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Identification of a 35-Kilodalton *Mycobacterium tuberculosis* Protein Containing B- and T-Cell Epitopes

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Screening of a *Mycobacterium tuberculosis* genomic DNA library in the lambda gt11 expression vector was carried out by using, as probes, sera from tuberculous patients and murine monoclonal antibody H61.3 recognizing a mycobacterial 35-kilodalton protein present only on the *M. tuberculosis* complex. The recombinant β-galactosidase-fused protein present in the crude lysate induced the proliferation of T lymphocytes from patients with tuberculous pleuritis. As the recombinant insert contains an internal *Eco*RI restriction site, it was possible to identify two fragments, one proximal to the *lacZ* gene and 1.7 kilobases (kb) in size and the other distal to the *lacZ* gene and 2.2 kb in size. Southern blot analysis showed that both of them hybridized with the genomic DNA from *M. tuberculosis* and *M. bovis* but not with the DNA from other mycobacterial species. To perform extensive immunological studies, the amount of β-galactosidase-fused protein being very low, we fused the 1.7-kb fragment to the N-terminal part of the gene coding for the DNA polymerase of bacteriophage MS2 in the expression vector pEx34. The fusion protein was partially purified, and subsequent Western blotting (immunoblotting) and T-cell proliferation experiments confirmed the presence of B- and T-cell mycobacterial epitopes. Furthermore, to isolate the chromosomal region containing the 35-kilodalton gene, we constructed another mycobacterial genomic library in the lambda 2001 vector by cloning 15 to 20 kb of foreign DNA. Screening of this library was carried out by using 1.7- and 2.2-kb recombinant fragments as probes. Restriction maps of some clones isolated were determined.

Tuberculosis is an infectious disease which is caused mainly by *Mycobacterium tuberculosis* and which affects over 30 million people worldwide (29). Pathogenicity and immunity to this infection are mediated by the host T-cell immune response, which is directed against a variety of mycobacterial antigens (9, 11, 13, 19, 20, 30). Until recently, efforts to isolate biochemically single antigens specific for the tuberculous complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) have been largely unsuccessful (4).

In an attempt to develop new diagnostic and vaccine tools for the control of tuberculosis, the antigens of *M. tuberculosis* and *M. bovis* have been cloned into expression vectors by workers in several laboratories (25, 32) and the T-cell-mediated immune response has been analyzed with recombinant proteins (10, 13–15, 19).

A genomic DNA library of *M. tuberculosis* has been constructed by Young et al. (32) with the lambda gt11 expression vector. Several screenings of this library have been performed with murine monoclonal antibodies specific for mycobacterial antigens in the last 4 years (5). Three recombinant proteins have been produced and studied in detail regarding the presence of T-cell epitopes. A 65-kilodalton (kDa) protein has been sequenced (22), and mapping of T-cell epitopes has been done by both subcloning and peptide synthesis (14). Recombinant mycobacterial proteins recognized by monoclonal antibodies directed against 19- and 14-kDa proteins present on the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) have been produced and studied regarding their ability to induce T-cell activation in vitro and in vivo (10, 15). Moreover, screening of the lambda gt11 library performed with a polyclonal rabbit serum has indicated the immunodominance of the 65-, 71-, and 14-kDa mycobacterial proteins (31).

By a preliminary screening of the lambda gt11 *M. tuberculosis* library performed in our laboratories with sera from tuberculous patients, we have isolated a colony producing a recombinant protein recognized by murine monoclonal antibody H61.3. We have shown that this antibody recognizes a 35-kDa protein present only on the *M. tuberculosis* complex (3). This protein contains a T-cell epitope(s), as determined by a T-cell proliferation assay (28). The presence of this protein within the lung macrophages of tuberculous patients (G. Barbolini, A. Bisetti, V. Colizzi, G. Damiani, M. Migaldi, and D. Vismara, Hum. Pathol., in press) suggests that it might be relevant in the induction of a specific immune response to *M. tuberculosis*. The purposes of this study were (i) to isolate and characterize the gene coding for this antigen, (ii) to perform Southern blot analysis with DNA from several mycobacterial species to confirm, at the DNA level, the species specificity of the 35-kDa protein, and (iii) to produce sufficient amounts of the recombinant epitope for immunological analysis and for subsequent determination of its protective role in vivo.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The *Escherichia coli* strains used in this work were Y1088, Y1089, and Y1090 (33); K-12 ΔH1 Δ*trp*, containing a heat-sensitive repressor (21); Q359 to screen the lambda 2001 library (7); and LE392 to grow the positive recombinant phages selected from the lambda 2001 library (16). The recombinant DNA library of *M. tuberculosis* genomic DNA in the lambda gt11 expression

