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(71) Applicant: CENTER FOR INNOVATIVE TECHNOLOGY [US/US]; CIT Building, Suite 600, 2214 Rock Hill Road, Herndon, VA 22070 (US).

(72) Inventors: DERTZBAUGH, Mark, T. ; 1907 F Hopkins Road, Richmond, VA 23224 (US). MACRINA, Francis, L. ; 405 Smoketree Circle, Richmond, VA 23236 (US).

(74) Agents: PATTERSON, Herbert, W. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, 1300 I Street, N.W., Washington, DC 20005-3315 (US).

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(54) Title: CHIMERIC PROTEINS

(57) Abstract

A chimeric peptide is provided which includes at least a portion of the B subunit of cholera toxin and an epitope region of a desired antigen fused to the N-terminal end of the B subunit of cholera toxin. The epitope region includes an antigenic determinant of the desired peptide. According to certain preferred embodiments, such chimeric peptides are used as a vaccine to elicit an immune response in a subject to a desired antigen. Recombinant-DNA mediated methods for the production of a chimeric peptide, DNA sequences, vectors and host organisms used in such methods are also provided.

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Description

CHIMERIC PROTEINS

BACKGROUND OF THE INVENTION

Technical Field

The mucosal surfaces of the gut and oral cavity are constantly exposed to a large number of organisms which include pathogenic bacteria, fungi, viruses, and protozoa. Many of these pathogens use the mucosa as the principal portal of entry into the host, whereupon the systemic immune system eliminates the agent. Other pathogens do not have to invade the mucosa in order to be pathogenic, and systemic immunity is ineffective in these cases. The mucosal-associated lymphoid tissue (MALT) probably developed in response to the need to protect the exterior surfaces of the host. The concept of a common mucosal immune system is relatively new (McGhee and Michalek, 1981). The predominant form of immunity at these sites is the release of dimeric secretory IgA (sIgA).

Background Art

Secretory IgA can prevent adherence of pathogens to the mucosa, neutralize viruses, and inactivate toxins (Tomasi, 1984). Induction of an immune response in the gut confers secretory immunity at distant sites of the mucosa such as the lungs, oral cavity, and the genitourinary tract. Antigen-primed lymphocytes are disseminated from the gut by the lymphatics to these sites where they mature into functional effector cells (McGhee and Michalek, 1981).

Attempts to stimulate MALT by oral immunization have had limited success. This has been attributed, in part, to the induction of tolerance, a state of specific immunologic unresponsiveness that develops upon exposure to an antigen. The development of tolerance is dependent upon the antigen, dosage, length of exposure, route of immunization, and genetic background of the host (Webb and Winkelstein, 1984).

A condition of oral tolerance develops to many antigens presented to MALT (Challacombe and Tomasi, 1980; Nossal, 1983). This phenomenon is believed to be a natural mechanism for preventing the immune system from responding to the large load of innocuous antigens, from food and normal microbial flora, that it is exposed to daily.

It was previously thought that a sIgA response could occur in the absence of systemic immunity, and this was observed with certain antigens (Suzuki et al, 1986). However, a recent study indicated that keyhole limpet hemocyanin (KLH), an antigen that causes systemic tolerance, also induces tolerance in the secretory compartment (Elson and Ealding, 1984b). Other data has shown that the sIgA and IgG responses are under coordinate genetic control, and that the Peyer's patches contain both IgG and IgA producing B cells (Elson and Ealding, 1984a). Thus, most orally administered antigens probably induce tolerance in both the secretory and systemic immune compartments. The development of oral tolerance appears to be under the regulatory control of suppressor and contrasuppressor cells (Green et al., 1982; Richman et al, 1978). Although most antigens induce tolerance in these cases, there are examples of oral immunization conferring solid immunity to pathogens such as poliovirus (Tomasi, 1984) and Vibrio cholerae (Lycke and Holmgren, 1987).

Certain adverse health conditions require treatment or would be aided by a secretory immune response. Attempts to make oral subunit vaccines have met with limited success, however, due mainly to the failure of many protein antigens presented by this route to stimulate an adequate antibody response (Challacombe and Tomasi, 1980; Crabbe et al, 1969; Pierce and Gowan, 1975). Cholera toxin (CT) is unusual because it is one of the few proteins that elicit a strong immune response when given orally (Pierce and Gowan, 1975). When co-fed, CT has been demonstrated to act as an adjuvant for other proteins which normally are not immunogenic when given orally (Lycke and Holmgren, 1986; Liang et al, 1988;

Nedrud et al, 1987). In fact, Elson and Ealding (1984b) were able to abrogate tolerance to keyhole limpet hemocyanin (KLH) in mice that were fed this antigen mixed with CT. This resulted in the production of both sIgA and serum IgG to the antigen. The role of CT in these cases appears to be greater than simply increasing the immunogenic mass, as most carrier proteins do, but the mechanism of this action is unknown.

CT is produced by the enteric pathogen Vibrio cholerae and is composed of two subunits. The toxinogenic A subunit is responsible for the ADP-ribosylation of eucaryotic adenylate cyclase. This modification increases cAMP activity, resulting in the diarrhea and fluid loss observed in patients with cholera (Betley et al, 1986). The B subunit of cholera toxin (CTB) is composed of five identical subunits of 11.6 kDal joined together in a noncovalent association (Gill, 1976). The CTB pentamer binds to the monosialoganglioside GM_1 (Cuatrecasas, 1973), which appears to be the natural ligand of the protein. This ganglioside is found in abundance on the surface of intestinal epithelial cells and probably facilitates entry of the A subunit into the cell.

Although CTB is non-toxic, it is still immunogenic when given orally (Lycke and Holmgren, 1987). This has encouraged attempts to use CTB, instead of the holotoxin, as a carrier for chemically created mucosal vaccines. Bessen and Fischetti (1988) have induced protective immunity in mice that were intranasally immunized with an epitope from streptococcal M protein conjugated to CTB. Some studies have suggested that CTB may act as more than just a simple carrier for antigens (McKenzie and Halsey, 1984; Tamura et al, 1988). Moreover, it may be possible to increase the immunogenicity of the antigen even further by conjugating it to CTB. In a study by McKenzie and Halsey (1984), glutaraldehyde was used to covalently couple CTB to the oral toleragen horseradish peroxidase (HRP). When this conjugate was fed to mice, sIgA was present to both antigens at levels significantly greater than that found from feeding the two proteins as a mixture.

This in contrast, however, to the results reported by others (Lycke and Holmgren, 1986). Liang et al (1988) were unable to demonstrate an adjuvant effect for CTB when conjugated to Sendai virus.

There have also been reports of attempts to use gene fusion as an alternative to chemical conjugation. In such reports, gene fusion vectors have been reported which produce a chimeric protein including a large portion of the glucosyltransferase B enzyme (GtfB) produced by the cariogenic bacterium Streptococus mutans and CTB (Dertzbaugh and Macrina, 1987). That chimera, however, appeared to change the structure of CTB because the ability of CTB to bind to mucosal monosialoganglioside GM_1, was adversely affected. Thus, these constructs were not suitable for use as a vaccine.

Dental caries commonly occurs in people who consume sucrose as a part of their diet. Although the incidence of dental caries has been reduced in the general population, it is still a prevalent disease of man. For example, over 24 billion dollars was spent in the United States in 1984 to repair carious lesions (Loesche, 1986). Better oral hygiene has reduced the severity of the disease, but hygiene alone cannot eliminate the disease. The structure of the tooth surface contains fissures where S. mutans is more likely to accumulate and cause caries, even with good hygiene. Furthermore, proper mechanical debridement of the teeth is rather labor-intensive and, consequently, many people do not clean their teeth adequately. Currently, the only recourse to the problem of dental caries is to repair the damage after it has occurred. A more attractive approach is to prevent carious lesions from developing. A way in which to achieve this is to prevent colonization of the tooth surface by S. mutans.

One method for preventing colonization of the tooth surface is to vaccinate against S. mutans. Several studies have been performed using different components of S. mutans as a vaccine. The early vaccines were composed of whole

cells, and these were found to reduce colonization and caries formation in some cases (Gregory and Filler, 1987; Michalek et al, 1983; Russell et al, 1980). However, surface antigens of S. mutans have been found to cross-react with human heart tissue, and this has precluded the use of whole cells vaccines for safety reasons (Ayakawa et al, 1985; Ayakawa et al, 1988; Hughes et al, 1980). For this reason, recent efforts have been directed towards the development of subunit vaccines using purified components of S. mutans.

