

Protein-Protein Interactions of Proteins from the ESAT-6 Family of *Mycobacterium tuberculosis*

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In the present study, we demonstrate that, in analogy with the genes encoding ESAT-6 and CFP-10, the genes *rv0287* and *rv0288* from the ESAT-6 gene family are cotranscribed. Using Western-Western blotting and protein-print overlay methodologies, we demonstrate that ESAT-6 and CFP-10, as well as the protein pair Rv0288/Rv0287, interact pairwise in a highly specific way. Most notably, the ESAT-6 proteins interact directly with Rv3873, a possible cell envelope component of the ESAT-6 secretion pathway.

The 6-kDa early secretory antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10) from *Mycobacterium tuberculosis* are two dominant targets for T cells in the early phases of infection (1, 25, 27). Furthermore, ESAT-6 has recently been demonstrated to induce protective immunity when administered as either a subunit (5) or a DNA (12) vaccine. The genes encoding ESAT-6 and CFP-10 (*esx* and *lhp*, respectively) lie next to each other in an operon-like structure (4). A dual knockout of ESAT-6 and CFP-10 in *M. bovis* results in decreased virulence of the pathogen (29), indicating that the two molecules may play important roles in immunopathogenesis and virulence. Recent genomic and genetic studies have revealed that *lhp* and *esx* and their neighboring genes constitute a gene cluster (*esx* cluster 1), which encodes a cellular function that is fundamental for the virulence of *M. tuberculosis* (15, 19, 28).

Both ESAT-6 and CFP-10 are low-molecular-mass proteins that belong to a large protein family, the ESAT-6 family, which has 23 members in *M. tuberculosis*. When *M. tuberculosis* is grown in broth culture, these proteins are released into the surroundings (2, 4, 23) through a Sec-independent pathway, since none of the proteins contain common signal peptides. The genes encoding ESAT-6 family proteins are arranged in tandem pairs at 11 loci in *M. tuberculosis* H37Rv and are often preceded by a *pe-ppe* gene pair. At five loci, the *esx*-like gene pairs are part of larger gene clusters that have genetic contexts similar to that of the *esx* cluster 1. These clusters characteristically include a core region, consisting of a pair of *esx*-like genes next to a *pe-ppe* gene pair, flanked by open reading frames (ORFs) encoding potential membrane proteins, serine proteinases, and putative ABC transporters. It has been suggested that these gene clusters (the *esx* clusters) encode novel transporter systems that are responsible for the secretion of ESAT-6-like proteins (9, 18, 28). Experimental evidence that supports this theory is now available (20).

Two of the *esx* clusters are conserved in *M. leprae* (9); one is the *esx* cluster 1, while the other is the *esx* cluster 3 encoding ESAT-6 family members Rv0287 and Rv0288. As it has been

suggested that *M. leprae* may contain the minimum gene set required by a pathogenic mycobacterium (7), the function represented by the *esx* cluster 3 may thus also be essential for the pathogenesis of *M. tuberculosis*. In fact, although large numbers of genetic deletions have been identified in clinical isolates of *M. tuberculosis*, none of them occurred in the *esx* cluster 3 (13). Comparing gene expression in virulent *M. tuberculosis* H37Rv and the avirulent H37Ra, Rindi et al. showed that *rv0288* expression was markedly down-regulated in the attenuated strain (22), and the newly reported comprehensive identification of essential genes in *M. tuberculosis* included the *esx* cluster 3 in the list of 600 genes that are essential for in vitro growth (24). Recent immunological studies have demonstrated that Rv0288 is a potent T-cell antigen strongly recognized in *M. tuberculosis*-infected humans, as well as in cattle infected with *M. bovis* (1, 25, 26). As those to ESAT-6 and CFP-10, the immune response to Rv0288 is fully developed shortly after infection with *M. bovis* or contact with tuberculosis patients (1, 3), suggesting that this protein is produced and secreted and interacts with the host immune system at the early stages of infection. Information about Rv0287, on the other hand, is very limited, although this protein has been identified in the short-term culture filtrate of *M. tuberculosis* in proteomic studies (23). The present study demonstrates a close resemblance between ESAT-6/CFP-10 and Rv0287/Rv0288 protein pairs with respect to cotranscription and protein-protein interactions. Furthermore, we demonstrate the existence of direct interactions between the ESAT-6 proteins and the PPE protein encoded by the *esx* cluster 1.