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vector was constructed by Young et al. (32) and made available through the World Health Organization Program for Research in the Immunology of Tuberculosis. Plasmid vector pEx34 is a derivative of pEx29 (24). Phage lambda 2001 is a derivative of phage lambda 1059 (8). Manipulation of DNA was performed by standard procedures (16).

Human sera and murine monoclonal antibody. Sera from patients with pulmonary tuberculous were kindly provided by Forlanini Hospital, Rome, Italy. The sera were pooled and preadsorbed extensively to *E. coli* Y1090. H61.3 is a murine monoclonal antibody of the immunoglobulin G2b isotype which recognizes a 35-kDa antigen associated with the cell wall and expressed only on the *M. tuberculosis* complex (3). This antibody was used as ascites.

Immunoscreening of the lambda gt11 library. Screening was performed as described by Young and Davis (33) with only minor modifications. Phage-infected Y1090 was plated in top agar on Luria-Bertani plates (0.5×10^5 to 1.0×10^5 PFU per 150-mm plate) and incubated for 2.5 h at 42°C. Isopropyl-β-D-thiogalactoside-saturated nitrocellulose filters were overlaid on plates, and incubation was continued for 3.5 h at 37°C. Screening was done with sera from tuberculous patients; the filters were blocked in Tris buffered solution (TBS)–20% fetal calf serum, incubated overnight with antibodies, washed four times with TBS, and incubated again for 1 h in TBS–20% fetal calf serum containing biotinylated anti-human antibodies. The filters were rinsed four times, transferred to a solution containing streptavidin-peroxidase, and incubated for 1 h. After four washes with 50 mM Tris (pH 6.8), the peroxidase substrate solution (4-chloronaphthol) was added. Three rounds of reisolation of the phages were performed to ensure clonality.

Production of crude lysates containing the β-galactosidase-fused protein and purification of the MS2 polymerase-fused proteins. A crude lysate containing the recombinant antigen was prepared by expressing lambda gt11 recombinants as lysogens in *E. coli* Y1089 (33). Lysogens were grown to a high cell density on Luria-Bertani plates at 37°C and then incubated at 42°C for 20 min. Isopropyl-β-D-thiogalactoside was added to induce expression and synthesis of the β-galactosidase-fused protein. Recombinant proteins were extracted by cell sonication, and the suspension was resuspended in 1/20 of the original volume. The production and purification of MS2 polymerase-fused proteins were performed as described previously by Nicosia et al. (18) with only minor modifications. At the end of the procedure, recombinant proteins were suspended either in 5 ml of H_2O or in 7 M urea. In some cases, MS2 polymerase-fused proteins were loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, visualized by staining with Coomassie blue, sliced out of the gel, and purified by electroelution from the SDS-polyacrylamide gel for 16 h at 150 V and 2 mA (Economo-Columns). With this system it was possible to obtain 5 to 30 mg of purified protein from 1 liter of culture (data not shown; 18).

Western blotting (immunoblotting). To detect antigens present in lysates containing β-galactosidase- or MS2 DNA polymerase-fused proteins, we mixed the samples with 1 volume of SDS loading buffer and loaded them on 6 or 15% SDS-polyacrylamide gels for SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (12). The polyacrylamide gels were stained with Coomassie blue or transferred to nitrocellulose filters as reported by Towbin et al. (26).

