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Identification of a Protein-Binding Sequence Involved in Expression of an Esterase Gene from *Streptomyces scabies*

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Expression of an esterase gene from *Streptomyces scabies* is regulated by zinc in both *Streptomyces scabies* and *Streptomyces lividans*. A specific protein-binding site was identified on an esterase promoter fragment by using an S-30 extract from *S. scabies*. The location of the protein-binding site was determined by gel shift assays of promoter deletion fragments and by DNase I footprinting analysis. The protein-binding site maps from nucleotides −59 to −81 relative to the start of transcription. An esterase gene construct cloned and expressed in *S. lividans* was used to assess the importance of the protein-binding site. Deletion of the 23-bp protein-binding site resulted in a 10-fold decrease in esterase production when cells were grown in zinc-inducing conditions. The protein-binding site may represent a region involved in positive regulation of the *S. scabies* esterase gene.

The gram-positive soil bacterium *Streptomyces scabies* is the causative agent of scab disease which affects a variety of underground vegetables including the potato (5). The underground portions of these plants are coated with a waxy polyester, suberin, which acts to control moisture loss and perhaps acts as a barrier to invasion by plant pathogens (15, 21). Invasion by the pathogen induces the proliferation of suberin by the host at the site of the infection. Enzymes involved in the breakdown of suberin or other components on the surface of the potato tuber may play an important role in invasion by *S. scabies*.

In previous studies, it was demonstrated that pathogenic isolates of *S. scabies* produce heat-stable extracellular esterases when grown in minimal medium supplemented with suberin extracted from potato peel (21). Zinc associated with the suberin was identified as the critical component involved in the induction of esterase activity. When *S. scabies* was grown in minimal medium with the addition of suberin or 2.0 μM Zn(SO₄)₂, esterase activity was induced 20- to 50-fold (21). The esterase gene from the pathogenic *S. scabies* isolate FL1 has been cloned and sequenced, and the transcriptional start site has been determined by nuclease S1 mapping (24). The amount of esterase transcript detected from *S. scabies* FL1 cultures grown in the presence of zinc was greatly enhanced compared with that from cells grown without zinc. The results have suggested that zinc regulation of the esterase gene may be at the level of transcription. One model consistent with the data involves positive regulation of esterase gene transcription mediated by the binding of a regulatory protein. Zinc may be involved in the binding and/or activation of this regulatory protein.

In this article we report the identification of a specific protein-binding site which is 5' proximal to the esterase promoter sequence. Site-directed deletion analysis has been used to demonstrate the importance of this protein-binding sequence to the regulation of esterase gene expression.

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MATERIALS AND METHODS

**Bacterial strains and plasmids.** *S. scabies* FL1, a pathogen of the potato, was isolated from a scab lesion as described previously (20). The esterase gene was subcloned as a 2.5-kb *SmaI* fragment into the *SmaI* site of pUC119 (33). The clone containing the esterase gene in the same orientation as *lacZ* is pJW27; the construct with the esterase gene in the opposite orientation is pJW9 (24). Deletion subclones of the esterase gene were made in pJW9 or pJW27 (24). *Escherichia coli* JM109 (35) was used to maintain *E. coli* plasmids. The esterase gene was transformed into *Streptomyces lividans* TK24 (13) with streptomycete vector pIJ486 (34).

**Media and growth conditions.** *S. scabies* FL1 was grown on oatmeal agar (28) at 30°C and maintained as spore suspensions in 20% glycerol at −20°C. *S. scabies* cultures used in an S-30 cell lysate preparation were grown in NMM minimal medium, a modification of NMMP medium which does not contain polyethylene glycol (12). Esterase induction requires the addition of 2.0 μM Zn(SO₄)₂. All liquid cultures were grown at 30°C with shaking in flasks containing springs.

*S. lividans* TK24 was grown on R2YE agar medium and maintained as spore suspensions in 20% glycerol at −20°C (12). For selection of resistant *S. lividans* transformants containing pIJ486 derivatives, thiostrepton was incorporated into solid medium at 40 μg/ml and liquid medium at 5 μg/ml (12).

*S. lividans* TK24 was grown on antibiotic medium 2 agar plates (Difco Laboratories, Detroit, Mich.) and in LB (26) liquid cultures at 37°C. For selection of JM109 transformants containing pUC119 and derivatives, ampicillin was incorporated into the medium at 50 μg/ml (26).