At present, two components of S. mutans have been identified as the best candidates for a subunit vaccine. A component of the cell wall of S. mutans has been independently identified by two groups and designated either antigen I/II (Russell and Lehner, 1978) or SpaA (Holt et al, 1982). Vaccination with this purified protein has been shown to reduce colonization and dental caries in monkeys (Lehner et al, 1980; Lehner et al, 1981), and it does not appear to induce heart cross-reactive antibodies (Bergmeier and Lehner, 1983). The antibody response elicit was reported to be predominately serum IgG, which reaches the oral cavity via leakage into the gingival crevicular fluid. However, immunizations were performed using adjuvants which may not be practical for human use. Furthermore, parenteral immunization with antigen I/II did not induce significant sIgA responses in these animals. This was not surprising, considering that secretory immunity requires local stimulation in order to develop. Another group of proteins that have been evaluated for use in subunit vaccines are glucosyltransferases. These enzymes catalyze the formation of glucans from sucrose, and these polymers mediate the adherence of S. mutans to the tooth surface. Enzyme preparations have been used to immunize rodents both perorally (Smith et al, 1978; Smith et al, 1979) and parenterally (Bahn et al, 1977). Vaccination with these proteins has also prevented colonization and caries formation in these animals. The advantage of oral immunization was demonstrated by these studies, because both sIgA and serum

IgG were elicited to glucosyltransferase. This vaccination route confers both systemic and secretory antibodies which both can play a role in host defense against S. mutans. However, these studies demonstrated the current problem with oral vaccines to S. mutans, specifically that in order to induce and maintain high levels of antibody, the animals had to be immunized repeatedly, over a long period of time.

In general, these experiments have demonstrated that oral vaccination with soluble antigens of S. mutans may be an effective method of preventing colonization and caries formation. However, the vaccines used required repeated booster immunizations in order to maintain protective levels of antibody, due to the unique nature of the secretory immune system. The use of an oral adjuvant may improve the immune response to these vaccine components.

The present inventors have surprisingly found that peptide sequences composed of only an epitope when fused to the N-terminal end of CTB elicit an antibody response, giving rise to a sustained antibody response with antibodies directed against the entire protein of interest. For example, a 15 amino acid epitope of GtfB fused to CTB gives rise to antibodies against the GtfB protein which is over 1000 amino acids in composition.

Disclosure of the Invention

It is an object of the present invention to provide an oral vaccine capable of eliciting both a secretory immune respsonse and a systemic immune response, thereby causing a host to produce sIgA and IgG.

Another object of the present invention is to provide chimeric peptides not toxic to a host which can be produced by recombinant DNA methods.

Yet another object is to provide a universal vaccine carrier that can be fused to form a chimeric peptide with any number of antigens or epitopes to elicit a secretory immune response to that specific antigen or epitope when the chimeric peptide is administered orally to a host.

It is still another object to provide the recombinant methods, vectors and host organisms that produce peptides for effective oral vaccine compositions.

These objects and other objects are achieved by providing a chimeric peptide including the B subunit of cholera toxin and an epitope region of a desired antigen fused to the N-terminal end of the B subunit of cholera toxin. The epitope region includes an antigenic determinant of the desired peptide. Such chimeric peptides can be used for an oral vaccine according to certain preferred embodiments in which the epitope fused to the B subunit of cholera toxin gives rise to an immune response, producing antibodies directed against the peptide of interest. Recombinant methods for producing a chimeric peptide, and the DNA sequences, vectors and hosts used in such recombinant methods are also provided.

According to other advantageous features according to certain preferred embodiments of the present invention, an epitope of the GtfB peptide is fused to the CTB peptide to provide an oral vaccine for the prevention of dental caries.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate various embodiments of the invention and, together with the description, serve to explain the principles of the invention.

Brief Description of the Drawings

Fig. 1. Construction of the ctxB fusion vectors. Plasmid pVA1662 which contains the ctxB gene (shaded area) was digested with EcoRI and NdeI to delete the 5′ end of the gene. Then synthetic linkers with compatible ends were inserted and ligated to create the fusion vectors shown.

Fig. 2. Construction of pVA1555. Plasmid pHK7, which contains a 1.9 kb insert encoding a truncated portion of the gtfB gene of Streptococcus mutans (stippled area), was

inserted into pVA1542 as a PstI-EcoRI fragment upstream of the ctxB gene (shaded area). The resulting plasmid, pVA1555, expresses a gtfB::ctxB fusion protein of 58 kDal in E. coli.

Fig. 3. Construction of pVA1782. Plasmid pVA1542 contained a promoterless version of the ctxB gene of Vibrio cholerae (stippled area) that lacked DNA encoding the first 17 amino acids of the leader sequence of the protein. The synthetic oligonucleotide encoding gtfB.1 (filled area) was inserted into pVA1542 as an EcoRI-NdeI fragment to create pVA1599. The resulting gtfB.1::ctxB gene fusion could be inserted easily into a number of expression vectors as an EcoRI-BamHI fragment, if desired. The secretion vector pINIII ompA2 contained DNA encoding the leader sequence of ompA (striped area), an outer membrane protein of E. coli, under control of the inducible lac promoter. Promoter activity is inhibited by repressor, the product of the laci gene, until induction by IPTG. Insertion of the gtfB.1::ctxB gene into this vector resulted in expression of a chimeric protein of 14.4 kDal upon induction with IPTG.

Fig. 4. Linker sequences of the fusion vectors. These oligonucleotides were used as a translational junction between gtfB and ctxB. The number above each linker designates the plasmid vector containing the sequence. Bold-faced letters correspond to sequences actually encoded by the synthetic linkers. Important restriction endonuclease sites are shown above each linker; amino acid sequences are located below each linker.

Fig. 5. The sequence of the gtfB.1 peptide. The hydrophilicity plot is shown for a portion of the glucosyltransferase B enzyme of the cariogenic bacterium Streptococcus mutans. The region encoding amino acid residues 345-359, corresponding to peptide gtfB.1, is shown in black. The gtfB.1 peptide (shaded area) was inserted between the leader sequence for the ompA gene of E. coli and the B subunit gene of cholera toxin (ctxB). The cleavage site for ompA is indicated by the arrow. The numbers above the amino acid sequence correspond to the residue number of

the mature, secreted form of the chimeric protein. The corresponding DNA sequence is listed above, along with important restriction sites. Bold letters indicate the sequence encoded by the synthetic oligonucleotide used to construct the gene fusion.

Fig. 6. Analysis of CTB and the chimera by circular dichroism. Mean residue ellipticity (M.R.E.) is expressed in deg cm^2/mole. The minima for the oligomeric form of the chimera (dotted line) was shifted relative to native CTB (solid line), indicating some differences. However, the spectra of the monomer (dashed line) was significantly different from both the oligomer and CTB.

Fig. 7. Immunogenicity of the chimera. Protein samples were fractionated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose sheets. Replicate blots were probed with antiserum to either CTB, GtfB, or chimera. Arrows denote immunoreactive proteins of interest. Lane 1, extracellular proteins form S. mutans GS-5; V1792 chimera; lane 3, CTB.

Fig. 8. Kinetics of enzyme inhibition. Total glucan synthesis was measured over time (in hours). The enzyme was preincubated in either PBS (control), normal rabbit serum (N.R.S.), anti-GtfB serum, or anti-Chimera serum before addition to the reaction mixture. All sampling was performed in triplicate.

Fig. 9. Differential inhibition of glucan synthesis. Protein samples preincubated in either PBS (control), anti-GtfB, or anti-chimera serum were assayed for differential glucan synthesis. The reactions proceeded for 8 h at 37°C before measuring activity. The samples were assayed in triplicate for both total glucans and water-insoluble glucans. Total glucans were precipitated with methanol. Water-insoluble glucans were precipitated with deionized water. The amount of water-soluble glucans produced was determined by subtracting insoluble glucans from total glucans. The activity of the samples in each assay was

determined relative to the control. The activity of the control was normalized to 100%.

