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Molecular Cloning, Expression, Purification and Immunological Characterization of Proteins Encoded by Regions of Difference Genes of *Mycobacterium tuberculosis*

Shumaila Nida Muhammad Hanif, Rajaa Al-Attiyah and
Abu Salim Mustafa
Department of Microbiology, Faculty of Medicine, Kuwait University
Kuwait

1. Introduction

Tuberculosis (TB) is an international infectious disease problem, but people in the developing countries are the major sufferers (World Health Organization, 2010). The disease in humans is primarily caused by a pathogenic mycobacterial species known as *Mycobacterium tuberculosis*. The current estimates suggest that about 1/3rd of the world population is latently infected with *M. tuberculosis*, 9 million people develop the active disease and 1.7 million die of TB each year (World Health Organization, 2009). Among infectious diseases, TB is the second top most killer of adults, after HIV/AIDS, and is among the overall top 10 causes of death in the world (World Health Organization, 2008). The global problem of TB is so serious that it has been declared "a global emergency" by the World Health Organization (WHO), the first declaration of its kind by WHO. Several factors have contributed in the deterioration of the global problem of TB, which include the increase in the incidence of drug-resistant TB (multi and extensive drug resistance), migration of people, due to political and economic reasons, from poor and highly endemic countries to countries with low endemicity, and co-infection with *M. tuberculosis* and HIV (Borgdorff et al., 2010; Chiang et al., 2010; Semba et al., 2010). The worldwide control of TB requires cost-effective, sensitive and specific methods to diagnose latent as well as active TB, and vaccines that can provide protection in all human populations irrespective of their immune status, geographical locations and environmental conditions (Mustafa, 2009, 2010).

Tuberculin skin test (TST) is considered the standard test for the diagnosis of infection with *M. tuberculosis*. The test involves intradermal injection of purified protein derivative (PPD), which is a poorly defined and crude mixture of secreted and somatic proteins present in the culture filtrate of *M. tuberculosis*. For TST, PPD is injected in the forearm and the ensuing delayed type hypersensitivity (DTH) skin reaction (induration) is read after 48 to 72 h. A DTH reaction with induration of 10 mm or greater is considered positive in immunocompetent subjects (Mahadevan et al., 2005; Moffitt & Wisinger, 1996). Although, PPD has been used for TB diagnosis and epidemiological studies for more than half a century, it cannot distinguish between active disease, prior sensitization by contact with *M.*

tuberculosis, BCG vaccination, or cross-sensitization by environmental mycobacteria (Hill et al., 2006; Leung et al., 2005). This is because PPD contains a large number of mycobacterial antigens (>200), some of which are shared among all the pathogenic mycobacteria belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*), environmental non-tuberculous mycobacteria (NTM), and the substrains of *M. bovis* bacille Calmette-Guerin (BCG) vaccines. Thus, although response to PPD is an important aid in the diagnosis of TB and can give an indication of exposure to mycobacteria, it is often impossible to distinguish BCG vaccination and exposure to NTM from *M. tuberculosis* infection. Thus, TST has poor diagnostic value, especially in geographic areas where BCG vaccine is routinely used or the environmental burden of non-tuberculous mycobacteria is high (Kurup et al., 2006). Therefore, there is a need to identify and produce *M. tuberculosis*-specific antigens, which can be useful as skin test reagents for specific detection of infection with *M. tuberculosis.*

Vaccination is considered the most important tool to provide protection against TB, and attempts have been made to vaccinate humans, against TB, since the discovery of the TB bacillus by Koch in 19th century. However, the best vaccine available till date is the live attenuated strain of *M. bovis*, i.e. BCG, developed by Calmette and Guerin in 1921. Since then BCG has been used as a vaccine to prevent TB. In spite of its widespread application for about 90 years and many advantages like being inexpensive, safe at birth, given as a single shot, and provision of some protection against childhood TB and severe manifestations of the disease (Trunz et al., 2006), BCG vaccine remains the most controversial vaccine in current use (Fine, 2001). This is because its protective efficacy has varied widely in different parts of the world (Haile & Kallenius, 2005). In particular, BCG has failed to provide protection in developing countries against pulmonary TB, the most common manifestation of TB, in adults (Crampin et al., 2009; Narayanan, 2006). Furthermore, the widespread use of BCG vaccination faces two additional problems. First is the interference with TST in the diagnosis of TB because BCG vaccination induces a DTH response that cannot be distinguished from infection with *M. tuberculosis*, and therefore it becomes difficult to use TST for diagnostic or epidemiological purposes (Mustafa & Al-Attiyah, 2003). Second, BCG, being a live vaccine, cannot be used in all groups of people because it may cause disease by itself in immunocompromised people (Hesseling et al., 2007). The WHO has recommended that children with symptoms of HIV or AIDS should receive all the vaccines except BCG (World Health Organization, 2007). An ideal vaccine against TB should be safe and induce protective immunity both in immunocompetent and immunocompromised individuals. The development of a better BCG vaccine or alternative vaccines requires the identification and production of *M. tuberculosis*-specific antigens recognized by protective immune responses.

2. Identification of *M. tuberculosis*-specific genomic regions and encoded antigens

The existence of three *M. tuberculosis* / *M. bovis*-specific genomic regions deleted in BCG, i.e. regions of difference (RD)1, RD2 and RD3, was first described by Mahairas et al. in 1996 by employing subtractive genomic hybridization to identify genetic differences between *M. tuberculosis*, *M. bovis* and BCG (Mahairas *et al*., 1996; Table 1). Later, in 1999, studies using comparative genome analysis identified 16 RDs that were present in *M. tuberculosis* H$_{37}$Rv (RD1 to RD16) but deleted/absent in some or all strains of *M. bovis* and/or BCG (Behr et al., 1999; Table 1). Among these, 11 regions (RD1, RD4 to RD7, RD9 to RD13 and RD15)

covering 91 open reading frames (ORFs) of *M. tuberculosis* $H_{37}Rv$ were deleted from all vaccine strains of BCG (Table 1). It was suggested that the identification of antigens encoded by the genes in *M. tuberculosis*-specific RDs may identify new *M. tuberculosis*-specific antigens with diagnostic and /or vaccine potential (Mustafa, 2001, 2002).