**DNA manipulations and bacterial transformations.** Plasmid DNA was isolated as previously described (1). Restriction enzymes were obtained and reactions were carried out according to the manufacturer’s instructions (Gibco Bethesda Research Labs, Gaithersburg, Md., and New England Biolabs, Beverly, Mass.). DNA restriction fragments were electrophoresed on a 1.0% agarose gel with Tris-borate-EDTA buffer (pH 8.0; TBE buffer) (26) and purified by electroelution with an electroelution apparatus (Interna-
Maxam-Gilbert sequencing reactions of the end-labelled fragment (19) or dideoxy-sequencing reactions of pUC19 with universal primer (17, 27) were used as DNA size standards. After electrophoresis, the gels were dried and subjected to autoradiography.

**Site-directed mutagenesis.** The pJW27 plasmid was used to construct a deletion of the protein-binding sequence from nucleotide (nt) −58 to nt −81 relative to the transcriptional start site in the esterase gene by using the oligonucleotide 5′-GGTGATCAAACCGGGGTGTCCATCICACC3′. Site-directed mutagenesis was performed by the methods of Kunkel et al. (18) by using the Mutagen-Gen in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). The deletion of the protein-binding region was confirmed by sequence analysis of the resulting construct, pJL129.

**Esterase assay and protein determination.** Esterase activity was measured in culture filtrates by a spectrophotometric assay with p-nitrophenyl-butyrate as the substrate (21). All cell filtrates were heat treated at 65°C for 30 min to inactivate heat-labile esterases (21). The protein concentration of the S-30 extracts was determined by the method of Bradford (2).

**RESULTS**

**Evidence for a specific protein-binding site.** The first approach to identify regions of the esterase gene important in the regulation of expression was to generate deletions in the promoter-proximal sequence. Deletions were made in the esterase gene with exonuclease III after digestion of pJW9 with KpnI and NruI (24). The resulting deletion subclones are shown in Fig. 1. The precise endpoints of these deletions were determined by DNA sequencing. The EcoRI site within the polycloning region and the EcoRI site in the esterase coding sequence were used to isolate purified DNA fragments containing the various-length deletions. EcoRI fragments of the deletion constructs U1 to U5 were used in experiments to localize a protein-binding site that was postulated to be necessary for the expression of the S. scabies esterase gene.

*S. scabies* was grown in NMM with 2 μM Zn(SO$_4$)$_2$ to the late exponential phase to prepare S-30 cell extracts for gel retardation experiments. The S-30 extract was used to demonstrate that a protein produced by *S. scabies* binds to a specific region of the esterase sequence. When the 32P-labelled U1 EcoRI fragment (Fig. 1) was incubated with the S-30 extract (Fig. 2A, lane 1), its migration on a polyacrylamide gel was retarded when compared with the migration of the unbound fragment (Fig. 2A, lane 6). The protein-binding activity was detected in cell lysates prepared from *S. scabies* cultures grown with or without 2.0 μM zinc.

To demonstrate that the interaction of the protein with the esterase sequence is specific, increasing amounts of unlabelled U1 EcoRI fragment were added to the DNA-protein-binding reaction mixtures (Fig. 2A, lanes 2 to 5). The unlabelled fragment competed with the labelled fragment for binding by the protein. Protein binding to the labelled fragment was not interrupted by the addition of nonspecific dI-dC DNA equal to the amount of unlabelled competitor DNA that was added in the experiment whose results are shown in panel A (Fig. 2B).

**Location of the protein-binding site.** To determine the location of the protein-binding site within the sequence 5′ to the esterase gene, 32P-labelled EcoRI fragments of deletion constructs U1, U2, U3, U4, and U5 were used in the DNA-protein binding reactions (Fig. 3). During electrophoresis, the migration of the U1, U2, and U3 fragments was
EcoRI fragment.

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FIG. 1. Restriction map of the S. scabies esterase 5' sequence in pJW9. Exonuclease III-generated deletions were made in the esterase upstream sequence after cleavage with NruI and KpnI. The KpnI site is within the polycloning region in the pJW9 construct. The NruI site is located 556 bp upstream of the esterase gene transcriptional start site. Subclones U1 through U5 contain increasing lengths of deletion. The endpoints of each deletion are numbered relative to the transcriptional start site. The start of transcription is indicated as +1. The hatched boxes represent the beginning of the esterase open reading frame at the ATG codon. The open reading frame extends for another 1,007 bp beyond the EcoRI site (24). The closed box represents sites available in the polycloning region of pUC119.