Extraction of mycobacterial genomic DNA. The mycobacterial species and strains used were *M. tuberculosis* (H37RV and Jhonston strains), *M. bovis* (BCG strain), *M. kansasii*, *M. fortuitum*, *M. intracellulare*, *M. avium*, and *M. scrofulaceum*. Bacteria were grown in broth at 37°C for 2 to 3 weeks. The cells were harvested by filtration, homogenized, and suspended in about 5 ml of 25% sucrose–50 mmol of Tris (pH 8). Lysozyme (50 μl of a 40-mg/ml solution), proteinase K (10 μl of a 20-mg/ml solution), and EDTA (0.5 M) were added for 10 min each on ice. The cells were lysed with Sarkosyl (10%) and heat inactivated (50°C for 16 h). Genomic DNA was extracted with phenol-chloroform, precipitated with ethanol, centrifuged, and suspended in distilled water.

Southern blotting with mycobacterial genomic DNA. Purified fragments of mycobacterial recombinant DNA were obtained from digested pEx34. Random primer radiolabeling with [^{32}P]dATP and [^{32}P]dCTP was performed, and the fragments were used as probes in Southern blotting analysis (23) with *Bam*HI-digested mycobacterial genomic DNA. The stringency conditions were 65°C and 0.1% SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate); reduced-stringency conditions were not used.

Construction and screening of a mycobacterial genomic library with the phage lambda 2001 vector. High-molecular-weight *M. tuberculosis* genomic DNA was prepared by using a CsCl gradient (16). After partial digestion with restriction endonuclease *Sau*3AI and size fractionation of restriction fragments on a 10 to 40% sucrose gradient, 15- to 20-kilobase (kb) DNA fragments were ligated to *Bam*HI-digested lambda 2001 DNA and packaged in vitro (16). Recombinant phages were selected by plating on *E. coli* Q359 (16). Amplification and screening of the library and restriction mapping of selected positive clones were performed by standard procedures (16).

Preparation of lymphocytes and proliferation assay. Mononuclear cells from the ascitic effusion of a patient with tuberculous pleuritis and from the peripheral blood of three healthy contacts positive in a purified protein derivative skin test were isolated by centrifugation on a discontinuous density gradient of Ficoll-Hypaque (27). The cells were suspended in complete medium (RPMI 1640 supplemented with 10% heat-inactivated pooled human serum, 2 mM L-glutamine, and 100 IU each of penicillin and streptomycin per ml). Cells (10^6/ml) were cultured in 96-well flat-bottomed microdilution plates for 5 days with different concentrations of soluble purified protein derivative (Serum Institute, Copenhagen, Denmark), with lysates containing β-galactosidase-fused protein, or with MS2 DNA polymerase-fused recombinant proteins at different levels of purification. After 5 days of incubation in a humidified atmosphere of 5% CO_2–95% air at 37°C, the cells were pulsed with [^{3}H] thymidine (1 μCi; Amersham International) for 16 h and automatically harvested onto glass fiber filters. Proliferation was correlated with thymidine incorporation, expressed as the mean counts per minute ± standard deviation of the average counts of duplicate or triplicate cultures.

RESULTS

Identification of a recombinant mycobacterial protein by screening of the lambda gt11 *M. tuberculosis* library. A preliminary screening of 10^6 PFU of the *M. tuberculosis* genomic library with a pool of sera from tuberculous patients allowed the isolation of several plaques giving positive signals.

Crude lysates were prepared from lysogenic clones corresponding to three distinct recombinant clones (Fig. 1A, lanes