RD designation	No. of ORFs	ORFs designation	ORFs deleted / absent in	
			M. bovis strains	BCG strains
RD1	8 9 14	ORF1A-ORF1K Rv3871-Rv3879 ORF2-ORF15	Not deleted	All strains
RD2	11	Rv1978-Rv1988	Not deleted	Connaught , Danish, Frappier, Glaxo, Pasteur, Phipps, Prague, Tice
RD3	14	Rv1573-Rv1586c	3/8	All strains
RD4	3	Rv0221-Rv0223c	8/8	All strains
RD5	5	Rv3117-Rv3121	8/8	All strains
RD6	11	Rv1506c-Rv1516c	8/8	All strains
RD7	8	Rv2346c-Rv2353c	8/8	All strains
RD8	4	Rv0309-Rv0312	Not deleted	Connaught, Frappier
RD9	7	Rv3617-Rv3623	8/8	All strains
RD10	3	Rv1255c-Rv1257c	8/8	All strains
RD11	5	Rv3425-Rv3429	8/8	All strains
RD12	4	Rv2072c-Rv2075c	8/8	All strains
RD13	16	Rv2645-Rv2660c	4/8	All strains
RD14	8	Rv1766-Rv1773c	Not deleted	Pasteur
RD15	15	Rv1963c-Rv1977	8/8	All strains
RD16	6	Rv3400-Rv3405c	Not deleted	Moreau

Table 1. Designation of *M. tuberculosis* RDs deleted/absent in *M. bovis* and/or BCG, the number of ORFs predicted in each ORFs designations.

To analyze the *M. tuberculosis*-specific RDs for cell mediated immune (CMI) responses involving T helper (Th)1 cells that are associated with protection against TB, a novel approach of overlapping synthetic peptides was used (Al-Attiyah & Mustafa, 2008). Each peptide was 25 aa in length and overlapped with the neighboring peptides by 10 aa. Since the length of Th1 cell epitopes is usually between 8 to 10 aa (Mustafa, 2000, 2005a), the

overlapping synthetic peptide strategy minimized the possibility of missing the potential Th1 cell epitopes. A total of 1648 peptides were synthesized and peptide pools corresponding to each RD were tested for reactivity with human PBMC in Th1-cell assays, i.e. antigen-induced proliferation and IFN-γ secretion. The results showed that RD1 was the most important region encoding proteins with Th1-cell reactivity (Al-Attiyah & Mustafa, 2008, 2010; Mustafa & Al-Attiyah, 2009; Mustafa et al., 2011). The analysis of individual peptide pools of RD1 ORFs identified three major (Rv3873, Rv3874 and Rv3875) and three moderate (Rv3871, Rv3872 and Rv3876) antigens stimulatory for Th1 cells in antigen-induced proliferation and IFN-γ assays (Hanif et al., 2008; Mustafa 2005b; Mustafa et al., 2008). However, Rv3873 and Rv3876 proteins were equally good stimulators of Th1 cells from TB patients and healthy donors, whereas Rv3872, Rv3874, Rv3875 proteins were better stimulators in TB patients and Rv3871 protein in healthy donors (Mustafa et al., 2008). When tested with PBMC in a cattle model of TB, Rv3872, Rv3873, Rv3874 and Rv3875 were also found to be major to moderate stimulators of Th1 cells present in the peripheral blood of *M. tuberculosis*-infected Cattle (Mustafa et al., 2002). The overall results showed that *M. tuberculosis*-specific proteins of RD1 Rv3872, Rv3873, Rv3874 and Rv3875 are major Th1-cell antigens and therefore could be useful as antigens in specific diagnosis and developing new vaccines against TB. However, for these applications, it was essential that the genes encoding these proteins are cloned and expressed in suitable vectors and the recombinant proteins are obtained in a purified form.

The predictions for all ORFs with Rv numbers are according to the predictions of Behr et al. (Behr et al., 1999), whereas RD1 ORF1A to ORF1K and ORF2 to ORF15 are according to the predictions of Mahairas et al. and Mustafa, respectively (Mahairas et al., 1996; Mustafa, 2005c). These RDs are expected to encode a total of 134 ORFs, of which 91 ORFs are predicted in RD1, RD4 to RD7, RD9 to RD13 and RD15, which are present in all strains of *M. tuberculosis*, but absent/deleted in all strains of BCG (Mustafa, 2001). RD3, although deleted in all BCG strains, is also deleted from some clinical strains of *M. tuberculosis* as well (Behr et al., 1999).

3. Molecular cloning, expression, purification and immunological characterization of proteins encoded by RD genes

In order to immunologically characterize the putative RD proteins encoded by *M. tuberculosis*-specific genes deleted/absent in BCG, previous studies attempted to clone and express six ORFs of RD1, i.e. ORF10 to ORF15 (Amoudy et al., 2006), as recombinant proteins in *E. coli*. However, these studies were successful in expressing five of the six targeted proteins as fusion proteins, fused with glutathione S-transferase (GST), by using the commercially available pGEX-4T vectors, but only two of them could be purified using glutathione-Sepharose affinity columns (Ahmed et al., 1999a, Amoudy et al., 2007; Amoudy & Mustafa, 2008). The problems included degradation of the mycobacterial proteins, non-binding of fusion proteins to affinity columns and the presence of contaminating *E. coli* proteins in purified preparations (Ahmed et al., 1999b, Amoudy & Mustafa, 2008). These studies have shown that even though high level expression of several mycobacterial proteins could be achieved by fusing them with GST, their purification was unsatisfactory and sometimes difficult due to improper folding of the fusion proteins and co-elution of *E. coli* proteins from the column along with the mycobacterial proteins. This is mainly due to the fact that the binding of GST containing fusion proteins on glutathione-Sepharose column