Fig. 10. Inhibition of fructan synthesis. Extracellular protein from S. mutans GS-5 was preincubated for 1 h at 37°C in either PBS (control), normal rabbit serum (N.R.S.), anti-GtfB serum, or anti-chimera serum. The samples were incubated in substrate containing ^{14}C-(fru)-sucrose for 8 h at 37°C. Total fructan synthesis was measured by precipitating the polymer in methanol.

Fig. 11A. The DNA sequence encoding the GtfB.1/CTB chimeric peptide. Fig. 11B. The amino acid sequence of the GtfB.1/CTB chimeric peptide.

Best Mode for Carrying Out the Invention

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention. Amino acid abbreviations used herein are customary and are defined, for example in Stryer, Biochemistry, second edition, W.H. Freeman and Company (1981). Vaccines encompassed by the present invention can be used for any living organism, and the term "subject" is used to define any human or non-human animal. All references cited herein are hereby incorporated by reference.

The present invention shows that, using recombinant DNA techniques, plasmids can be constructed that can be transfected into hosts and chimeric, immunogenically active peptides are expressed which include an epitope of the peptide of interest fused to the N-terminal end of CTB. The relative small size of the epitope amino acid sequence fused to the CTB reduces the chance that the structure or function of CTB will be altered. Large peptides fused to CTB are more likely to adversely affect the binding of CTB to mucosal tissue. Such alterations adversely affect the ability of the chimeric CTB peptide to elicit a secretory immune response. The inventors have surprisingly shown that peptides as small as an epitope fused to CTB elicits the desired immune response.

As defined in the present invention, an epitope should have the following characteristics. An epitope is defined as an antigenic determinant or that portion of an antigenic molecule that interacts by molecular complementarity with the antigen combining site of an immunoglobulin molecule. An epitope is a discrete portion of an entire antigen molecule which can trigger the immune response. As defined in the present invention, the epitope need not actually trigger an immune response by itself, but includes the particular region of the antigen which brings about the proper interaction to raise antibodies against that antigen when the epitope is fused to CTB. Moreover, the epitope, according to the present invention, should have little to no impact on CTB interaction with mucosal tissue when the epitope is fused to the CTB. In order to ensure that the epitope according to the present invention does not interfere with CTB interaction, the epitope should be no greater than 100 amino acids long and no greater than 11 kDal in size. According to certain preferred embodiments, the epitopes according to the present invention are in the range of about 10 to 30 amino acids long, or 15-20 amino acids long.

There are standard procedures that one of ordinary skill in the art knows how to employ to locate the epitopes of an antigen molecule. Epitopes or antibody binding sites of proteins have been correlated with regions of hydrophilicity (Hopp and Woods, 1981; Lerner, 1982; and Tamura, 1983) and with high segmental mobility regions or random coil regions (Geysen et al., 1987; and Westhoff, 1984). High segmental mobility is characterized by a peptide devoid of a highly organized secondary structure. Domains with these properties would probably be located on the surface of the protein, where they would be accessible to an antibody, and would have a highly malleable secondary structure, thus allowing them to conform to a pre-existing antibody specificity. Epitopes possess these predicted characteristics.

By synthesizing an oligonucleotide corresponding to a domain of a protein with these properties, an antibody binding site or epitope can be identified. Available algorithms, such as those included in a software program known as MICROGENIE, from BECKMAN INSTRUMENTS, Palo Alto, California, may assist in finding epitope regions. First, the hydrophilicity of regions of a protein can be determined using the algorithm of Hopp and Woods (1983). Secondary structure predictions of this region can also be made using the algorithm of Garnier et al. (1978). Domains that are identified which have both high segmental mobility and hydrophilicity can then be screened to ensure that these regions comprise an antigenic determinant or epitope. By following the methods of the present invention, such regions can be screened by fractionating proteins into peptides, isolating the peptides containing the putative epitope of interest, and determining its reactivity with antisera to the protein of interest. Alternatively, expression vectors could be prepared that produce chimeric peptides, including the regions to be screened fused to CTB, and testing the chimeric peptides to determine if such peptides raise antibodies directed against the protein of interest.

The following is a non-exhaustive list of certain peptides which could be used for fusion to CTB for use as an oral vaccine. The listed peptides are known to elicit immune responses that reduce and/or eliminate the severity of the respective disease.
Such peptides could be fused to the CTB as described in the present application to form an oral vaccine.

Residues 141-158 and 200-213 of virus coat protein I (VPI) form the Kaufbeuren strain of Foot and Mouth Disease Virus, a disease of cattle (DiMarchi, et al., Science 232:639-641, 1986). The conserved region of the M Protein of group A streptococci (residues 216-235, 248-269, 275-284), responsible for pharyngitis (Bessen and Fischetti, Infect. Immun. 56:2666-2672, 1988). The conserved tetrapeptide region (Asn-Ala-Asn-Pro) of the circumsporozoite protein of

Plasmodium falciparum, the causative agent of malaria (Que, et al., Infect. Immun. 56:2645-2649, 1988). Subunits of gonadotropin for use as an anti-fertility vaccine. (Alam, et al., Vaccine 7:129-131, 1989). Synthetic peptide encoding entire preS2 region of hepatitis B virus surface protein (Emini, et al., J. Med. Virol. 28:7-12, 1989). The M genome segment of Rift Valley Fever virus (Schmaljohn et al., Virol. 170:184-192). The pilin subunit of Haemophilus influenza (Brinton, et al., Pediatrc. Infec. Dis. 8:(Suppl) S51-61. Fimbrial subunit of enterotoxic colibacillosis in neonatal piglets (Greenwood, et al., Vaccine 6:389-92, 1988). Active site residues of Pseudomonas aeruginosa exotoxin A (Lukac, et al., Infect. Immun. 56:3095-3098, 1988). Peptides corresponding to the cleavage region of VP3 protein of rotavirus which induce neutralizing antibodies (Streckert et al., J. Virol. 62:4265-4269, 1988).

Another nonexhaustive list below includes further antigens which include epitopes which can be fused to CTB to produce oral vaccines. Although epitopes of the following list have not been characterized, one of ordinary skill in the art should be able to determine those regions using the methods described herein. Those methods include, but are not limited to, the use of algorithms to locate areas which are hydrophilic and which have high segmental mobility, preparing chimeric proteins with plasmids according to the present invention and screening those chimeric proteins for their ability to elicit an immune response. Alternatively, peptide fragments identified by the algorithm method can be tested for cross-reaction with antisera to the protein of interest to screen for appropriate epitopes for use in the present chimeric peptide, oral vaccines.

Diphtheria toxin subunits A (toxin subunit) and/or B (binding subunit) from Corynebacterium diphtheriae. Greenfield et al., Proc., Natl. Acad. Sci. 80:6853-6857 (1983). Pertussis toxin subunits A (toxin subunit) and/or B (binding subunit) from Bordetella pertussis, the causative agent of whooping cough. Black et al., Science 240:656-659

(1988). *spa*A, a surface protein antigen from Streptococcus mutans, the causative agent of dental caries. *gtf*C, the glucosyltransferase C enzyme produced by Streptococcus mutans, the causative agent of dental caries. *ftf*, the fructosyltransferase enzyme produced by Streptococcus mutans, the causative agent of dental caries. Fimbrial subunit (an adherence protein) of Bacteroides gingivalis, a bacterium associated with periodontitis. Type I fimbrial subunit (an adherence protein) of Streptococcus sanguis, a bacterium involved in early stages of dental plaque formation. Tetanus toxin of Clostridium tetani, the causative agent of tetanus. Invasin protein of Yersina pseudotuberculosis. The hemagglutinin (HA) antigen of influenza virus, the causative agent of flu, Tamura et al., Vaccine 6:409-413 (1988). The pilin subunit of Bacteroides nodosus, the causative agent of foot rot of sheep. Surface antigens of human papilloma virus, the causative agent of genital warts. Envelope proteins of herpes simplex virus. Tumor-associated antigens in melanomas and carcinomas. The serum and pilin opacity proteins of Neiserria gonorrheae, the causative agent of gonorrhea. Surface proteins of Giarida lamblia, a causative agent of diarrhea in man. Dengue 1 virus structural proteins, Bray et al., Virol. 63:2853-2856 (1988). F protein of rinderpest virus, Barrett et al., Virol 170:184-192 (1989). P28 antigen of Schistisoma mansoni, Wolowazuk et al., J. Immunol, 142:1342-1350 (1989). Avian influenza virus hemagglutinin antigen. Merozoite antigen of Eimeria acervulina, the causative agent of coccidiosis in chickens, Kim et al., Infect. Immun. 57:2434-40 (1989).

