-GAT · IGPS · PRAI-COOH.

Plasmids carrying 3.4-kb inserts were also capable of complementing different *E. coli* mutants lacking PRAI activity. Complementation of a *trpC* mutation in *E. coli* was not dependent on the cloning vectors or the orientation of the insert in the vectors (Table 2). This implies that the sequences required for transcription and translation initiation in *E. coli* occur fortuitously within the coding sequence of the *A. parasiticus trpC* gene. The PRAI activity encoded by the *A. parasiticus trpC* gene was apparently initiated in *E. coli* from within the coding region and not from the *A. parasiticus* promoter, since the 5′ end of the gene could be eliminated and the PRAI activity was still retained. In support of this inference, clone lambda AptrpM1, which contained only the C-terminal portion of the *A. parasiticus trpC* gene, was able to complement the PRAI deficiency in *E. coli*. The fact that pJH34 encoded a complete *A. parasiticus trpC* gene but that only PRAI activity could be expressed in *E. coli* also supported this inference. This same pattern was observed for expression of the *A. nidulans trpC* gene (32) and the *N. crassa trp-1* gene (26) in *E. coli*. Expression of both PRAI and IGPS activities encoded by pLH23 in *E. coli* suggested that transcription from this construct was initiated at the *Serratia trp* promoter. However, the *A. parasiticus* DNA insert must have provided a Shine-Dalgarno sequence (ribosome-binding site) for translation, because both pRK9 and the *Serratia trp* promoter lack this sequence (26).

Expression of the trifunctional *trpC* genes is regulated differently among fungi. For instance, in *A. nidulans*, the level of the *trpC* transcript from cultures grown in minimal medium was considerably higher than those grown in tryptophan-rich medium (31). In contrast, cultures of *C. heterostrophus* (28) and *P. blakesleeanus* (24) grown in minimal or rich medium showed no significant differences in the amount of *TRP1* mRNA. In *A. parasiticus*, the amount of the *trpC* mRNA did not appear to be affected by the presence or absence of tryptophan in the culture medium.

To our knowledge, this report represents the first successful cloning of a gene from an aflatoxin-producing strain of *A. parasiticus*. This is particularly significant in our task of analyzing the aflatoxin biosynthetic pathway of this fungus at the molecular level. For example, there exist several *A.*

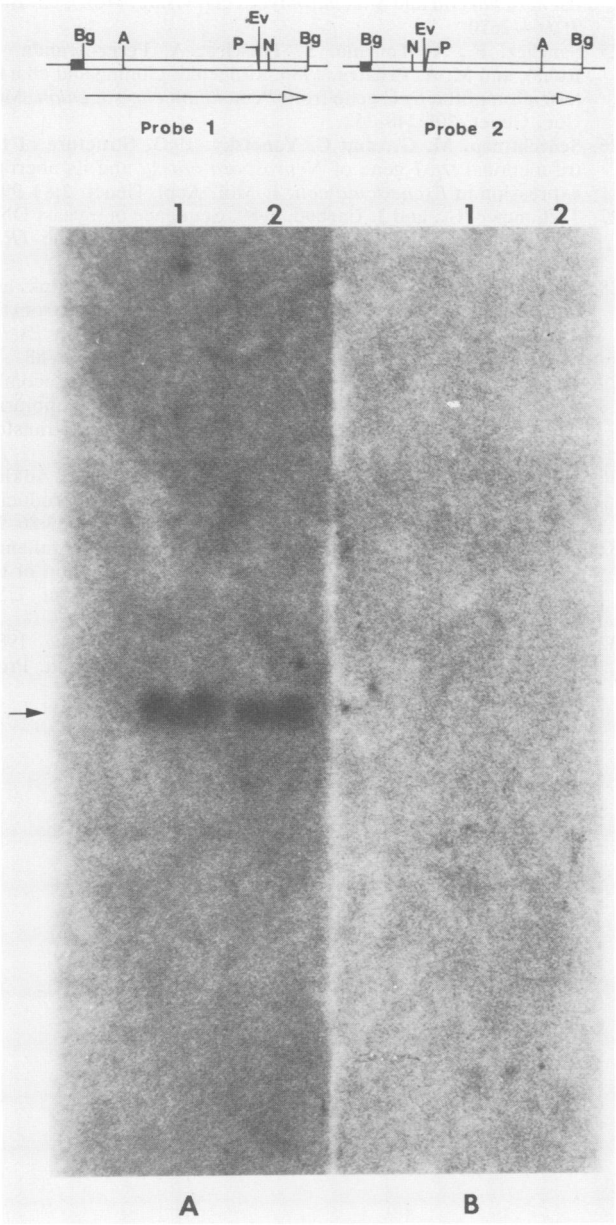

FIG. 6. Mapping the *A. parasiticus trpC* transcript. Poly $(A)^+$ RNA isolated from *A. parasiticus* NRRL 5862 grown in minimal medium (Czapek Dox broth; lane 1, 10 μg) or rich medium (Czapek Dox broth supplemented with 100 μg of L-tryptophan per ml; lane 2, 10 μg) was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde (12), blotted onto a GeneScreen Plus membrane, and hybridized to ^{32}P-labeled probe 1 (A) or probe 2 (B) generated with an SP6 transcription system (Riboprobe System II; Promega Biotec). The templates for SP6 RNA polymerase were prepared by inserting a 0.5-kb *Bgl*II fragment purified from the *trpC*-coding region in plasmid pJH34 in the two possible orientations into Riboprobe plasmid vector pSP64. The resulting recombinant plasmids were linearized and subjected to in vitro transcription with SP6 RNA polymerase by the procedure of Melton et al. (20). Specific activities of the probes were $>10^8$ cpm/μg of RNA. A solid block in the diagram indicates the SP6 promoter region. The size of the RNA transcript was estimated with a 0.24- to 9.5-kb RNA ladder from Bethesda Research Laboratories. The 2.7-kb *A. parasiticus trpC* transcript is indicated by an arrow. For abbreviations of restriction enzymes, see the legend to Fig. 3.

parasiticus mutants blocked at various stages of aflatoxin biosynthesis (1). The *trpC* gene vector could be used in complementation studies to isolate aflatoxin biosynthetic genes. This cloned *trpC* gene will offer a useful selectable marker in the development of a host-vector system for *A. parasiticus*. Experiments to isolate *A. parasiticus trpC* mutants to facilitate DNA-mediated transformation of *A parasiticus* with the *trpC* gene as a selectable marker are in progress.

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