is dependent on the proper folding of the GST tag and some *E. coli* proteins bind to the column non-specifically. However, binding of proteins fused with the 6x His tag to Ni-NTA agarose is not affected by the conformation of the expressed proteins and, consequently, proteins containing this tag can be purified even under denaturing conditions (Hendrickson et al., 2000). Therefore a new vector, pGES-TH-1, was developed using pGEX-4T-1 vector as the backbone (Ahmad et al., 2003). In addition to the features offered by the pGEX-4T vectors for high level expression of fusion proteins, the new vector was expected to allow relatively easy purification of recombinant proteins on the highly versatile Ni-NTA-agarose affinity matrix. The experimental utility of the new vector was demonstrated by expressing and purifying to homogeneity two proteins of RD1 (Rv3872 and Rv3873) and three proteins of RD15 (Mce3A, Mce3D and Mce3E) of *M. tuberculosis* (Ahmad et al., 2003, 2004, 2005). All these proteins were expressed in *E. coli*, in fusion with GST. The recombinant RD proteins were purified and isolated, free of GST, by affinity purification on glutathione-Sepharose and/or Ni-NTA-agarose affinity matrix and cleavage of the purified fusion proteins by thrombin protease. All recombinant proteins were more than 90% pure and were immunologically reactive with antibodies present in sera of TB patients and/or immunized animals (Ahmad et al., 2003, 2004, 2005; El-Shazly et al., 2007). These results demonstrated the utility of the newly constructed pGES-TH-1expression vector with two affinity tags for efficient expression and purification of recombinant RD proteins expressed in *E. coli*, which could be used for further diagnostic and immunological investigations both in animals as well as in humans.

In a recent study, we extended the work to achieve high level expression and purification of three low molecular weight proteins encoded by *M. tuberculosis*-specific genomic regions of RD1 and RD9, i.e. Rv3874, Rv3875 and Rv3619c, using the pGES-TH-1 expression vector (Hanif et al., 2010a). The complete cloning, expression and purification strategy for these proteins is shown in figure 1, by using Rv3875 as an example (Fig. 1). In brief, DNA corresponding to each gene was amplified using gene-specific forward (F) and reverse (R) primers and genomic DNA of *M. tuberculosis* by polymerase chain reaction (PCR). In addition to gene-specific sequences, in order to facilitate cloning in the pGEST-TH-1 vector, the F and R primers had *Bam*H 1 and *Hind* III restriction sites at their 5′ ends, respectively. The PCR-amplified DNA were analyzed by agarose gel electrophoresis, which showed the amplified fragments corresponded to the size of the respective genes. The PCR-amplified DNA were ligated to pGEM-T Easy vector DNA, yielding recombinant plasmids pGEM-T/Rv3874, pGEM-T/Rv3875 and pGEM-T/Rv3619c. The identities of the cloned DNA fragments were determined based on size by digestion of the recombinant plasmids with *Eco*R I. Furthermore, The DNA fragments corresponding to *rv3874*, *rv3875* and *rv3619c* genes were excised from recombinant pGEM-T Easy using the restriction enzymes *Bam*H 1 and *Hind* III and subcloned in the expression vector pGES-TH-1, which was predigested with the same restriction enzymes. The identity of each gene in recombinant pGES-TH-1 was confirmed by restriction digestion and DNA sequencing. *E. coli* cells were transformed with recombinant pGES-TH-1 and induced for the expression of fusion proteins, which were detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using lysates of induced *E. coli* cells. The results showed the expression of proteins that corresponded to the size of GST/Rv3874, GST/Rv3875 and GST/Rv3619c. The identity of the expressed fusion proteins was established by Western immunoblotting with anti-GST and anti-penta His antibodies.

The SDS–PAGE analysis of cell-free extracts and pellets of sonicates of induced *E. coli* cells containing pGES-TH/Rv3874, pGES-TH/Rv3875 and pGES-TH/Rv3619c showed that recombinant GST-Rv3874 and GST-Rv3875 proteins were present in the soluble fraction, whereas the recombinant GST-Rv3619c protein was present in the pellet, which could be solublized best in 4 M urea. To purify the recombinant RD proteins, the soluble/solublized fractions were loaded on to glutathione-Sepharose affinity matrix and the GST-free RD proteins were released from the GST fusion partner bound to the column matrix by cleavage with the enzyme thrombin protease. The cleaved RD proteins were eluted and analyzed for purity by SDS–PAGE and staining with Coomassie Blue. The results showed that the recombinant Rv3874 and Rv3875 proteins were contaminated with another protein of nearly 70 kDa, whereas Rv3619c protein was nearly homogeneous (more than 95% pure). The partially purified Rv3874 and Rv3875 proteins were further purified on Ni-NTA agarose affinity matrix, and the analysis of eluted fractions showed the presence of a single sharp band in SDS–PAGE gels, which suggested that Rv3874 and Rv3875 preparations became free of the 70-kDa contaminant and were nearly homogeneous (more than 95% pure) (Hanif et al., 2010a). These results further strengthen the suggestion that pGES-TH-1 is useful for high level expression and efficient purification of recombinant mycobacterial proteins (Ahmed et al., 2003; 2004). The reason for Rv3619c requiring only one column (glutathione-Sepharose) for purification could be the presence of the fusion protein GST-Rv3619c in the pellet of induced *E. coli* cultures, which lacked the contaminating *E. coli* protein of 70 kDa. On the other hand, GST-Rv3874 and GST-Rv3875 proteins were present in the soluble fraction that also contained *E. coli* protein of 70 kDa, which was capable of binding to glutathione-Sepharose column nonspecifically, and was eluted from the column along with Rv3874 and Rv3875. However, the subsequent use of Ni-NTA matrix efficiently removed the contaminating *E. coli* protein and made the recombinant Rv3874 and Rv3875 proteins homogeneously pure (Hanif et al., 2010a).