The utility of the fusion vectors for producing chimeric peptides has been demonstrated by the construction according to the present invention of the V1555 chimeric protein containing CTB and a portion of the GTF protein from S. mutans. Construction of CTB fusion vectors, using synthetic oligonucleotide linkers, has been shown here to be a practical method of producing chimeric proteins for use as vaccines. The use of synthetic linkers provides a convenient

method of inserting a spacer of any length, rigidity, and composition between the two domains of the chimera. The following examples show the use of vectors for the construction of gene fusions using DNA sequences from S. mutans. However, the vectors may be used to make fusions to other DNA as well.

Once the DNA sequence encoding the antigen or epitope is known, an oligonucleotide specifying the immunologically important domains could be synthesized and inserted into the vectors. The use of these peptide sequences would increase the specificity of the immune response, and impose minimal conformational changes on CTB which could affect its immunogenicity.

An ideal fusion vector would contain an inducible promoter and ribosomal binding site, followed by a polylinker cloning site, and then the ctxB gene. This construct would allow almost any DNA sequence to be inserted into it and express a CTB fusion protein.

Although the V1555 chimeric protein demonstrated the feasibility of constructing gene fusions with CTB, there were several problems which affected the usefulness of this protein. First, immunoblotting analysis revealed the presence of 2 major species of 58 and 72 kDal, instead of the 58 kDal protein predicted by the nucleotide sequence data. Sufficient DNA is located upstream of the start codon for gtfB to encode a protein of that size. There may have been additional translational start sites within the DNA encoding the truncated gtfB gene that were recognized by E. coli. This would have resulted in the translation of a larger protein sharing the same sequence as the 58 kDal chimera. The fact that the 72 kDal protein cross-reacted to both antisera would support this hypothesis, since it shares the same epitopes as the 58 kDal protein. Furthermore, cell lysates of V1619 also contained additional immunoreactive polypeptides which were larger than the 46 kDal protein predicted from the nucleotide sequence data (Fig. 5). V1619 is an E. coli strain that contains pHK7 and expresses the

truncated gtfB gene used in the fusion. Second, the leader sequence of gtfB was not recognized in *E. coli*, which resulted in the protein being trapped in the cytoplasm. This made extraction of the protein difficult and contributed additional contaminants which had to be removed. Third, because the protein could not be exported from the cytoplasm, it was degraded by intracellular proteases. The resulting degradation products were co-eluted on the GM_1 affinity column, and produced a heterogeneous mixture of proteins which could not be easily resolved from one another. Fourth, the addition of the 46 kDal GtfB moiety to CTB appeared to affect the structure of the protein, based on the reduced affinity of the V1555 chimera for GM_1. This was not surprising, since the GtfB moiety is over 3 times the size of CTB. Genetic fusion of such a large protein to CTB appeared to interfere with its function which, in turn, could affect the putative adjuvant properties of CTB. For these reasons, it was concluded that the V1555 chimera was not an ideal model system for vaccine studies.

To overcome these drawbacks, the present inventors constructed the V1782 chimera. The present inventors believed that fusion of smaller peptides than those expressed from the V1555 construct to CTB would probably minimize the effect they had on the structure and function of the protein. Furthermore, a putative antibody binding site of the GtfB enzyme had been identified by the present inventors as a result of the degradation of the V1555 chimera (see Example II(D)). However, the precise location of this site was unknown and was too large to synthesize a corresponding oligonucleotide to the entire degradation product.

According to the methods described herein, including the use of the Hopp and Woods algorithm and the Garnier algorithm, regions of the protein being hydrophilic and having high segmental mobility were located. It was assumed that the epitope possessed these predicted characteristics.

By synthesizing an oligonucleotide corresponding to a domain of GtfB with these properties, an epitope (antibody

binding site) was identified. Genetic fusion of this peptide to CTB resulted in expression of a chimeric protein which was recognized extremely well by antiserum to GtfB. This technique may be helpful for identifying antigenic domains of other large peptide fragments that cannot be located by other means.

The secretion vector system used to make V1782 chimera provided a convenient method of constructing and expressing small peptides fused to CTB. It improved an earlier system by using the inducible lac promoter and a signal peptide sequence to secrete the fusion protein into the periplasm of E. coli. This system is similar to one which has been used to overproduce CTB for production of cholera vaccine (Sanchez and Holmgren, 1989). Placement of the chimera under control of an inducible promoter such as lac permitted overproduction of the protein upon induction with IPTG. By fusing the chimera to the peptide leader sequence of ompA, the protein could be transported to the periplasm where it was easier to isolate and purify. As verified by the N-terminal sequence data, the ompA leader was properly recognized in E. coli, even though it was fused to a foreign protein. The secretion of foreign proteins using the ompA leader has been previously described (Ghrayeb et al, 1984). Because of the way in which the ompA leader was fused to the chimera, two additional amino acids were left at the N-terminus upon processing by E. coli. Although these additional residues could be removed by oliogonucleotide-directed mutagenesis (Ghrayeb et al, 1984), they did not appear to interfere with the antigenicity or structure of the chimera, and were thus left intact.

Other vectors that can be used to express the chimeric peptide fusions once they are made in pVA1542, pVA1543 or pVA1544, with or without the ompA vector, include pKK223-3 (J. Brosius, Gene 27:151-160, 1984), and pET translation vectors. These examples are in no way limiting and any number of vectors could be used for expressing the chimeric peptide.

The present invention shows that peptides can be fused to CTB with minimal effect on the structure of the protein. Furthermore, chimeras can be constructed that retain the antigenicity of each moiety, which is important in the use of these proteins as subunit vaccines. The ability to oligomerize and to bind to GM_1 shows the liklihood that this protein will be selected out of the intestinal milieu and will induce an immune response in the Peyer's patches. The V1782 chimera retains these properties, as well as much of the native structure of CTB.

To summarize, a complete system for constructing, expressing, and purifying CTB chimeras by genetic fusion is provided. Using this system, an antigenic segment of the GtfB enzyme of S. mutans was identified and genetically fused to the N-terminus of CTB. The structure of this chimeric protein was characterized extensively, and demonstrated that 15 amino acids can be added to the N-terminus of CTB with minimal effect on the structure or biological activity of the protein. Antiserum raised to the chimeric protein recognized the native GtfB enzyme as well as CTB. Furthermore, it was demonstrated in vitro that the antiserum was able to inhibit glucosyltransferase activity of S. mutans. This is the first case in which anti-peptide antiserum has been demonstrated to inhibit enzyme activity in S. mutans, and suggests that this may be a useful approach for a subunit vaccine against dental caries.

Although the present examples are directed to the fusion of a portion of GtfB to CTB, the CTB vectors described herein can be used to fuse any number of suitable epitope regions to CTB to produce effective oral vaccines. Moreover, the particular 15 amino acid sequence of GtfB included in the chimeric peptide expressed by V1782 is in no way a limiting example. Extending the length of either end of that 15 amino acid sequence or shifting the span of that sequence on the GtfB sequence are also contemplated. For a vaccination

regimen, it is also contemplated to administer a combination of different chimeric peptides which include different portions of an antigen.