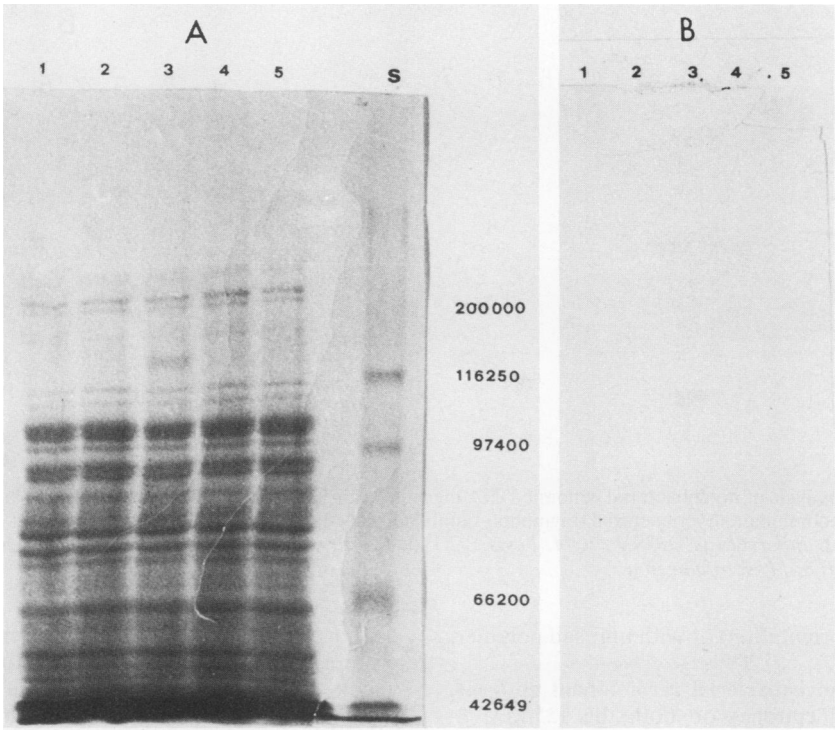

FIG. 1. (A) Polyacrylamide slab gel of recombinant lambda gt11-infected *E. coli* stained with Coomassie blue. The polyacrylamide gel was loaded with crude lysates, and the molecular masses of proteins were determined with the following standards (Bio-Rad Laboratories) (lane S): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (42 kDa). (B) Western blotting of recombinant lambda gt11-infected *E. coli* incubated with murine monoclonal antibody H61.3. Lanes: 1, 2, and 3, distinct recombinant clones; 4, wild-type lambda gt11-infected *E. coli* Y1089; 5, uninfected *E. coli* Y1089.

1, 2, and 3, corresponding to clones 1, 2, and 3, respectively), from lysogenic *E. coli* Y1089 infected with wild-type lambda gt11 (lane 4), and from uninfected *E. coli* Y1089 (lane 5). The proteins present in these crude lysates were separated according to their mobility in SDS-PAGE and stained with Coomassie blue. There was a band of 125 kDa in lane 3 which was absent in the other recombinant lysates (lanes 1 and 2) and in the negative control preparations (lanes 4 and 5). The proteins were also transferred to nitrocellulose filters and probed with a panel of murine monoclonal antibodies. The only positive signal was obtained with murine monoclonal antibody H61.3 (Fig. 1B). Western blotting performed with this antibody revealed a single band of 125 kDa in lane 3 but not in the other lanes, indicating that the 125-kDa band could correspond to an *M. tuberculosis* fusion protein. Antibody H61.3 recognizes an epitope present on the 35-kDa protein of the cell walls of the *M. tuberculosis* complex organisms (3; Barbolini et al., in press).

Southern blot analysis with *M. tuberculosis* genomic DNA and other mycobacterial species. To confirm the specificity of the identified *M. tuberculosis* DNA clone, we used two DNA fragments of 1.7 and 2.2 kb obtained after *Eco*RI digestion of recombinant lambda gt11 clone 3 as probes in Southern blotting experiments. Mycobacterial DNA was extracted and digested with *Bam*HI. Both the 1.7-kb (Fig. 2A) and 2.2-kb (Fig. 2B) probes specifically recognized a band of 4.2 kb in *M. tuberculosis* H37Rv (lane 1) and Jhonston (lane 3) and *M. bovis* BCG (lane 2). The 4.2-kb band was predicted by a restriction map determined from the recombinant clones isolated from the lambda 2001 library (see below). No positive signals were observed with the other mycobacterial species (*M. kansasii*, *M. fortuitum*, *M. intracellulare*, *M. avium*, and *M. scrofulaceum*) (Fig. 2).

Expression and purification of MS2-fused mycobacterial protein in expression vector pEx34. Expression vector pEx34 contains a coding region for the N-terminal part of the MS2 polymerase under the control of the p_L promoter of phage lambda. Since the 1.7-kb fragment is located proximal to the *lacZ* gene in lambda gt11, this fragment must be the one coding for the mycobacterial fusion protein. Therefore, this 1.7-kb fragment was cloned in both orientations into pEx34B, which contains the same reading frame of the *Eco*RI site as does lambda gt11. The two recombinant proteins were purified and analyzed by SDS-PAGE. Fig. 3A shows total *E. coli* cell lysates analyzed by SDS-PAGE before (lanes 1 and 2) and after (lanes 3 and 4) partial purification. Lanes 5 and 6 show that the same recombinant proteins eluted from the gel. A major band of 24 kDa was present in lanes 1, 3, and 5. Another band of 12 kDa was present in lanes 2, 4, and 6. These two bands corresponded to the MS2 polymerase-fused proteins. Lane 7 shows the partially purified polymerase (10 kDa); lane 8 shows molecular weight markers.