The immunogenicity of all the three pure recombinant proteins (Rv3874, Rv3875 and Rv3619c) was evaluated in rabbits. The animals were immunized with the purified RD proteins and the sera were tested for antibody reactivity with the full-length recombinant proteins, pools of synthetic peptides covering the sequence of each protein and their individual peptides, as described previously (Hanif et al., 2010a). In brief, polyclonal antibodies were raised in rabbits against the purified and GST-free Rv3874, Rv3875 and Rv3619c recombinant proteins by emulsifying purified proteins (50 µg/ml) with an equal volume of incomplete Freund's adjuvant and injected intramuscularly in the right and left thigh. The rabbits were boosted twice with the same amount of protein at 2 weeks intervals. The animals were bled from the ear vein before the immunization and 2 weeks after the last immunization. The sera were tested for antigen-specific antibodies using Western immunoblotting. The results showed that pre-immunized rabbit sera did not have antibodies to any of these proteins, and the sera from immunized rabbits had antibodies reactive with the immunizing proteins only, thus confirming the specificity of the polyclonal antibodies to the immunizing antigens. In addition, the results suggested that the rabbits used were not exposed to *M. tuberculosis* and the antibody epitopes of a given protein were not crossreactive with other proteins. Moreover, ELISA was performed to detect antibodies in rabbit sera against full-length purified recombinant proteins and overlapping synthetic peptides corresponding to each protein. The ELISA results further showed specificity of the antibody reactivity with the immunizing proteins and positivity with synthetic peptides, which suggested that linear epitopes of each protein were recognized by B cells, after immunization with full-length proteins. Furthermore,

testing of sera with individual peptides of each protein demonstrated that rabbit antibodies recognized several linear epitopes that were scattered throughout the sequence of each protein (Hanif et al., 2010a). Interestingly, previous studies using pools of synthetic peptides have shown that all of these proteins (Rv3874, Rv3875 and Rv3619c) are major T cell antigens in humans, and the linear T cell epitopes were scattered throughout the sequence of these proteins (Hanif et al., 2008; Mustafa et al. 2000, 2003, 2008). A further analysis of the sequence of these proteins for B cell and T cell epitope prediction using appropriate prediction servers further suggested that B-cell and T-cell epitopes were scattered throughout the sequence of each protein (Hanif et al., 2010a; Mustafa et al., 2008; Mustafa and Shaban, 2006). Thus, both prediction and experimental results confirm the strong immunogenicity of Rv3874, Rv3875 and Rv3619c proteins for inducing antigen-specific immunological reactivity.
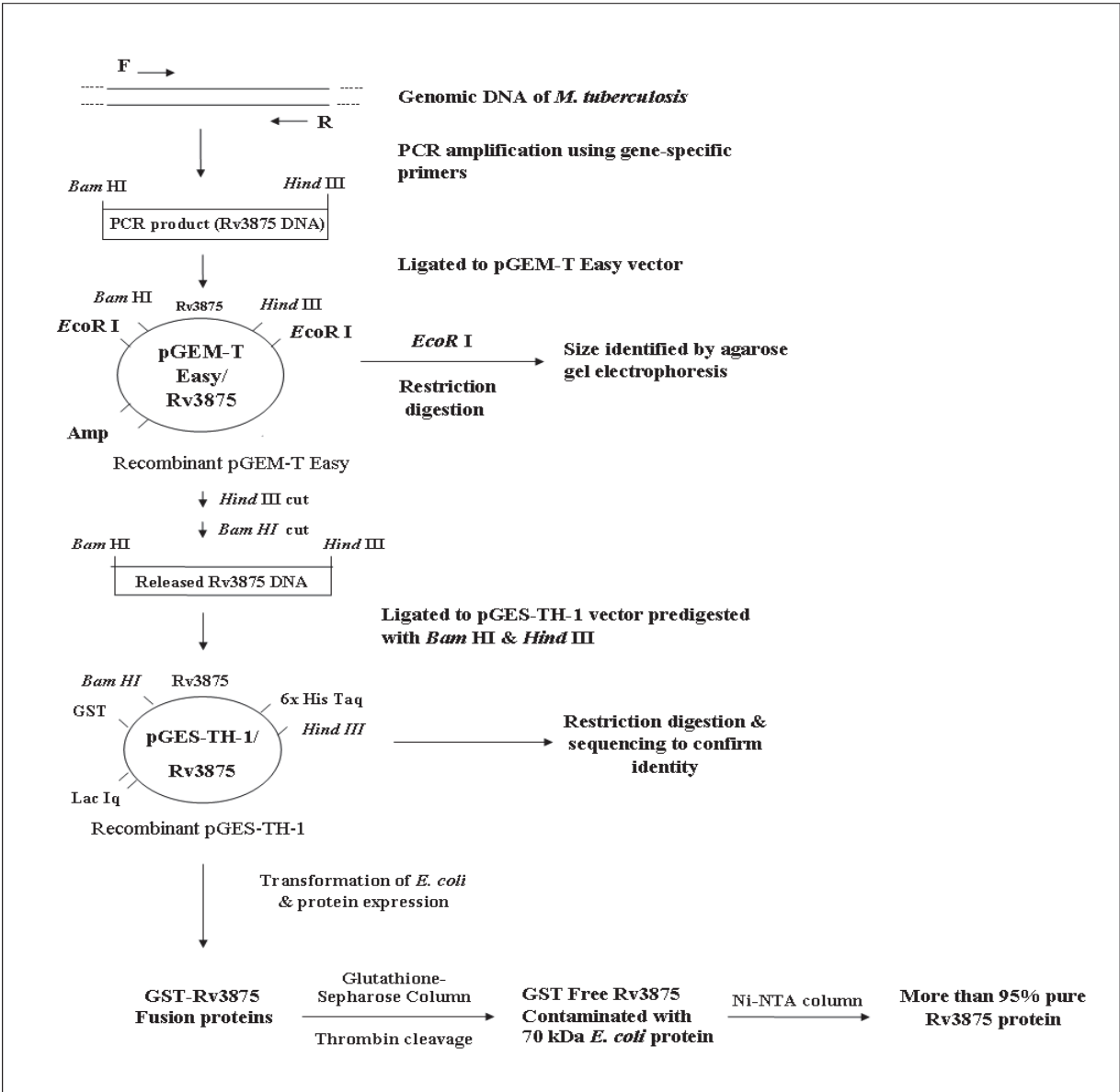
Fig. 1. Strategy and the schematic presentation of the steps involved in the cloning of *rv3875* gene in pGES-TH-1 vector, followed by expression of the fusion protein (GST-Rv3875) and purification of GST-free recombinant Rv3875 protein.