EXAMPLES

I. MATERIALS AND METHODS

A. Enzymes and Reagents

Restriction endonucleases and T4 ligase (400 unit/ul) were purchased from New England Biolabs (Beverly, MA). Enzymatic reactions were performed according to the manufacturer's directions. A 5' end-labeling kit was purchased from Bethesda Research Laboratories (Gaithersburg, MD). DNA polymerase I (Klenow fragment) and dNTP mix were purchased from International Biotechnology, Inc. (New Haven, CT). [gamma-^{32}P]dATP (4286 Ci/ mmol) and ^{14}C-sucrose was obtained from New England Nuclear (Boston, MA). Isopropyl-beta-D-galactoside (IPTG), CTB, and GM_1ganglioside were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-dextran, T10 dextran, and Sephadex G-100 were purchased from Pharmacia (Piscataway, NJ). Spherosil XOC-005 was purchased from Supelco (Bellefonte, PA). Goat anti-CTB was purchased from Calbiochem (LaJolla, CA). Phosphatase-labeled second antibodies and BCIP/NBT substrate were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Pooled, normal rabbit sera was purchased from Flow Laboratories (McClean, VA). The BCA assay (Pierce Chemical Co.; Rockford, IL) was used for the quantitation of total protein. A silver stain kit was purchased from Bio-Rad (Richmond, CA) and used as recommended by the manufacturer.

B. Bacteria and Plasmids

Bacterial strains and plasmids used in this paper are listed in Table II. Plasmid pJBK30, containing a promoterless version of the ctxB gene from the El Tor strain of Vibrio cholerae, was obtained from J.B. Kaper, University of Maryland School of Medicine, Baltimore, MD (Kaper et al, 1984). Plasmid pHK7 was provided by H.K. Kuramitsu, Northwestern University, Chicago, IL. This plasmid encoded the amino terminal one-third of the gtfB gene of S. mutans

GS-5 (Aoki et al, 1987). Plasmid DNA was prepared by SDS-high salt lysis (Guerry et al, 1973) followed by dye-buoyant density equilibrium centrifugation (Welch et al, 1979). E. coli HB101 was transformed with plasmid DNA by the $CaCl_2$-heat shock method (Morrison, 1977). The cells were grown at 37°C in M-9 media (Miller, 1972) supplemented with 10 mg/ml casamino acids (Difco), 20 g/ml leucine and proline, and 2mg/ml thiamine. V1555 was grown to an O.D. 660 = 0.6, washed twice in phosphate-buffered saline (PBS, pH 7.3), and then lysed in a French pressure cell (SLM Aminco; Urbana, IL). The lysate was clarified by centrifugation before use. For fermenter scale growth of V1782, cells were grown to an O.D. 660 = 0.6 before the addition of 1 mM IPTG to induce the lac promoter. Cultures were allowed to grow for an additional 2 h at 37°C before harvesting the contents of the periplasm by osmotic shock (Neu and Heppel, 1965). Cells were washed twice at 25°C in buffered saline (30 mM NaCl, 10 mM HEPES, pH 7.3), then resuspended for 10 min in 0.1 volume of hypertonic solution (15% sucrose, 50 mM EDTA, 50 mM Tris, pH 8.0). The cells were pelleted and suspended in 1.0 volume of cold deionized water for 10 min. The shock fluid was clarified of cells by centrifugation, and then was adjusted to pH 6.8 using 0.1 volume of a 10X concentrate of wash buffer (10 mM $NaPO_4$, 200 mM NaCl, 0.02% NaN_3, pH 6.8).

C. **Enzyme Preparation**

Extracellular proteins of S. mutans were obtained as follows. Strain GS-5 (Bratthall serotype c) was grown in 3 1 Todd-Hewitt broth, under anaerobic conditions, to an O.D. 660=0.6. The cells were removed by centrifugation, and ammonium sulfate was added to 60% saturation. The precipitate containing extracellular proteins was collected by centrifugation, resuspended in 300 ml PBS (pH 7.3), and then dialyzed 3 times against PBS. Approximately 30 ml of this material was aliquoted and stored at -70°C. This material was used for all enzyme assays. The remaining sample was diluted in PBS + 6 M urea and subjected to diafiltration through a YM-100 ultrafiltration membrane

(Amicon; Danvers, MA). The clarified sample was concentrated by ultrafiltration to 20 ml and then dialyzed in PBS (pH 7.3) in order to remove the urea. The samples were aliquoted and stored at -70°C. This preparation was used as a control for immunoblotting analyses.

D. Antisera

Rabbit antiserum to the glucosyltransferase B enzyme of S. mutans GS-5 was obtained from H.K. Kuramitsu, Northwestern University, Chicago, IL. Antiserum to the purified, monomeric form of the V1782 chimeric protein was raised in female New Zealand White rabbits. One mg of protein was emulsified in complete Freund's adjuvant and injected subcutaneously into the hind quarters of the animal. Three weeks later, the rabbit was boosted with the protein emulsifed in incomplete Freund's adjuvant. Then, one week later, the rabbit was bled. The serum was collected, aliquoted, and stored at -20°C.

E. Oligonucleotides

Oligonucleotides and their complementary strands were synthesized on an Applied Biosystems 380A synthesizer. The linkers were isolated by thin-layer chromatography on Kieselgel 60 F_{254} silica gel plates (Merck; Darmstadt, W. Germany) in 1-propanol, ammonium hydroxide, and water (55:35:10). The DNA bands were visualized by a hand-held UV light and then scraped off the plate. The DNA was extracted by washing the silica gel three times with water, and then concentrated by evaporating the aqueous phase to dryness. The larger oligonucleotides were separated by using a 20% polyacrylamide gel containing 8 M urea. Gel fragments containing the DNA were minced and extracted 3 times with an equal volume of extraction buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0). The oligonucleotides were purified using a Nensorb affinity column (Dupont, Wilmington, DE) before use. The forward reaction of T4 kinase was used to phosphorylate the 5' ends of the oligonucleotides in order to improve the efficiency of the ligation reaction (Maniatis, et al., 1982). Unincorporated dATP was removed by purifying the

oligonucleotides with a Nensorb column. One pmol of purified vector was mixed with 21 pmol of each complementary linker in annealing buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol). The sample was heated to 2, 90°C for 10 min and then incubated overnight at 42°C to reanneal the DNA. Unincorporated oligonucleotides that could interfere with the ligation were removed by washing the sample in annealing buffer using a Centricon 30 ultrafiltration unit (Amicon; Danvers, MA). The retentate was suspended in ligation buffer with T4 ligase (400 units) for 6 h at 25°C.

F. Construction of the Fusion Vectors

Plasmid pJBK30, containing the promoterless ctxB gene, was prepared for insertion of the linkers by deleting two restriction sites that could interfere with their placement. The NdeI site, located near the origin of replication of the plasmid, and the EcoRI site located at the 3' end of the ctxB insert were both deleted from the plasmid (Fig. 1). Plasmid pJBK30 was partially digested by restriction enzyme and the linearized DNA was isolated by electroelution from agarose gels (Maniatis et al, 1982). The 5' ends were filled-in using DNA polymerase I (Klenow fragment) and these blunt ends were ligated together using appropriate reaction conditions (Maniatis et al, 1982). The plasmid containing the two deleted restriction sites was designated pVA1662. Plasmid pVA1662 was digested with NdeI and EcoRI and the larger of the two resulting fragments was isolated by electroelution from an agarose gel. The linkers were inserted into this fragment as described in Example I(E) (also see Fig. 4). The plasmid was transformed into E. coli HB101, and clones that were tetracycline (Tc)- and ampicillin (Ap)-resistant were selected and screened for insertion of the linker by restriction mapping. Plasmids containing each linker were isolated and designated pVA1542, pVA1543, and pVA1544. These vectors contained a truncated version of the ctxB gene in

which the 5' end of the gene was deleted to remove the ribosomal binding site and DNA encoding the leader sequence of the protein.

G. **Gene Fusions**

V1555 was constructed as shown in Figure 2. Plasmid pHK7 contained a 1.9 kb PstI-EcoRI fragment from S. mutans GS-5, Bratthall serotype c (Hamada and Slade, 1980), that encoded a truncated portion of gtfB. The PstI-EcoRI fragment from pHK7 was directionally cloned into each fusion vector. E. coli transformants that were Tc-resistant and Ap-sensitive were screened for expression of a protein that was recognized by antiserum to CTB. A clone was selected, containing a plasmid derived from pVA1542, and designated V1555.

The strategy used to construct V1782 is shown in Figure 3. The oligonucleotides were inserted into the fusion vector pVA1542 at the 5' end of ctxB as previously described in part I(E). Proper insertion of the oligonucleotides into pVA1542 was monitored by restriction enzyme digestion of plasmid DNA prepared from minilysates (Macrina et al, 1982). In order to express the translational fusion protein, the chimeric ctxB gene was transferred to the secretion vector pINIII ompA2.