Western blotting of three distinct extracts was performed with monoclonal antibody H61.3 (Fig. 3B). Lane 1 contains the unfused MS2 polymerase, lane 2 contains the recombinant 12-kDa protein, and lane 3 contains the recombinant 24-kDa protein. The 24-kDa protein band was recognized by H61.3, indicating that this protein is the product of the 1.7-kb fragment cloned into pEx34 in the correct orientation. No positive signals were observed with the 12-kDa band (pro-

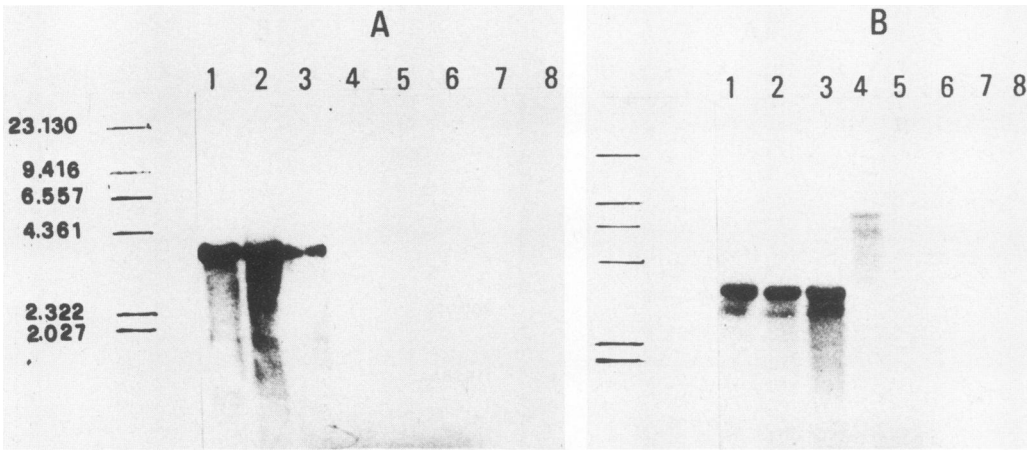

FIG. 2. Southern blot analysis of mycobacterial genomic DNA digested with *Bam*HI restriction endonuclease and hybridized with labeled 1.7-kb (A) and 2.2-kb (B) recombinant mycobacterial fragments. Lambda DNAs digested with *Hin*dIII were used as molecular mass standards (in kilobases). Lanes: 1, *M. tuberculosis* H37RV; 2, *M. bovis* BCG; 3, *M. tuberculosis* Jhonston; 4, *M. kansasii*; 5, *M. fortuitum*; 6, *M. intracellulare*; 7, *M. avium*; 8, *M. scrofulaceum*.

duced by the incorrect orientation) or with unfused polymerase.

T-cell recognition of mycobacterial recombinant antigens. The presence of T-cell epitopes on both the 125-kDa β-galactosidase-fused protein and the MS2 polymerase-fused protein was detected by a T-lymphocyte proliferation assay. For this purpose, a T-cell population from the ascitic effusion of a patient with tuberculous pleuritis was tested with the three distinct clones from the lambda gt11 recombinant library. The recombinant crude lysate from clone 3 was able to activate T cells, while the lysates from uninfected *E. coli* Y1089, from *E. coli* Y1089 infected with wild-type lambda gt11, and from clones 1 and 2 failed to do so (Fig. 4A). Thus, the recombinant protein produced by clone 3 contains a T-cell epitope(s).

The T-cell recognition pattern of the 24-kDa recombinant protein produced in the pEx34 vector is shown in Fig. 4B, which reports the data for one such experiment. The 24-kDa recombinant protein induced a significant level of DNA synthesis, while the 12-kDa recombinant protein and the unfused polymerase failed to do so.