Coexpression of *rv0287* and *rv0288* in *M. tuberculosis*. Both Rv0287 and Rv0288 are secreted into the surroundings by *M. tuberculosis* grown in vitro (23). The ORFs encoding the proteins, *rv0287* and *rv0288*, are 291 and 288 bp, respectively, separated by a 35-bp DNA fragment (Fig. 1A). The short intergenic space suggests that these two genes may be transcribed as one mRNA unit. This was investigated by reverse transcriptase-PCR (RT-PCR) with *M. tuberculosis* grown in vitro. A specific primer set was designed so that the forward primer (F-primer) carried a sequence from the 5' region of *rv0287* and the reverse primer (R-primer) was 650 bp downstream and had a sequence complementary to that of the 3' terminus of *rv0288* (Fig. 1A). RNA was isolated from *M. tu-*

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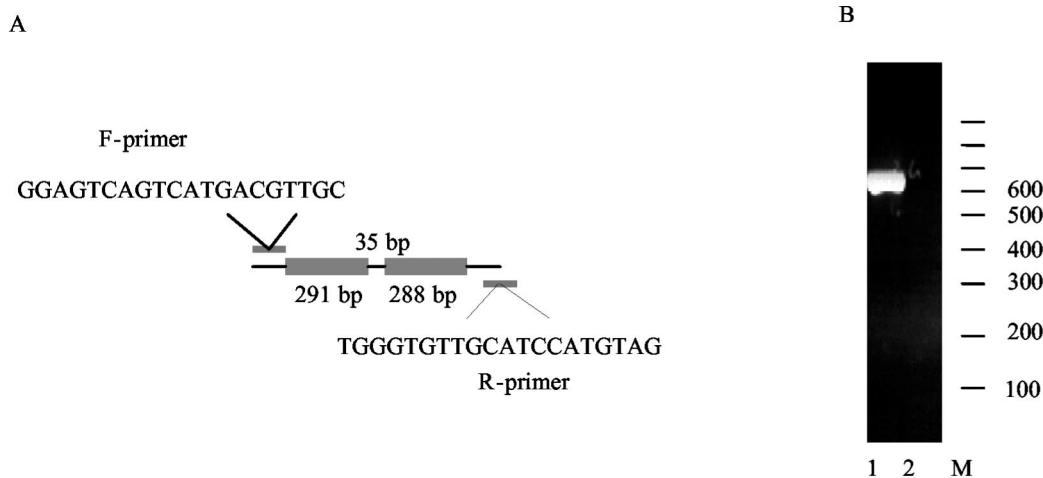


FIG. 1. Cotranscription of *rv0287* and *rv0288*. (A) Sequences and locations of the primers used for RT-PCR. (B) Lanes 1 and 2, results of RT-PCRs with and without reverse transcriptase, respectively. The 100-bp ladder is indicated (M).

berculosis H37Rv after growth for 1 week in broth culture (17); prior to cDNA synthesis, RNA was treated with RNase-free DNase I (Boehringer Mannheim) at 37°C for 20 min. Reverse transcription was carried out with the R-primer, and the resulting products were amplified by PCR. A single amplicon of the expected size was obtained (Fig. 1B), and it was confirmed by nucleotide sequencing that the amplicon carried the *rv0287-rv0288* gene pair (data not shown). No amplicon was obtained in the absence of reverse transcription.

Study of interaction between ESAT-6 proteins by Western-Western blotting. Recently, using pull-down assays and different spectroscopy techniques, Renshaw et al. observed a strong, direct interaction between ESAT-6 and CFP-10 (21). In the present study, we developed a Western-Western blotting assay to study the interactions between the ESAT-6 family members. In this assay, proteins are electro-separated and immobilized onto a nitrocellulose membrane, which is subsequently overlaid with a solution containing the protein of interest. A specific antibody against the protein in the solution then detects binding to the immobilized proteins. We decided to compare the interaction between ESAT-6 and CFP-10 with that between Rv0287 and Rv0288.