H. **Screening**

Plasmid DNA was obtained from cells by a rapid minilysis procedure (Macrina et al, 1982). The DNA was checked for plasmid content by agarose gel electrophoresis and restriction enzyme analysis. For protein analysis, a whole-cell lysate of the clones was prepared by a modification of the DNA minilysis procedure. Cells containing the secretion vector were screened for expression of a chimeric protein by first cultivating them on M-9 media containing 1 mM IPTG. After digestion in lysozyme, the cells were suspended in 0.5 ml of a hypotonic solution (10 mM Tris, 30 mM NaCl, pH 7.3) and then subjected to a rapid freeze-thaw. Insoluble material was removed by centrifugation, and the lysates were assayed for expression of the chimeric protein by an enzyme-linked immunosorbent assay (ELISA) that

used GM_1 ganglioside-coated microtiter wells as the solid phase and antiserum to CTB as the primary antibody (Sack et al, 1980).

I. Purification

A GM_1 ganglioside affinity column was prepared and used to purify the CTB chimeras according to the method of Tayot et al (1981). Lyso-GM, was prepared from 50 mg of GM_1 and then covalently coupled to 15 g of DEAE-dextan coated Spherosil. The samples were recirculated through the column for 24 h at 4°C and at a linear flow rate of 1.5 ml/min. The column was washed with 10 volumes of wash buffer (10 mM $NaPO_4$, 200 mM NaCl, pH 6.8) and then the chimera was released using elution buffer (50 mM citrate, 200 mM NaCl, pH 3.0). The eluate was adjusted to pH 7.3 and then concentrated by diafiltration in PBS (10 mM $NaPO_4$ 140 mM NaCl, pH 7.3) using a YM10 ultrafiltration membrane (Amicon; Danvers, MA). The retentate was quantitated for total protein as well as for reactivity in the ELISA. Native CTB was used as the reference standard for the ELISA. A 1.6 x 200 cm Sephadex G-100 column was prepared and used to separate monomeric and oligomeric fractions of the V1782 chimera. A 5 mg sample was loaded onto the column and eluted in PBS (pH 7.3) at a linear flow rate of 0.13 ml/min.
Fractions were collected in 2 ml volumes and analyzed for total protein content and immunoreactivity.

J. Protein Analysis

The N-terminal sequence of the monomeric protein (40 g) was determined using an Applied Biosystems Model 470A Sequencer, with in-line PTH-amino acid analysis.

The procedure of Gross and Witkop (1962) was used to cleave protein by treatment with cyanogen bromide. The protein was dissolved in 200 1 of 70% formic acid and deaerated by flushing with nitrogen. A 100-fold molar excess of cyanogen bromide over methionine then was added. The reaction was allowed to proceed for 13 hours in the dark at

room temperature in a tightly sealed tube. The reaction was terminated by removal of the reagents in a Savant Speedvac Concentrator.

The peptides produced by cyanogen bromide cleavage were separated by high performance liquid chromatography on a C-18 reverse phase column (Varian), using a linear gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 50% acetonitrile. A flow rate of 1 ml/min and a total time of 60 min was used. The effluent was monitored at 215 nm. Peaks were collected, and analyzed by amino acid analysis.

Samples of protein or peptides were hydrolyzed in sealed, evacuated tubes with constant boiling HCl for 24 hr at 110°C. Amino acid analyses were performed with a Durrum MBF amino acid analyzer using o-phthaldehyde as the detection reagent. In some cases, the samples were alkylated with iodoacetamide prior to hydrolysis. Each sample was reacted with iodoacetamide (1 mole sample/10 moles iodoacetamide) in 50 mM Tris (pH 8.0) for 1 h at 25°C.

The monomer, oligomer, and CTB were analyzed by circular dichroism. Samples were extensively dialyzed against PBS (pH 7.3) using a Centricon 30 (Amicon). The filtrate was saved for use as the buffer blank. A 1.5 ml sample (0.15 mg/ml) of each protein was loaded into a cell and analyzed on a Jasco J-500C spectropolarimeter.

Analytical polyacrylamide gel electrophoresis was performed with a BioRad slab gel apparatus with a 15% polyacrylamide gel. A 4% stacking gel was used. The gel and buffer formulations were those of O'Farrell (1975), in some cases modified to include 8 Murea in the gel and sample buffer.

K. **Immunoblotting Analysis**

Protein samples were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose sheets (Towbin et al, 1979). Nonspecific binding of antibody was prevented by blocking the sheets for 1 h at 25°C with a 5% solution of chicken serum in tris-buffered saline (TBS; 20 mM

Tris, 500 mM NaCl, pH 7.5). The sheets were incubated for 6 h in primary antisera diluted 1:1000 in TBS with 5% chicken serum. The sheets were washed 3 times for 10 min in TBS with 0.05% Tween 20 (TTBS), and then incubated for 2 h in enzyme-labeled second antibody. After washing again 3 times in TTBS, the sheets were developed with substrate.

L. Enzyme Assays

Glucosyltransferase activity was determined by measuring the amount of ^{14}C-glucose converted into glucan polymer from specifically labeled sucrose. Fructosyltransferase activity was measured by a similar means, except that sucrose labeled with ^{14}C in the fructose moiety was used as the substrate. The reaction mixture was incubated at 37°C and consisted of 10μl enzyme, 5μl T10 dextran (5 mg/ml), 5μl labeled substrate (261 mCi/mmol, 20 Ci/ml), and 80μl substrate buffer (10 mM imidazole, 10 mM sucrose, 0.02% sodium azide, pH 6.5). A 100μl sample of the reaction mixture was pipeted onto 2.4 cm glass fiber filters(Whatman GFA; Maidstone, England). All samples were performed in triplicate. Total polymer was collected onto the filters by precipitation in methanol. The filters were washed 6 times with methanol using a vacumn manifold apparatus (Millipore; Bedford, MA). Water-insoluble polymer was collected by washing the filters 4 times with deionized water and then 2 times with methanol. The filters were air-dried, placed into vials with 5 ml scintillation cocktail (Amersham OCS; Arlington Heights, IL), and then counted for 2 min in a scintillation counter (Beckman LS-1800). The mean counts per minute (CPM) of each sample were subtracted from a background control containing no enzyme. For inhibition assays, enzyme was pre-incubated with an equal volume of serum for 1 h at 37°C prior to addition to the reaction mixture. Enzyme was pre-incubated with an equal volume of PBS for use as a positive control. Sera was diluted in PBS, when necessary.

M. Animal studies

The protocol to be used is approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Male C57Bl/6 mice can be obtained from Charles River (Wilmington, MA). The mice are used at 6-8 weeks of age, and should be fasted overnight before feeding the protein. The chimera is administered using an intragastric feeding needle (G. Tiemann & Sons, Long Island, NY). The GM_1-purified chimera is diluted in 0.2 M Na_2HCO_3 and is delivered in a volume of 0.5 ml. Control animals receive saline solution. For comparison, the antigen is injected intraperitoneally into some animals. After the antigen is diluted in MAALOX, it was injected in a volume of 0.1 ml. Mice are immunized on day 0 and 14. On day 21, serum and intestinal secretions are collected and assayed to assay for specific antibody. Microtiter wells are coated with either CTB or synthetic peptide composed of the same 15 amino acid sequence as found in the chimera. The assay for detecting peptide-specific antibody in intestinal washings should be sensitive enough such that 0.01 ng/ml of peptide-specific antibody may be detected.

A protocol for testing the effectiveness of the chimeric peptide vaccines for preventing dental caries, according to the present invention, is set forth in Morisaki et al., Infection and Immunity, 40:577-591 (May 1983).