The specific esterase DNA sequence involved in protein binding was determined by DNase I footprinting experiments (Fig. 4). Either the noncoding or the coding DNA strand was end labelled and incubated with various amounts of the S-30 extract prior to DNase I treatment. The site protected by protein binding was from nt −61 to nt −77 on the noncoding strand (Fig. 4A) and from nt −59 to nt −81 on the coding strand (Fig. 4B) relative to the start of transcription (Fig. 5). A hypersensitive site for DNase I cleavage was observed on the coding strand DNA when bound by S-30 protein. The hypersensitive site was mapped to position −76 (Fig. 4B, lanes 2 to 4). These results are consistent with the gel shift assays in which the EcoRI fragments containing the 23-bp protein-binding site (U1 to U3) showed a shift when bound by the protein whereas fragments with the site deleted (U4, U5) did not. The esterase upstream sequence and the location of the DNase I-protected region are shown in Fig. 5.

FIG. 2. Gel retardation assay. The DNA-protein-binding reaction mixture contained 0.2 ng of 32P-labelled U1 EcoRI fragment and 5 µg of an S-30 extract prepared from a culture of S. scabies grown in NMM containing 2.0 µM Zn(SO4)2. The reaction mixtures used in panel A contain increasing amounts of competing unlabelled U1 EcoRI fragment at 0 (lane 1), 10 (lane 2), 20 (lane 3), 40 (lane 4), and 120 (lane 5) ng. The control reaction mixture used in lane 6 of both panels contained no S-30 extract. The reaction mixtures used in panel B are identical to those used in panel A except that 0 to 120 ng of nonspecific dIdC DNA was added instead of the unlabelled U1 EcoRI fragment.

FIG. 3. Localization of the DNA-binding activity. 32P-labelled EcoRI fragments of deletion subclones U1, U2, U3, U4, and U5 were used in gel retardation assays with (+) or without (−) 5 µg of an S-30 extract from S. scabies.

The hypersensitive site on the observed the protected by protein binding was (Fig. 5). the coding in the 120 but not within the portion of the sequence common to the U4 fragment (Fig. 1).

The specific esterase DNA sequence involved in protein binding was determined by DNase I footprinting experiments (Fig. 4). Either the noncoding or the coding DNA strand was end labelled and incubated with various amounts of the S-30 extract prior to DNase I treatment. The site protected by protein binding was from nt −61 to nt −77 on the noncoding strand (Fig. 4A) and from nt −59 to nt −81 on the coding strand (Fig. 4B) relative to the start of transcription (Fig. 5). A hypersensitive site for DNase I cleavage was observed on the coding strand DNA when bound by S-30 protein. The hypersensitive site was mapped to position −76 (Fig. 4B, lanes 2 to 4). These results are consistent with the gel shift assays in which the EcoRI fragments containing the 23-bp protein-binding site (U1 to U3) showed a shift when bound by the protein whereas fragments with the site deleted (U4, U5) did not. The esterase upstream sequence and the location of the DNase I-protected region are shown in Fig. 5.

Zinc regulation of the S. scabies esterase gene in S. lividans. Since an esterase mutant of S. scabies was not available, the S. scabies esterase gene was cloned and expressed in the nonpathogenic streptomyces S. lividans TK24. S. lividans TK24 was chosen as the host strain for in vivo studies of esterase gene expression for two reasons. First, S. lividans TK24 does not produce a heat-stable esterase when grown under zinc-inducing conditions, thereby facilitating detection of the cloned S. scabies esterase (data not shown). Second, gel shift experiments using the U1 and U4 esterase EcoRI fragments and S-30 extracts prepared from S. lividans...
FIG. 4. DNase I footprinting analysis. The DNase I-protected region for both strands of the S. scabies esterase regulatory region was determined. The regions bracketed on the gels are portions of the sequence protected from treatment with DNase I. The numbers to the right of the brackets in panels A and B correspond to the nucleotide positions in the sequence relative to the transcriptional start site (see Fig. 5). (A) The EcoRI-NraI fragment end labelled at the EcoRI site was used to footprint the noncoding strand of the DNA. Protein-DNA binding reactions were carried out with this fragment and 0 (lanes 1 and 6), 50 (lane 2), 25 (lane 3), 10 (lane 4), and 5 (lane 5) μg of S-30 extract. After the binding reaction, the DNA was treated with DNase I and run on a 6.0% polyacrylamide-urea gel. Maxam-Gilbert sequencing reactions (A+G, C+T) were used to identify the DNase I-protected region of the fragment. (B) The EcoRI-NraI fragment labelled by filling in the EcoRI site with [α-32P]dATP was used to footprint the coding strand. Reaction mixtures contained 0 (lane 1), 5 (lane 2), 2.5 (lane 3), and 1 (lane 4) μg of S-30 extract. Sequenase reactions (C, T, A, and G) using universal primer and pUC19 were used to identify the position of the DNase I-protected site (17, 27). The DNase I-hypersensitive site is marked with an arrow.