4. Potential of the purified recombinant RD proteins as *M. tuberculosis*-specific skin test antigens

Although, PPD in tuberculin skin test has been widely used as a diagnostic and epidemiological tool for tuberculosis monitoring in humans and animals for many years, there are many practical and theoretical problems related to the skin test, as stated above in the introduction of this chapter. To overcome the antigenic crossreactivity of PPD, we have tested four purified recombinant RD1 proteins (Rv3872, Rv3873, Rv3874, Rv3875) and one RD9 protein (Rv3619c) of *M. tuberculosis* for their ability to induce DTH skin responses in guinea pigs immunized with *M. tuberculosis* (Hanif et al., 2010b). Like RD1, RD9 is also a region that has been suggested to be deleted in all strains of BCG (Behr et al., 1999). However, using overlapping synthetic peptides, Rv3619c protein has been previously shown to induce moderate T-cell responses both in TB patients and healthy subjects (Mustafa and Al-Attiyah, 2004). Furthermore, Rv3619c belongs to the same family of proteins as Rv3874 and Rv3875 of RD1, i.e. ESAT6-family (Mustafa and Al-Attiyah, 2004). The evaluation of these proteins for induction of DTH responses as well as their specificity to *M. tuberculosis*-injected animals is important in the context of their potential usefulness as reagents for specific diagnosis to replace PPD, which has both *M. tuberculosis*-specific as well as crossreactive antigens.

In order to investigate the diagnostic potential of the purified recombinant proteins Rv3872, Rv3873, Rv3874, Rv3875, and Rv3619c, DTH skin responses were studied in guinea pigs injected with heat killed *M. tuberculosis* and live BCG, *M. avium* and *M. vaccae*. Two to four weeks later, the guinea pigs were challenged intradermally in the flank region with 1 µg of mycobacterial sonicates and purified recombinant proteins. The DTH responses were quantitated by measuring erythema at the sites of injections after 24 h. The results showed that all mycobacterial sonicates induced positive DTH responses in *M. tuberculosis*, BCG, *M. avium* and *M. vaccae* injected guinea pigs, which, like PPD, have crossreactive antigens. The purified recombinant proteins Rv3872, Rv3873, Rv3874 and Rv3875 elicited positive DTH responses in *M. tuberculosis* injected group, but not in BCG, *M. avium* and *M. vaccae* injected guinea pigs; whereas Rv3619c elicited positive DTH responses in *M. tuberculosis* and BCG injected animals but not in *M. avium* and *M. vaccae* injected guinea pigs (Hanif et al., 2010b). These results confirm the expression of all the proteins in *M. tuberculosis* and furthermore demonstrate their *in vivo* immunogenicity in guinea-pigs, which is the most sensitive animal model of TB. In addition, the induction of species-specific DTH responses suggests that the use of *M. tuberculosis*-specific recombinant RD1 antigens (Rv3872, Rv3873, Rv3874 and Rv3875) may lead to the development of DTH skin tests that can differentiate between tuberculous infection verses BCG vaccination and exposure to environmental mycobacterial species of *M. avium* and *M. vaccae*. In addition, the use of *M. tuberculosis* complex-specific Rv3619c protein in skin tests may differentiate between infection with *M. tuberculosis* and BCG vaccination verses exposure to environmental mycobacteria. However, these suggestions should be followed-up and studies should be extended to natural hosts of TB, including humans, for evaluation of the identified recombinant proteins as species-specific skin test antigens to replace PPD.

5. Cloning, expression and immunogenicity of RD genes in DNA vaccine vectors

The failure of BCG vaccine in humans has prompted the research to develop alternative vaccines against TB. Among the novel vaccine candidates, plasmid DNA-based TB vaccines have drawn close attention because of their unique features compared to conventional live or subunit vaccines, including induction of strong Th1 based CD4+ responses as well as cytotoxic T cell responses (Huygen, 2006). Therefore potency of plasmid DNAs expressing a variety of immunogenic *M. tuberculosis* antigens has been intensively evaluated in the past (Romano & Huygen., 2009). Unfortunately, their performance is generally not superior to BCG, especially in large animals. However, the licensure of a DNA vaccine in horses highlights the potential of DNA vaccine technology in the prevention of TB infection (Ulmer et al., 2006). Besides, it is generally believed that novel TB vaccines will be tested in the context of the widely used BCG and perhaps different kinds of vaccines are needed for the eradication of TB (Mitsuyama & McMurray, 2007; Sander & McShane, 2007). As a result, enhancement of TB DNA vaccine efficacy has become the active field of current research (Okada & Kita, 2010). In this context, Baldwin et al. have shown that inclusion of the secretion signal peptide from tissue plasminogen activator (tPA) into a DNA vaccine construct resulted in stronger immune responses to Ag85A, and provided sustained protection upon *M. tuberculosis* challenge in mice, as compared to the DNA vaccine construct based on the parent plasmid lacking tPA (Baldwin et al., 1999). Furthermore, a plasmid DNA vaccine expressing the heat shock protein 65 (HSP65-DNA vaccine) provided improved protective and therapeutic effects in mice when fused with human interleukin-2 (hIL-2) (Changhong et al., 2009).