II. RESULTS

A. Fusion Vectors

A set of cloning vectors have been developed for constructing and expressing _ctx_B gene fusions in _E. coli_. The plasmid pJBK30 was used as the basis of construction of the fusion vectors because it contained a promoterless _ctx_B gene and did not express this protein in _E. coli_. By making CTB the C-terminal end of the chimera, an ELISA could be used to detect successful fusion, because the gene would only be expressed if it were translationally in-frame with sequences located upstream. The region located between the _Eco_RI and _Nde_I sites of _ctx_B encoded a putative hydrophobic leader

which was not present in the mature protein (Lockman and Kaper, 1983). By eliminating this region from the fusion vectors, the chance of abberant cleavage or folding of the chimeric protein was minimized. The linkers were designed to be compatible with any DNA that contained an EcoRI site within its open reading frame (Fig. 4). The 3' end of the linkers encoded a sequence compatible with an NdeI site located within the open reading frame of ctxB. Only one reading frame in each linker allowed translation of ctxB to occur. Although translation relative to the EcoRI site occurred in a different reading frame, the length of the linkers was adjusted so that the last codon terminated at the junction of the NdeI site. Sequencing data has shown that this junction separates codons translated within ctxB (Lockman and Kaper, 1983). A unique restriction site was encoded into each linker to serve as a marker. These were very helpful for determining which linker was inserted in the vector (Fig. 4). The linkers contained codons that have been translated by other genes expressed in E. coli. Small, polar neutral amino acids were encoded whenever possible in order to minimize conformational changes in the proteins. DNA encoding an aspartyl-prolyl amino acid sequence was included in each linker to allow post-translational cleavage of the chimera, if desired. This sequence is found rarely in proteins, and contains a peptide bond susceptible to acid hydrolysis (Landon, 1977).

B. Construction of V1555

A 1.9 kb fragment, encoding the glucosyltransferase B gene (gtfB) of S. mutans GS-5 (Bratthall serotype c), was used to construct a fusion with ctxB. This fragment, contained in pHK7 (Fig. 2), constitutively expressed a non-enzymatically active 48 kDal truncated polypeptide that cross-reacted with antiserum to GtfB. The immunoblotting analysis of the V1555 chimera peptide was performed by purifying the peptide by GM_1 ganglioside affinity chromatography. A clarified whole cell lysate from E. coli strain V1555 was loaded onto the column (lysate). Bound

material was eluted from the column by decreasing the pH stepwise (pH 4, pH 3). The column was cleaned by washing it with 0.1 M NaOH. Duplicate blots were probed either with antiserum to glucosyltransferase B (anti-GTF) or to the B subunit of cholera toxin (anti-CTB). Purified CTB and a lysate of V1619 were used as positive controls for the antisera. E. coli strain V1619 contains the plasmid pHK7 which expresses a 46 kDal truncated GTF protein. The fragment was inserted into all three fusion vectors, but immunopositive clones were isolated from only one, pVA1542, indicating that the reading frame of gtfB was compatible to the linker contained in this plasmid. This result was verified by the nucleotide sequence data for gtfB (Shiroza et al, 1987). One of the clones was selected and designated V1555. Plasmid DNA from this clone was mapped by restriction enzyme analysis to verify construction of the gene fusion (Fig. 2). The results demonstrated that the fusion vectors could be successfully used for constructing CTB chimeras.

C. Purification of the V1555 Chimera

Analysis of V1555 revealed that the chimeric protein was trapped in the cytoplasm of E. coli. The leader sequence for GtfB was not recognized by the host, even though this protein is normally secreted by S. mutans. This phenomenon has been observed with other extracellular streptococcal proteins that are expressed in E. coli (Aoki et al, 1986). In order to liberate the protein from the host, the cells had to be lysed in a French pressure cell. Once the cells were lysed, the protein was ready to be purified by affinity chromatography.

CTB has a high affinity for GM_1 ganglioside (Sillerud et al, 1980). The use of a GM_1 ganglioside affinity column has been shown previously to be a rapid and efficient method of purifying cholera toxin (Tayot et al, 1981). In order to optimize binding of the V1555 chimera to the GM_1 affinity column, the lysate was recirculated through the column overnight at 4°C. The majority of the activity was eluted at pH 4, which was higher than that required to elute native

cholera toxin from the column (Tayot et al, 1981). Immunoblotting analysis of the column eluate revealed the presence of two novel proteins of 58 kDal and 72 kDal that cross-reacted with antisera to either GtfB or CTB. Based on the published DNA sequences of both genes, the chimera was predicted to be 58 kDal in size. The presence of the 72 kDal protein currently cannot be accounted for, but it does cross-react with antisera to both GtfB and CTB. Passage of the V1555 lysate through the affinity column was effective for removing many of the contaminating proteins from E. coli. Cleaning the column with 0.1 M NaOH only eluted a small amount of additional immunoreactive material, indicating that elution of the chimera was complete. Smaller immunopositive polypeptides were observed on the Western blot, particularly to GtfB, and these were postulated to be proteolytic degradation products. Proteolysis of foreign proteins in E. coli has been documented (Goff et al, 1984), and such fragments would co-elute with the chimera as long as they retained the GM_1 binding site of CTB. The addition of protease inhibitors to the lysate did not alter the pattern observed on SDS-PAGE and, therefore, appeared to occur in the host prior to lysis. The ability to elute the chimera from the GM_1 column at a higher pH than native cholera toxin suggested that the conformation of the CTB moiety was altered by the gene fusion. However, the extent of the change was not determined.

D. Construction of V1782

An antigenic segment of the glucosyltransferase B enzyme of S. mutans was identified from immunoblotting analysis of the V1555 chimeric protein. One of the peptide fragments cross-reacted with antisera to either GtfB or CTB, indicating that it contained antigenic segments from both proteins. The molecular weight of this fragment was slightly larger than that for CTB. Therefore, assuming that translation of the V1555 chimera terminated at the stop codon for CTB, the fragment contained the entire length of the CTB protein and a small portion of the GtfB protein which was

located near the junction of the fusion. Once identified, the sequence of the gtfB gene (Shiroza et al, 1987) was used to create a hydropathy plot of this antigenic region, based on the algorithm of Hopp and Woods (1983). Secondary structure predictions of this region were also made using the algorithm of Garnier et al (1978). Several domains of this region were identified which had both high segmental mobility and hydrophilicity.

One such domain, gtfB.1, is shown in Figure 5. This domain was relatively hydrophilic and was devoid of a highly organized secondary structure. A set of synthetic oligonucleotides were synthesized which were compatible with the amino acid sequence of this region. The gtfB.1 oligonucleotides were genetically fused to the promoterless ctxB gene in pVA1542 (Fig. 3). Insertion of gtfB.1 into pVA1542 did not result in expression of ctxB, due to the lack of a promoter and ribosomal binding site. This was provided by the secretion vector pINIII ompA2, which encoded the leader peptide sequence of the E. coli ompA gene (Ghrayeb et al, 1984). Transcription of ompA was controlled by the inducible lac promoter. Transfer of the ctxB gene fusions into pINIII ompA2 resulted in expression of chimeric proteins that could be detected in whole-cell lysates using an ELISA assay for CTB. The secretion vector containing the ctxB chimera was designated pVA1782 (Fig. 3). E. coli strains containing this plasmid were able to express a chimeric protein that was immunoreactive with antiserum to CTB or to GtfB. Immunoblotting analysis of the V1782 chimera was performed by preparing lysates from independently isolated clones from cells grown on M-9 agar plates containing 1 mM IPTG. The samples were separated by 15% SDS-PAGE under reducing conditions and then electrophoretically transferred to nitrocellulose sheets. Proteins that reacted with antiserum to the glucosyltransferase B enzyme are indicated by arrows. The V1555 lysate contains two truncated versions of GtfB, and was used as a control. Immunoreactive proteins were detected in the lysates from V1555 and the clones

expressing gtfB.1, but not from V1784 which was used as the background control.