His-tagged recombinant ESAT-6, CFP-10, Rv0287, and Rv0288 had been constructed previously (4, 11, 25; P. Andersen and R. Skjoet, 18 January 2001, international patent application, PCT publication no. WO0104151). The recombinant proteins were purified by a three-step procedure, which has been described elsewhere (17). For Western-Western blotting, 5 µg each of purified His-tagged recombinant Rv0287 (rRv0287), rRv0288, rCFP-10, and rESAT-6 was loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 2A), and after electrophoresis the proteins were electro-transferred onto nitrocellulose membranes, which were subsequently overlaid with binding solution (phosphate-buffered saline [pH 7.4] supplemented with 0.1% Tween 20 and 1% skimmed milk) containing 10 µg of rRv0288, rCFP-10, or rESAT-6/ml. The protein-protein interaction complexes were detected by specific antibodies against Rv0288, CFP-10, or ESAT-6, respectively. As shown in Fig. 2C, under the experimental conditions,

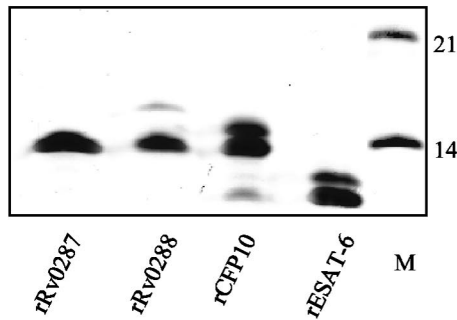
rESAT-6 and rCFP-10 bound to each other and rRv0288 bound to rRv0287. In the control experiment, neither the primary nor the secondary antibodies showed any cross-reactivity (Fig. 2B). The rCFP-10 preparation contained a minor protein band with a mobility corresponding to about 8 kDa. This band reacted with anti-His antibody (data not shown), indicating that the C-terminal region was missing. Matrix-assisted laser desorption ionization mass spectra analysis suggested that this CFP-10 band lacked the C-terminal 23 residues (P. Højrup, personal communication).

Study of interaction between ESAT-6 proteins by protein-print overlay. A drawback of Western-Western blotting is that the amounts of proteins transferred onto the membrane are not well defined, as the transfer efficacy varies from experiment to experiment and from protein to protein. We therefore developed a more standardized system using protein-printed membrane strips as a basis for overlay assays. rESAT-6, rCFP-10, rRv0287, and rRv0288 were printed onto the nitrocellulose membrane at concentrations ranging from 0.1 to 1 µg/cm. The membrane was subsequently cut into strips, which were then overlaid with rESAT-6 or rRv0288 at 10 µg/ml. Protein interaction patterns detected by this method were consistent with those obtained by Western-Western blotting, such that ESAT-6 interacted with CFP-10 and Rv0288 interacted with Rv0287. At the protein concentrations applied, the amount of protein captured correlated positively with the amount of immobilized protein (Fig. 3).

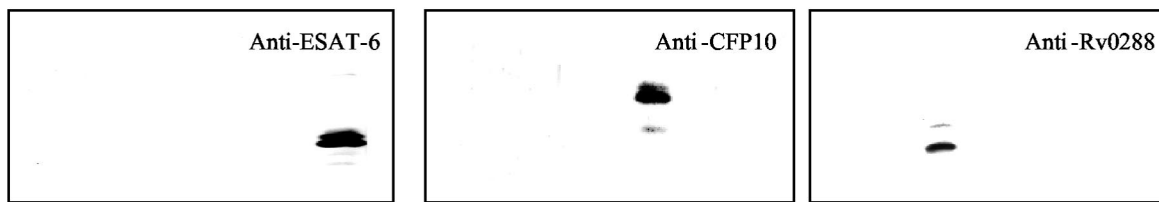
Results from both Western-Western blotting and protein-print overlay show that rESAT-6/rCFP-10 and rRv0287/rRv0288 interact in a pairwise and specific way so that rESAT-6 and rCFP-10 do not bind to rRv0287 and rRv0288 and vice versa.

Interaction of ESAT-6 proteins with other proteins encoded by the *esx* cluster 1. The genes encoding ESAT-6-like proteins often lie next to a *pe-ppe* gene pair, which is preceded by an ORF encoding a dimeric membrane-bound ATPase of the FtsK family. In the *esx* cluster 1, this ATPase is encoded by *rv3870* and *rv3871* and is required for the optimal transport of ESAT-6 (20). The PPE protein Rv3873 is associated with the