Construction and screening of a mycobacterial genomic library in lambda 2001 and restriction map of positive clones. High-molecular-weight genomic mycobacterial DNA was obtained and digested with *Sau*3AI restriction endonuclease. Fragments of 15 to 20 kb were ligated to *Bam*HI-digested lambda 2001 DNA. After in vitro packaging, the library was amplified in *E. coli* Q359. Screening was performed by plaque hybridization with ^{32}P-labeled 1.7- and 2.2-kb recombinant mycobacterial fragments as probes. Several recombi-

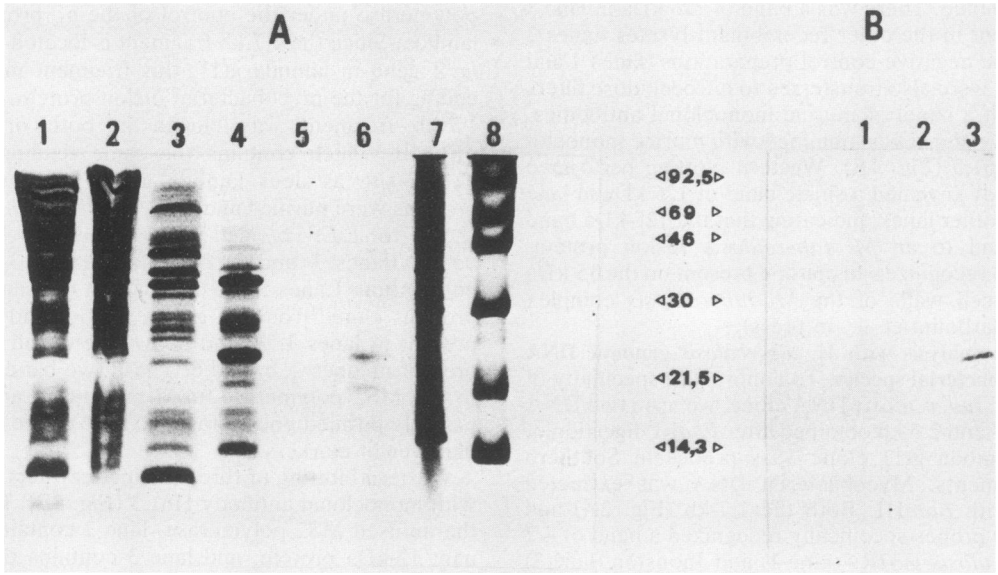

FIG. 3. (A) Expression and purification of MS2-fused proteins in pEx34. Lanes: 1 and 2, SDS-PAGE of total cell lysates; 3 and 4, partially purified fusion proteins; 5 and 6, electroeluted fusion proteins; 7, partially purified MS2 DNA polymerase from pEx34; 8, molecular mass standards (in kilodaltons). (B) Western blotting of recombinant MS2 polymerase-fused proteins incubated with murine monoclonal antibody H61.3. Lanes: 1, unfused MS2 polymerase; 2 and 3, recombinant MS2 polymerase-fused proteins (12 and 24 kDa, respectively).