TK24 gave results equivalent to those reported for the S-30 extracts of S. scabies (Fig. 3). These results indicated that S. lividans TK24 produced a similar DNA-binding protein that interacts with the esterase gene.

The esterase gene was originally cloned into pUC19 on a 2.5-kb SmaI fragment resulting in plasmid pJW27 (23). A HindIII digest of pJW27 was ligated to a high-copy-number streptomyces vector, pJ486, that had been cleaved with HindIII. E. coli JM109 competent cells were transformed with the ligation, and the transformants were screened for the proper construct. The resulting plasmid, pJLS130, is a bifunctional vector capable of replication in both E. coli and S. lividans. S. lividans TK24 was transformed with pJLS130 and assayed for zinc-regulated expression of the esterase gene.

Esterase activity produced by the S. lividans transformant containing pJLS130 was fivefold higher in the zinc-induced culture than in the culture grown without zinc (Fig. 6A). In a separate study, the esterase-specific mRNA produced in S. lividans carrying a similar esterase gene construct was assayed in cultures grown with and without zinc. The increase in esterase enzyme levels in the zinc-induced culture coincided with an equal increase in esterase transcript detected by dot blot analysis (7). In addition, the maximum production of esterase occurred as cells entered the late exponential and early stationary phases of growth. This timing of esterase synthesis is also similar to that observed in induced S. scabies cultures. The fivefold increase in zinc-induced esterase production in the S. lividans transformant is not as great as the typical 20-fold induction of esterase by zinc in S. scabies cultures (21). However, the evidence that S. lividans makes a protein which binds to the esterase DNA fragment together with the evidence for zinc-regulated expression of the esterase gene suggests that S. lividans contains the machinery required for zinc regulation of the S. scabies esterase gene. Thus, S. lividans can be used as an appropriate host strain for studying regulation of the S. scabies esterase gene.

Deletion of the protein-binding site and its effect on esterase gene expression in S. lividans. An esterase gene construct cloned and expressed in S. lividans was used to assess the importance of the specific protein-binding site that had been localized upstream from the esterase coding sequence (Fig. 5). Deletion of 23 bp containing the protein-binding site was accomplished by site-directed mutagenesis of pJW27 and confirmed by DNA sequencing. The resulting pJLS129 plasmid was ligated to pJ486 to produce the bifunctional plasmid pJLS131. The pJLS131 plasmid is identical to pJLS130 except for the 23-bp deletion within the esterase 5′ sequence. pJLS131 was introduced into S. lividans TK24 to determine the effect of the protein-binding site deletion on esterase production. TK24(pJLS131) was grown in the presence and absence of zinc, and heat-stable esterase production was monitored and compared with esterase production by parallel cultures of TK24(pJLS130) (Fig. 6). The pJLS131 transformant produced low levels of esterase when grown without zinc (about 0.2 nmol/min/μl) and showed an approximate twofold increase in activity when the culture was grown in the presence of zinc (Fig. 6B). This twofold increase occurred when the culture reached stationary phase, similar to when normal zinc induction of esterase production is observed (Fig. 6B). However, even the induced esterase levels seen in cells carrying the pJLS131
construct were at least 10-fold lower than the amount of esterase produced in *S. lividans* containing the intact esterase gene, pJLS130 (Fig. 6A). These results indicate that the 23-bp deletion containing a specific protein-binding site dramatically affects expression of the esterase gene. The protein that binds to this sequence may therefore play an important role in the regulation of esterase gene expression.

**DISCUSSION**

Our current model for zinc regulation of esterase gene expression involves the binding of a positive regulatory protein to the promoter region of this gene. In the presence of zinc, this regulatory protein activates transcription of the esterase gene. Gel retardation and DNase I protection experiments have demonstrated that *S. scabies* makes a protein that specifically binds to a nucleotide sequence adjacent to the RNA polymerase-binding site of the esterase gene. The protein-binding activity was detected in extracts prepared from *S. scabies* grown in the presence or absence of zinc (data not shown). Therefore, zinc may not be required for the expression of this binding protein. This protein-binding site plays an important role in esterase gene expression. Deletion of the binding site led to a 10-fold decrease in esterase production.
The addition of zinc to the binding assay was not required for the observed gel shift reaction. It is unclear, however, whether zinc is actually necessary for the protein to bind to the DNA. Either the protein is able to bind to the DNA in the absence of zinc or the buffers used in the S-30 extract contain contaminant levels of zinc sufficient for binding. It is not uncommon for positive regulatory proteins to bind to the target gene without the effector present. The regulatory protein of the mer operon in E. coli, MerR, binds to the merT promoter in the absence of mercuric ions (23). The MerR-promoter complex facilitates the binding of RNA polymerase in a closed complex incapable of transcription (6, 10). It is proposed that with the addition of mercuric ions, the MerR protein imposes a conformational change within the promoter sequence resulting in the formation of an open complex with RNA polymerase and the activation of transcription (6, 10). Similarly, the regulatory protein involved in oxidative stress in Salmonella typhimurium and E. coli, OxyR, binds to the regulatory regions of target genes but is an activator of transcription only when it is in its oxidized form (29).