A. Recombinant proteins



B. Western blots



C. Western-western blots

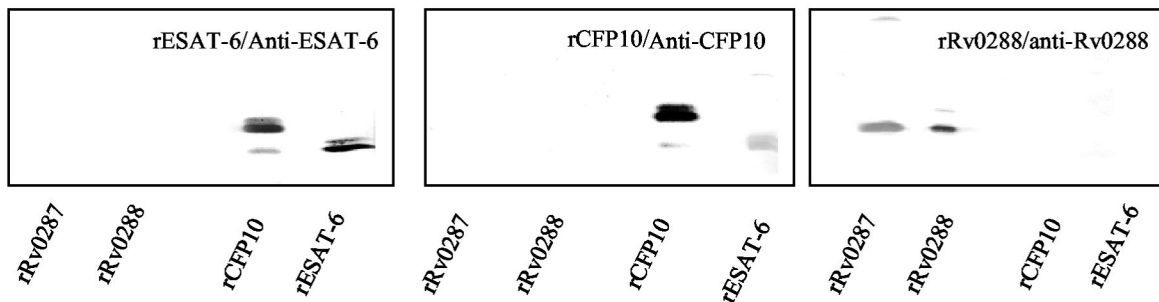


FIG. 2. Interaction between ESAT-6 proteins. (A) Coomassie-stained SDS-polyacrylamide gel with recombinant ESAT-6 family proteins. The 14- and 21-kDa molecular mass markers are indicated (M). (B) Nitrocellulose membranes overlaid with specific antibodies as indicated. (C) Nitrocellulose membranes which were overlaid first with recombinant ESAT-6 proteins as indicated and subsequently with specific antibodies.

cell envelope (17, 19). No data have so far been available as to the presence of the PE protein Rv3872 in *M. tuberculosis*. However, RNA microarray studies have detected induction of the corresponding RNA in a *sigE* mutant strain upon exposure

to SDS (16). We were interested in exploring whether ESAT-6 and CFP-10 interact directly with Rv3871, Rv3872, and Rv3873. In addition, as these three proteins are homologous to Rv0284, Rv0285, and Rv0286, respectively, of the Rv0287/

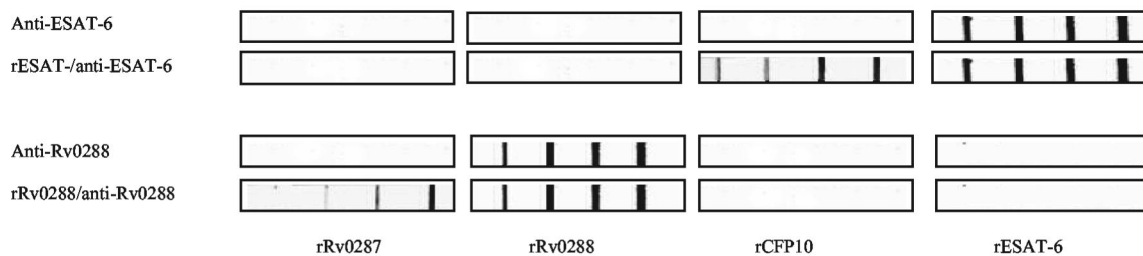


FIG. 3. Interaction between ESAT-6 proteins as demonstrated by protein-print overlay assays. ESAT-6 proteins at 0.1, 0.2, 0.5, and 1 $\mu\text{g}/\text{cm}$ were printed onto the nitrocellulose membrane strips by using the Linomat 5 sample application device. The reagents overlaid on the strips are indicated to the left.

TABLE 1. Amino acid sequence homology between components in the CFP-10/ESAT-6 and Rv0287/Rv0288 secretion pathways^a

CFP-10/ESAT6 pathway component(s)	Rv0287/Rv0288 pathway component	Description and/or possible function (reference[s])	% Homology
Rv3868	Rv0282	Members of CbxX/CfqX protein family; ATP binding (6, 23)	38
Rv3869	Rv0283	Transmembrane proteins (6, 23)	37
Rv3870, Rv3871	Rv0284	Dimeric membrane-bound ATPases of the FtsK family (6, 23)	33, 33
Rv3872	Rv0285	PE proteins (TubercuList)	32
Rv3873	Rv0286	Cell wall-associated PPE proteins (12, 14)	35
CFP-10	Rv0287	ESAT-6 proteins; secreted antigens (3, 20)	31
ESAT-6	Rv0288	ESAT-6 proteins; secreted antigens (20, 22)	29
Rv3877	Rv0290	Putative transmembrane transporter proteins (23)	28
Rv3883c	Rv0291	Serine proteases (6, 8)	44

^a Protein sequence comparisons were performed by BLASTP at the genome project website (<http://genolist.pasteur.fr/TubercuList/>).