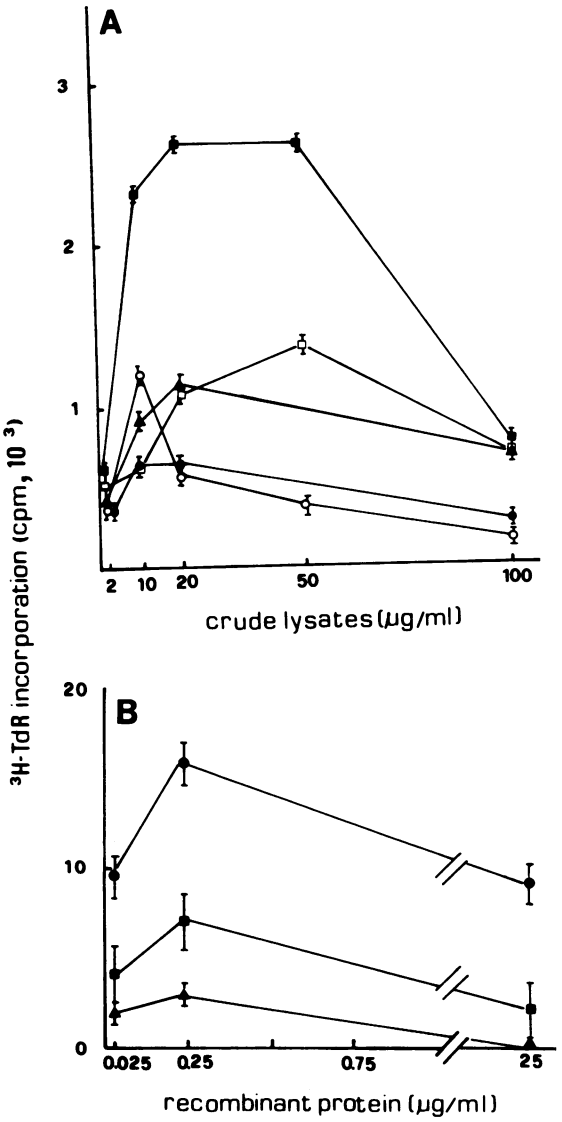

FIG. 4. Effect of recombinant proteins on the activation of *M. tuberculosis*-specific T lymphocytes. (A) Mononuclear cells were cultured for 5 days with different amounts of crude lysates from three different recombinant clones (1 [▲], 2 [□], and 3 [■]), from uninfected *E. coli* Y1089 (○), and from wild-type lambda gt11-infected *E. coli* Y1089 (●). T-cell cultures stimulated with 20 μg of purified protein derivative incorporated 28,385 ± 4,562 cpm in the same experiment. (B) Peripheral blood mononuclear cells were cultured for 3 days with different amounts of the 24-kDa (●) and 12-kDa (■) proteins purified by electroelution. Unfused polymerase (▲) was used as a negative control. T-cell proliferation was evaluated as radioactive incorporation of [^{3}H]thymidine (^{3}H-TdR), and results were expressed as counts per minute.

nant phages containing the genomic mycobacterial region of interest were isolated. Three of them (3_1, 4_1, and 4_8), including a 20-kb region of the *M. tuberculosis* chromosome, were mapped with the restriction endonucleases *Bam*HI, *Eco*RI, and *Xho* (Fig. 5).

DISCUSSION

Our study was begun with the goal of identifying T-cell epitopes specific for *M. tuberculosis* and relevant to the antitubercular immune response. For this purpose, we used the strategy of screening the recombinant *M. tuberculosis* lambda gt11 DNA library with human polyclonal antibodies from tuberculous patients, increasing the chance of detecting protein antigens relevant to this infection. The subsequent use of murine monoclonal antibodies and human T cells allowed a better definition of the B- and T-cell epitopes present in the fusion proteins. A β-galactosidase-fused protein of approximately 125 kDa has been identified by screening of an *M. tuberculosis* DNA lambda gt11 library first with human polyclonal sera and then with murine monoclonal antibodies and human T lymphocytes. This molecule is recognized by the murine monoclonal antibody H61.3, which specifically recognizes an epitope present on an *M. tuberculosis* complex-restricted 35-kDa protein associated with the cell wall of *M. tuberculosis* (3). More recent immunohistological studies have shown that this antibody binds the 35-kDa mycobacterial protein within the pulmonary macrophages of tuberculous patients (Barbolini et al., in press). The specificity of this epitope for the tuberculous complex has been confirmed at the DNA level. In fact, the recombinant fragments of 1.7 and 2.2 kb derived from positive clone 3 hybridized with *Bam*HI-digested *M. tuberculosis* and *M. bovis* DNAs (but not with DNAs of other species of mycobacteria) and recognized a *Bam*HI fragment (4.2 kb) predicted by the restriction map of lambda 2001-positive clones. Moreover, the finding that the recombinant proteins (fused to either β-galactosidase or MS2 polymerase) induced the proliferation of human T lymphocytes confirms previous observations on the presence of T-cell epitopes on 35-kDa mycobacterial proteins.