The protein-binding site identified in the 5' region of the esterase gene may be involved in general transcriptional activation or in zinc-regulated activation of esterase gene expression. By analogy to MerR (23) and OxyR (29), we propose that the binding of a regulatory protein to this binding site on the esterase DNA is not zinc dependent. The regulatory protein, however, would only act as a positive regulator of transcription in the presence of zinc. The pJLS130 construct contains the normal binding site. When cells containing this construct are grown without zinc, the protein may still bind to the DNA and promote some low-level transcription of the esterase gene. The apparent twofold increase in esterase activity observed between 25 and 38 h of growth could be accounted for by the approximate twofold increase in cell mass and subsequent accumulation of esterase produced continuously at a low level during this time period. This low-level production from the pJLS130 construct in the absence of zinc is in contrast to the greater-than-fivefold increase in esterase production observed when cells were grown in the presence of zinc.

The pJLS131 construct does not contain the protein-binding site, which would presumably preclude binding of the regulatory protein. When cells with pJLS131 were grown without zinc, esterase synthesis was lower than that observed for cells with pJLS130 grown without zinc. This could be due to the lack of regulatory protein binding to its primary binding sequence. There was an approximate twofold increase in esterase activity when the deletion construct was grown in the presence of zinc. This suggests that there may be a secondary weak binding site which allows limited protein binding and induction of esterase transcription in the presence of zinc. The protein involved in binding to the esterase gene sequence must be isolated to more fully understand its role in zinc regulation of esterase gene transcription.

The protein-binding site of the esterase gene was localized by gel retardation experiments with nested deletion fragments and by DNase I footprinting of both strands of the esterase DNA. The protein-binding site mapped from nt -61 to nt -77 on the noncoding strand and from nt -59 to nt -81 on the coding strand relative to the start of transcription of the esterase gene. A search of the GenBank data base did not reveal any homology between the esterase protein-binding sequence and other known DNA sequences.

The protein-binding site may represent a positive regulatory region which is 5' proximal to the esterase RNA polymerase-binding site. The position of the esterase regulatory sequence is similar to the location of the OxyR-binding site relative to the promoter sequence of its target genes (31, 32). The catabolite gene activator protein-binding site within the lac operon is also 5' and adjacent to the RNA polymerase-binding site (30). It has been demonstrated that the stimulation of transcription of the lac operon is dependent on protein-protein interaction between the catabolite gene activator protein and the RNA polymerase (30). In E. coli, the majority of activator protein-binding sites are proximal to the sigma 70 RNA polymerase-binding site (4). The close proximity of the regulatory protein-binding site and the RNA polymerase-binding site would facilitate interactions between these proteins.

In eucaryotes, zinc is required by several transcription activators which contain zinc finger motifs. The activators do not bind their target DNA and activate transcription in the absence of zinc. Examples of these activators include the Xenopus transcription factor ILA (22), human transcription factor Sp1 (14), and yeast ADR1, which is a regulatory protein required for transcriptional activation of the glucose-repressible alcohol dehydrogenase gene (9). The proposed transcriptional activator involved in the regulation of the esterase gene of S. scabies may also be a zinc-binding protein.

Zinc may also be involved in the expression of other genes in S. scabies. When S. scabies is grown in NMM minimal medium with zinc, a yellow compound is produced in the mycelium (unpublished results). The compound is produced only in the culture grown with zinc, and the appearance of this yellow compound coincides temporally with the production of esterase in S. scabies. Even though this yellow compound has not yet been identified, the dependence of its production on zinc suggests that zinc may have a more general effect on gene expression in S. scabies and is not limited to regulation of the esterase gene. In addition, expression of the S. scabies esterase gene is also regulated by zinc in S. lividans, indicating that the mechanism by which zinc regulates gene expression can function in other streptomycetes and is not limited to S. scabies. To further characterize regulation of the S. scabies esterase gene, regulatory mutants defective in zinc induction will be isolated, and the putative transcriptional activator protein will be purified and further characterized.

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