Rv0288 pathway (Table 1), we were also interested in knowing whether these proteins interact with Rv0287/Rv0288. His-tagged rRv3871, rRv3872, and rRv3873 were purified to high purity and were subsequently tested to determine whether they cross-react with the anti-ESAT-6, anti-CFP-10, and anti-Rv0288 antibodies. This was, unfortunately, the case for rRv3871; all three antibodies cross-reacted with this protein, preventing a meaningful interpretation of the overlay results (data not shown). Therefore, only rRv3872 and rRv3873 were evaluated further in interaction studies with the ESAT-6 proteins. rRv3872 was applied to a nitrocellulose membrane at 1, 0.5, and 0.2 $\mu\text{g}/\text{cm}$. To compensate for the higher molecular weight, rRv3873 was applied at 3.6, 1.8, and 0.72 $\mu\text{g}/\text{cm}$. The membrane strips were overlaid with rESAT-6, rCFP-10, or rRv0288. Interestingly, rRv3873 interacted with all three ESAT-6 proteins, whereas rRv3872 did not bind to any of the proteins tested (Table 2). To investigate whether rRv3873 is sticky so that it would have nonspecific binding to many *M. tuberculosis* proteins, we performed protein-print overlay assays with other recombinant *M. tuberculosis* proteins. Screening of a panel of nine monoclonal antibodies (from the Statens Serum Institut monoclonal antibody collections) for high reactivity with recombinant *M. tuberculosis* proteins showed that four of the antibodies (ST-3 against meromycolate extension acyl carrier protein [AcpM], HBT2 against thiol peroxidase, Hyb 76-5 against heat shock protein HspX, and C24b1 against MPT64 [Rv1980c]) displayed sensitivities comparable to those of the antibodies against the ESAT-6 proteins. HspX has been reported to be a major membrane protein (14), and MPT64 is a secreted protein (10). Under the experimental conditions, none of these proteins bound to rRv3873 in overlay assays (data not shown). Thus, the interactions between the ESAT-6 proteins and rRv3873 are specific.

TABLE 2. Interaction of the ESAT-6 proteins with Rv3872 and Rv3873

Protein	Reactivity with ^a :	
	Rv3872	Rv3873
CFP-10	–	+
ESAT-6	–	+
Rv0287	ND	ND
Rv0288	–	+

^a +, reactive; –, nonreactive; ND, not determined.

Conclusions. Our results demonstrating that *rv0287* and *rv0288* are cotranscribed in *M. tuberculosis* and the finding that there is direct interaction between rRv0287 and rRv0288 support the hypothesis that the *esx* gene pairs are transcribed as operons and that the proteins interact directly (4, 21). It has been suggested previously that the interaction between ESAT-6 proteins may not be limited to the proteins within a gene pair and that the formation of complexes may also occur between proteins from different ESAT-6 protein pairs (21). Under the present experimental conditions, the recombinant ESAT-6 proteins interacted in a pairwise-specific way and no protein-protein binding was observed across the ESAT-6/CFP-10 and Rv0287/Rv0288 protein pairs.

ESAT-6 and CFP-10 are components of a functional pathway encoded by the *esx* cluster 1 (Table 1). In the present study, we investigated whether ESAT-6 and CFP-10 interact directly with other protein components of this pathway and we found that Rv3873 interacts directly with ESAT-6 and CFP-10, indicating that Rv3873 may be an essential element of the ESAT-6/CFP-10 transportation system.

Most recently, Pym et al. have demonstrated that optimal secretion of ESAT-6 depends on the presence of an intact *esx* cluster 1 in *M. tuberculosis* (20), implying a substrate specificity of the pathway. However, recombinant *M. bovis* BCG lacking some of the components of the transport machinery was still able to secrete ESAT-6, albeit at a low level (20). This residual transport activity for ESAT-6 may be mediated through transport machineries encoded by other *esx* gene clusters. If such substrate cross-reactivity exists, one would expect direct protein-protein interactions between ESAT-6 proteins encoded by one *esx* cluster and components of the transport apparatus encoded by other *esx* clusters. In this regard, our study demonstrates that in addition to ESAT-6 and CFP10, Rv0288 encoded by *esx* cluster 3 interacts specifically with Rv3873. In this context, it should be mentioned that, although the overall homology between Rv3873 and its counterpart Rv0286 encoded by *esx* cluster 3 is only about 35% (Table 1), the homology of the N-terminal halves of the two molecules is much higher (50% identity). Whether this conserved region is directly involved in the interaction with the ESAT-6 proteins remains to be explored.

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