As lambda gt11 was not designed for high-level production of recombinant proteins in *E. coli*, the mycobacterial insert was subcloned in both orientations into the pEx34 expression vector and expressed as recombinant proteins fused to a fragment of DNA coding for the N terminus of the gene coding for the MS2 DNA polymerase under the control of the p_L promoter of phage lambda (18). The MS2 polymerase-fused proteins were produced in large amounts and purified by electroelution. The availability of these proteins has allowed the analysis of the T-cell-mediated immune response in vivo. In fact, preliminary observations in mice infected with *M. bovis* have shown that the MS2 polymerase-mycobacterium fusion protein is recognized by T cells in vivo by the development of a positive footpad skin test (unpublished observation).

A 35-kDa protein has also been identified by screening a plasmid *M. tuberculosis* library with rabbit polyclonal serum, and antibodies to this protein have been detected in 40% of patients with active tuberculosis (2). Moreover, a 38-kDa antigen has recently been isolated by affinity chromatography with a murine monoclonal antibody recognizing a B-cell epitope present in *M. tuberculosis* and *M. bovis*. However, in vivo results from skin tests and in vitro results from lymphocyte proliferation assays with cells from guinea pigs sensitized with *M. tuberculosis* and other mycobacterial species have revealed cross-reactions to antigens from nontuberculous mycobacterial species (6). Other studies with a 38-kDa protein purified with a different monoclonal antibody have shown that this protein contains multiple epitopes capable of inducing the proliferation of human T-cell clones, some of which were specific for *M. tuberculosis* and some of which were cross-reactive (17).

Andersen and Hansen (1) recently published the complete amino acid sequence of the 38-kDa b antigen (Pab) of *M. tuberculosis*, which is solely detected in virulent *M. tuber-*

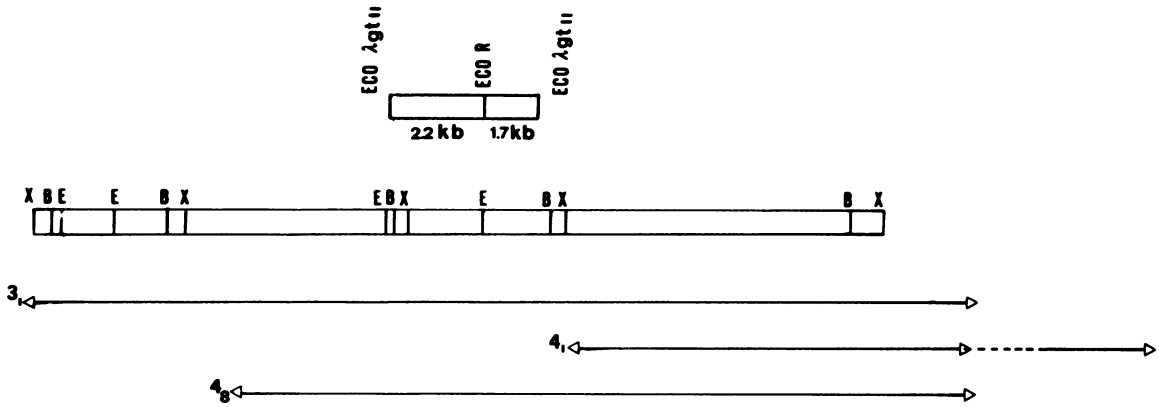

FIG. 5. Restriction endonuclease map of the *M. tuberculosis* chromosomal region containing the 1.7- and 2.2-kb fragments isolated from the lambda gt11 library. This region contains the gene coding for the 35-kDa *M. tuberculosis* protein. Shown are the *Bam*HI (B), *Eco*RI (E), and *Xho* (X) sites present in the mycobacterial region analyzed. Below the map are shown the lambda 2001 recombinant phages (3_1, 4_1, and 4_8) which were used to construct the map.

culosis and in lesser amounts in the vaccine strain *M. bovis* BCG. A restriction map comparison of the 35-kDa and 38-kDa *M. tuberculosis* proteins and an analysis of the entire 38-kDa amino acid sequence determined from the 2,000 base pairs of the 35-kDa protein sequenced so far have revealed no significant homology.

The construction and screening of the lambda 2001 mycobacterial library have allowed the isolation of the chromosomal region containing the gene coding for the 35-kDa protein. The sequencing is in progress, and more detailed immunological studies with the synthetic peptide approach and subcloning of restriction fragments will be performed.

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