To further study and compare the effect of tPA and hIL-2, we have used two plasmid vectors i.e. pUMVC6 and pUMVC7, to clone and express *M. tuberculosis*-specific RD proteins and studied antigen-specific cellular immune responses after immunization of mice with the recombinant plasmids (Hanif et al., 2010c). The plasmid vectors pUMVC6 and pUMVC7 are eukaryotic expression vectors, which have been prepared by University of Michigan Vector Core (UMVC) and made commercially available by Aldevron, USA. Both vectors have CMV promoter at 5′ end of the cloning site (Figs. 2, 3). pUMVC6 is characterized by having a secretion signal peptide from hIL-2 (hIL2 secretory peptide) as an immuno-stimulatory sequence (Fig. 2), whereas pUMVC7 has a signal peptide for targeting peptides to a secretory pathway by fusion to the tPA signal peptide (Fig. 3).

To prepare DNA vaccine constructs using the above plasmids, DNA fragments corresponding to five RD genes, i.e. *rv3872 (pe35), rv3873 (ppe68), rv3874 (esxA), rv3875 (esxB)* and *rv3619c (esxV),* were PCR amplified from genomic DNA of *M. tuberculosis* by using gene-specific forward (F) and reverse (R) primers. To facilitate cloning of PCR amplified DNA in pUMVC6 and pUMVC7 vectors, in addition to gene-specific sequences, the F and R primers had appropriate restriction sites at 5′ ends, i.e. *Bam*H I and *Bam*H I, and *Bam*H I and *Xba* I restriction sites for pUMVC6 and pUMVC7, respectively. The amplified DNA were first cloned into pGEM-T Easy vector, before subcloning into pUMVC6 and pUMVC7 (Figs. 2, 3 with Rv3875 DNA shown as an example). This was done to facilitate the subcloning of appropriate DNA into the eukaryotic expression vectors pUMVC6 and pUMVC7, as has been demonstrated previously for prokaryotic expression vectors pGEX-4T and pGES-TH-1 (Ahmed et al., 1999b, 2003; Hanif et al., 2010a). The cloning in pGEM-T Easy successfully yielded five recombinant plasmids, one for each gene, i.e. pGEM-T/Rv3872, pGEM-
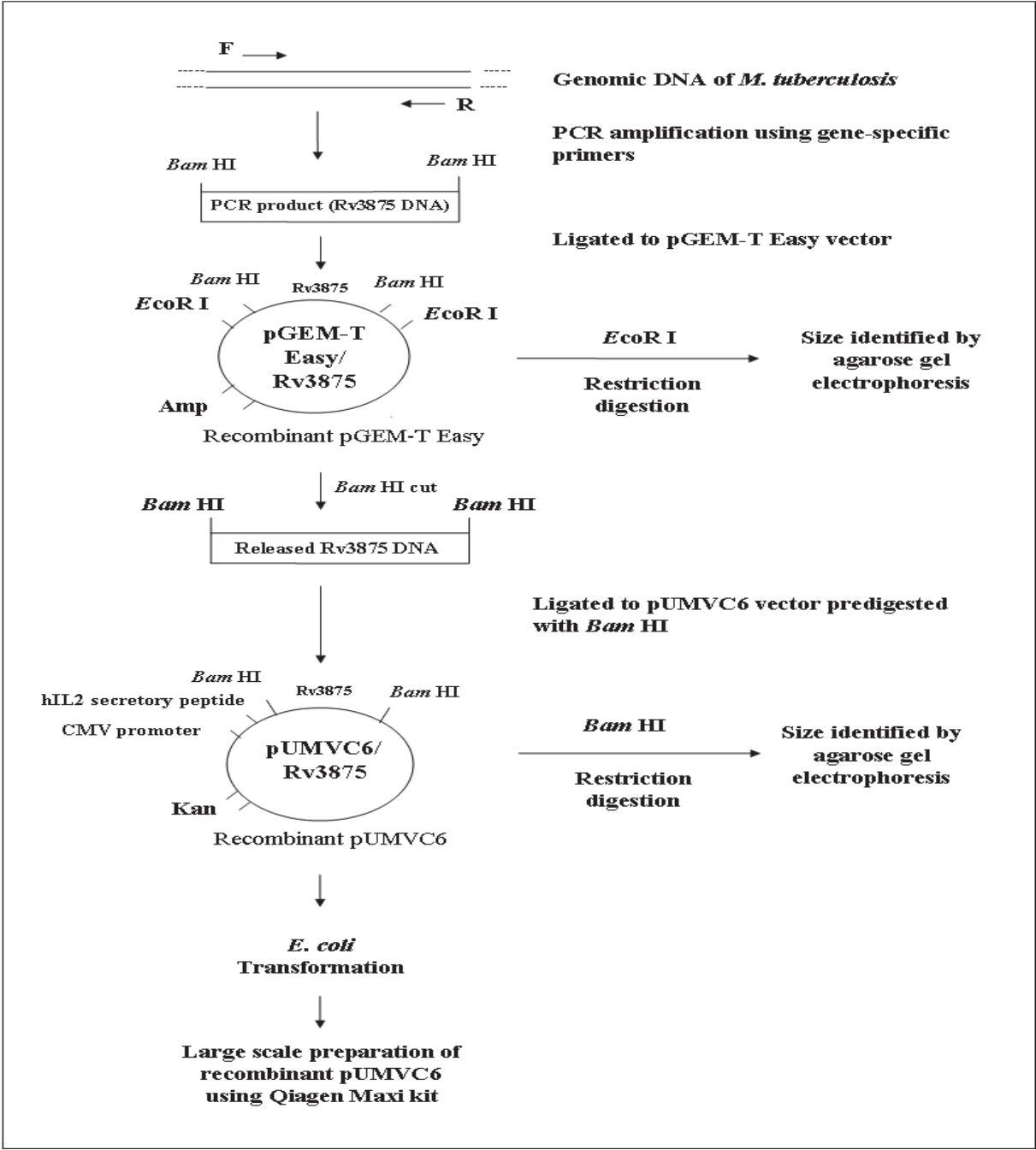
Fig. 2. The strategy and schematic presentation of the steps involved in the construction of recombinant plasmid pUMVC6/Rv3875, purification of recombinant plasmid DNA on a large scale.
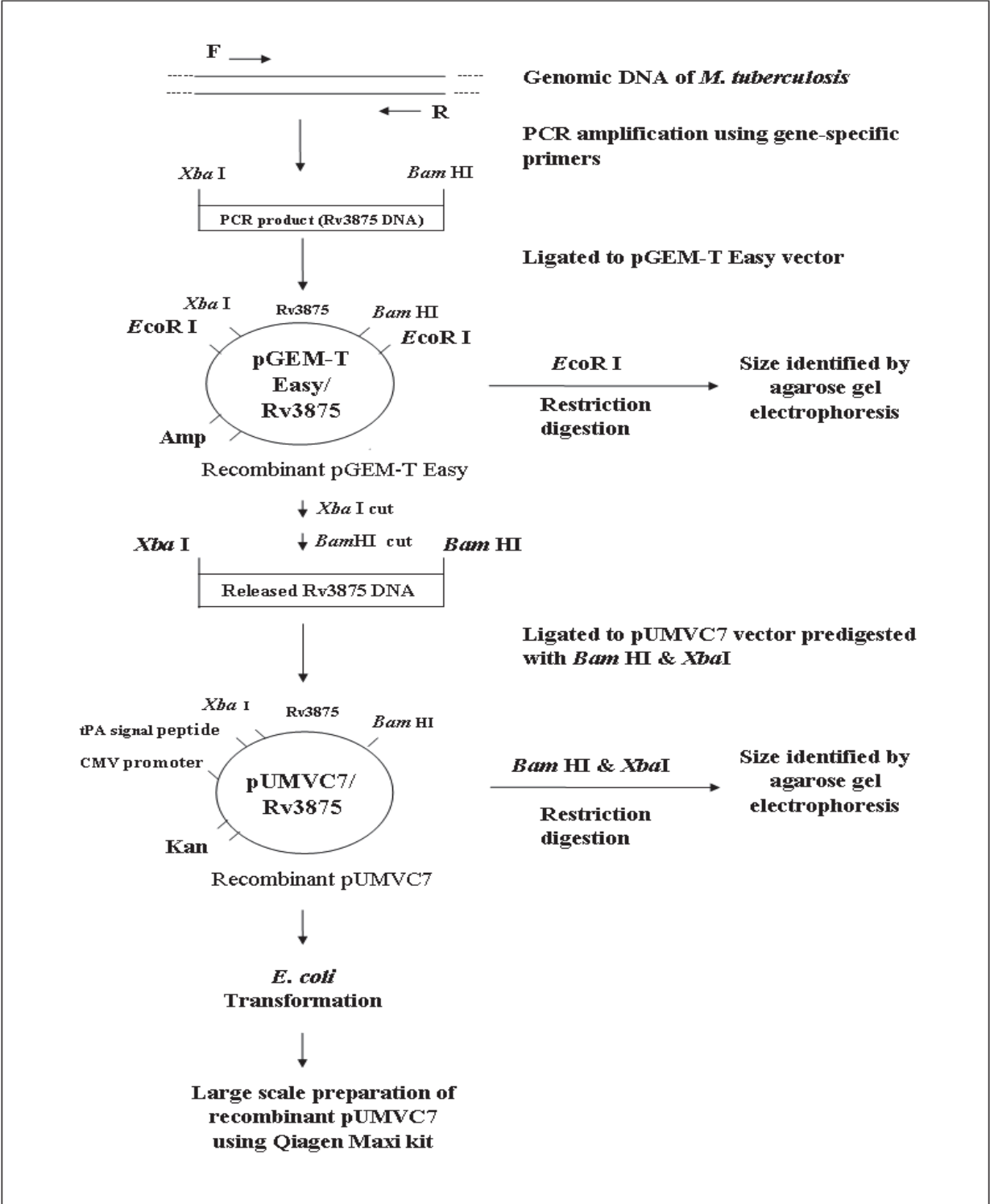
Fig. 3. The strategy and schematic presentation of the steps involved in the construction of recombinant plasmid pUMVC7/Rv3875, purification of recombinant plasmid DNA on a large scale.

T/Rv3873, pGEM-T/Rv3874, pGEM-T/Rv3875 and pGEM-T/Rv3619c. The analysis of DNA fragments released from the recombinant pGEM-T Easy plasmids after digestion with *Eco*R I showed that the cloned DNA corresponded to the expected molecular size of *rv3872, rv3873, rv3874, rv3875* and *rv3619c* genes. To prepare DNA vaccine constructs, the recombinant pGEM-Ts were single digested with *Bam*H I for subcloning into pUMVC6 and double digested with *Bam* HI and *Xba* I for subcloning into pUMVC7. These restriction digestions released the DNA fragments corresponding to *rv3872, rv3873, rv3874, rv3875* and *rv3619c* genes with *Bam*H I/*Bam*H I and *Bam*H I/*Xba* I cohesive ends. All the genes were subcloned into plasmid vectors by their ligation to pUMVC6 and pUMVC7 DNA predigested with *Bam*H I/*Bam*H I and *Bam*H I/*Xba* I, respectively. The identity of each cloned gene was determined by restriction digestion of recombinant plasmids with the restriction enzymes *Bam*H I for pUMVC6; and *Bam*H I and Xba I for pUMVC7, which released the cloned DNA corresponding to the size expected for each gene. *E. coli* cells were transformed with the parent and recombinant pUMVC6 and pUMVC7 plasmids, and large quantities of the plasmids were purified from the transformed *E. coli* cells grown in vitro by using Qiagen Endofree Mega kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The overall strategies of gene amplification, cloning and large-scale purification of recombinant pUMVC6 and pUMVC7 plasmid DNA are shown in figures 2 and 3, respectively, using *rv3875* as an example (Fig. 2, 3). In this way, a total of 10 recombinant DNA plasmids (five for each vector) were constructed (Hanif et al., 2010c).

To study the expression and immunogenicity of the RD1 and RD9 proteins cloned in the recombinant vaccine constructs of pUMVC6 and pUMVC7, immunization studies were performed in mice. Groups of 6–8 week old BALB/c mice (five mice in each group) were immunized intramuscularly with three doses of purified parent or recombinant plasmid DNA, each dose containing 100 µg of DNA, and given 3 weeks apart. After 3 weeks of the third immunization, spleen cells were isolated from each immunized mouse for cellular immune responses using antigen-induced proliferation as an indicator (Hanif et al., 2010c). The results demonstrated that spleen cells of mice un-immunized or immunized with the parent plasmids did not show positive antigen-induced proliferation responses to any of the antigens corresponding to the cloned genes (Hanif et al., 2010c). The results further showed that both the recombinant vectors induced antigen-induced cellular proliferation to Rv3872, Rv3874, Rv3875 and Rv3619c proteins. However, antigen-induced proliferation responses were observed in response to a given protein only with spleen cells of mice immunized with the recombinant DNA vaccine construct expressing that protein, except for Rv3873, which failed to induce proliferation responses in animals immunized with pUMVC6/Rv3873 or pUMVC7/Rv3873 (Hanif et al., 2010c). Moreover, recombinant pUMCV6 induced relatively better responses than recombinant pUMCV7. The improved responses with recombinant pUMCV6 suggest that hIL2 secretory protein acted as a better adjuvant and enhanced cellular immune responses, assessed by antigen-induced proliferation of spleenocytes, to the fused mycobacterial proteins more effectively than the tPA signal peptide. The relevance of antigen-specific cellular proliferation induced by DNA vaccine constructs with protection against *M. tuberculosis* challenge has been demonstrated in the mouse model of TB (Fan et al., 2009).

Although, the results of this study, showed induction of antigen-specific cellular immune responses to the antigens encoded by genes present in DNA vaccine constructs, are interesting; the work should be extended to demonstrate their protective efficacy in

challenge experiments, with *M. tuberculosis*, using various animal models of TB, e.g. mice, guinea-pigs, rabbits and monkeys etc. Such vaccines may be useful in both prophylactic and therapeutic applications in humans, If found effective in animals. Furthermore, DNA-based vaccines expressing *M. tuberculosis*-specific antigens may even be useful in BCG vaccinated subjects as preventive vaccines, because revaccination with BCG has not shown beneficial effects (Rodrigues et al., 2005, Roth et al., 2010), and may even be combined with BCG to improve its protective efficacy (Fan et al., 2007).

6. Conclusions

The modified pGES-TH-1 expression vector was extremely useful in obtaining highly purified recombinant preparations of Rv3872, Rv3873, Rv3874, Rv3875 and Rv3619c proteins of *M. tuberculosis*. All of these recombinant proteins were immunogenic in rabbits and the antibody epitopes were scattered throughout the sequence of each protein. These results suggest that pGES-TH-1 vector could be employed in obtaining pure recombinant proteins, predicted to be encoded by hypothetical genes present in *M. tuberculosis*-specific genomic regions, for their immunological characterization. Furthermore, the induction of species-specific DTH responses suggests that the use of *M. tuberculosis*-specific RD1 antigens may lead to the development of a DTH skin test that discriminates between tuberculous infection verses BCG vaccination and exposure to environmental mycobacteria (*M. avium* and *M. vaccae*). In addition, the use of *M. tuberculosis* complex-specific Rv3619c protein in skin tests may differentiate between tuberculosis infection and BCG vaccination verses exposure to environmental mycobacteria. Furthermore, DNA vaccine constructs encoding RD1 and RD9 genes induced antigen-specific cellular responses in immunized mice to the respective proteins, and thus could be useful as safer vaccines to immunize against TB. However, to confirm their usefulness as anti-TB vaccines, further studies with the DNA vaccine constructs of pUMVC6 and pUMVC7 may be performed to determine their protective efficacy in appropriate animal models of TB (mice, guinea-pigs, rabbits and monkeys etc.) after challenging the immunized animals with live *M. tuberculosis*.

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8. References

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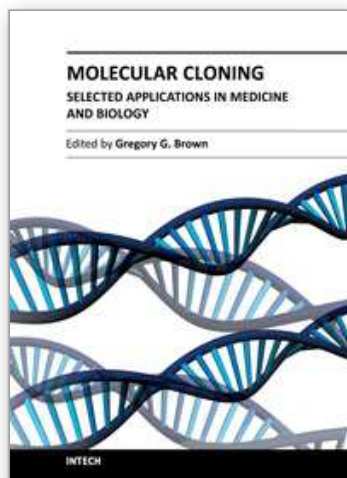
The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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