The ability to overproduce the chimeric protein is a desirable trait for large-scale vaccine production. Induction of expression in V1782. Cell lysates containing the V1782 chimera or V1784, containing the plasmid vector, were grown in M-9 broth to an O.D. 660 = 0.6 before induction with 1 mM IPTG. Upon induction, the cells were allowed to express for 2 h before harvesting the contents of the periplasm by osmotic shock. As controls, parallel cultures of each strain were grown in the absence of IPTG. Samples of the shock fluid were separated by 15% SDS-PAGE and then blotted to nitrocellulose. The blots were reacted with antiserum to CTB. The addition of IPTG resulted in elevated levels of chimera being produced. However, overexpression of foreign genes in E. coli can be deleterious to the host. The laci gene, encoding repressor for the lac promoter, was located on the same plasmid in order to maximize expression of repressor. The laci gene reduced promoter activity significantly, but it did not completely inhibit expression of the protein. Although not tightly regulated, promoter activity was sufficiently repressed, in the absence of IPTG, to allow normal growth of the host. Osmotic shock experiments demonstrated that most of the protein was secreted into the periplasm of E. coli, which was consistent with the chimeric protein being fused to the ompA leader. No extracellular activity was detected. Previous work with the V1555 chimera resulted in the protein being trapped in the cytoplasm of E. coli. This made extraction of the protein difficult, but the use of ompA allowed secretion of the protein into the periplasm where it could be extracted easily from the cell by osmotic shock. The chimeric protein derived from V1782 was used for all subsequent studies in this paper.

E. Purification of the V1782 Chimera

The chimeric protein was purified by GM_1 affinity chromatography, based on the affinity of the CTB moiety for GM_1. The column was used to concentrate the chimera from the

shock fluid by recirculating the fluid through the column. Saturation at 4°C was achieved within 24 h of initial loading of the column. The chimera was eluted from the column only upon decreasing to pH 3.0. The use of a higher pH did not result in elution of the protein. Table III lists the purification data from a typical experiment. Approximately 23 g of protein could be collected from one elution. Silver-staining of a sample from the column, separated by SDS-PAGE, revealed that most contaminating proteins were removed by this method. No proteolysis of the protein was observed as was seen in the V1555 chimera.

A sample of the affinity-purified chimera was loaded onto a gel filtration column (Sephadex G-100) and eluted in PBS. Gel filtration profile of the V1782 chimera. The GM_1 column-purified chimera was fractionated through a 1.6 x 200 cm column containing Sephadex G-100. Samples of the column fractions were reduced and then separated by 15% SDS-PAGE. Proteins were visualized by silver-staining. Activity was measured by an ELISA assay, which depends on the ability of the chimera to bind to GM_1-coated wells and to be recognized by antiserum to CTB. Total protein was measured using the BCA assay (Pierce). The sample was fractionated into two major size classes. Using native molecular weight standards, the proteins were estimated to be 54 and 17.4 kDal (CTB = 33 kDal). Samples from the column fractions were separated by SDS-PAGE, which revealed the presence of two species (16.4 and 17.4 kDal) that were common to both fractions. Immunoblotting analysis demonstrated that these species were immunologically identical regardless of whether antibody to CTB or to GtfB was used. Because the composition and immunoreactivity of the column fractions were identical, it was assumed that the fractions contained monomeric and oligomeric forms of the chimera. A high molecular weight protein was observed in the oligomeric fraction that did not react with antisera in the immunoblot, and was not present in

the fraction containing monomer. Biological activity, based on ELISA, indicated that most of the reactive material was associated with the oligomer.

F. Analysis of the V1782 Chimera

The monomeric form of the V1782 chimera was subjected to 25 cycles of Edman degradation. The amino acid sequence was found to be identical to that predicted by the construction of such a fusion protein (Fig. 5). According to the sequence data, the leader sequence of the ompA protein was absent from the periplasmic form of the protein. Fusion of the chimeric protein to the ompA leader did not appear to affect the ability of E. coli to recognize and cleave the signal sequence. The data also suggested the absence of any additional N-terminal sequences in the sample.

The amino acid composition of the C-terminal ends of monomeric and oligomeric forms of the chimera were also compared to that of native CTB. Protein samples were cleaved by reaction with cyanogen bromide and the peptide fragments were fractionated by HPLC. Elution of the protein was monitored by absorbance at 215 nm. The resultant peptides were then analyzed for their total amino acid composition (Table IV). DNA sequence data for the ctxB gene predicted that cyanogen bromide cleavage would release an ala-asn dipeptide fragment if translation of the genes terminated properly in E. coli (Lockman and Kaper, 1983). Table IV shows that peptide A had this composition. This dipeptide was isolated from all three samples. The amino acid composition of peptide B was found to match that expected for residues 61-92 of the chimera. Peptide C is apparently the unresolved mixture of the remaining cyanogen bromide peptides. For the chimera, peptides A and B were collected, analyzed, and shown to be present in essentially a 1:1 ratio, recovering 0.51 nmoles A/0.62 nmoles B. This was essentially identical to the amount of each obtained from the native CTB (0.96 nmoles A/1.2 nmoles B). Therefore, the possibility that the sample is a mixture of native CTB and chimera was ruled out. It can be concluded that, within the limits of

measurement, translation of the protein uniformly terminated at the stop codon normally recognized for ctxB.

G. **SDS-PAGE of the V1782 Chimera**

Analysis of the effluent from the gel filtration column by SDS-PAGE suggested that there were two forms of the chimera in the monomeric and oligomeric fractions of 17.4 and 16.4 kDal in size. Samples of CTB, monomer, and oligomer were reduced and alkylated, in order to block disulfide bond formation, and then fractionated by SDS-PAGE. The samples were fractionated by 15% SDS-PAGE and then stained with Coomassie blue. Some samples were reduced with 2-mercaptoethanol and then alkylated with iodoacetamide prior to loading them onto the gel. Certain samples were boiled for 5 min. in loading buffer containing 2.5% 2-mercaptoethanol and 1% SDS. Other samples were prepared similarly, except 8 M urea was included in the sample buffer and gel. Reduction and alkylation had no effect on the profile of these samples when fractionated by SDS-PAGE. When these same samples were fractionated by SDS-PAGE using gels containing 8 M urea, only a single species of 14.4 kDal was observed. This protein was the same size as the one predicted by the nucleotide sequence data for the monomeric form of the chimera.

H. **Disulfide Bond**

The monomeric and oligomeric forms of the chimera were analyzed for the presence of disulfide bonds by alkylation of the proteins with iodoacetamide. The alkylated samples were hydrolyzed and then analyzed for their amino acid composition. After alkylation with iodoacetamide, 2.2 moles of carboxymethylcysteine per mole of protein were found for the monomer, while none was detected in the oligomer. This was consistent with the monomer having two free sulfhydryl groups, and suggested that the monomeric fraction contained a reduced form of the chimera which lacked the proper intramolecular disulfide bond.

I. **Circular Dichroism**

Samples of monomer, oligomer, and CTB were subjected to analysis by circular dichroism. The spectra of these samples are shown in Figure 6. The results indicated a shift in the minima for the oligomer compared to that of native CTB. This suggested that the oligomer has less organized secondary structure than CTB, but this could not be correlated with a detectable difference in its activity. On the other hand, the conformation of the monomer was radically different from both CTB and the oligomer, and this could be correlated with a loss of activity.

J. **Immunoreactivity of the Chimera**

Previous immunoblots demonstrated that the V1782 chimera was antigenic for antisera either to CTB or to GTF. However, it was not clear that this protein was immunogenic or that antiserum raised to the chimera would recognize the native GTF enzyme. In order to determine this, replicate blots were prepared containing CTB, GTF, and chimera. The blots were probed with antiserum to either CTB, GTF, or chimera. The results demonstrated that the chimera elicited antibodies which reacted with CTB and to GTF (Fig. 7). The reaction of the anti-chimera antibody with GTF was weaker than that produced by the antiserum to GTF, but this was expected since the anti-chimera antibody is specific for relatively tiny region of the 150 kDal enzyme.

K. **Inhibition of Enzyme Activity**

Antiserum to the chimera was evaluated for its potential to inhibit glucosyltransferase activity _in vitro_. The specificity of the assay was verified before performing the inhibition studies. Extracellular proteins of _S. mutans_ GS-5 were assayed for glucosyltransferase activity by measuring the incorporation of ^{14}C-(glu)-sucrose into glucan polymer. Total polymer was precipitated in methanol and collected on glass fiber filters. In order to determine if enzyme activity was actually being measured, the assay was verified in two ways: (A), glucan synthesis was shown to increase linearly over 20 hours of incubation; (B), the

amount of glucan synthesized was inversely proportional to the amount of protein used. Sampling was performed in triplicate. The mean counts per minute for each sample was subtracted from a background control containing PBS. It was demonstrated that the assay increased linearly over time and that dilution of the enzyme resulted in a decrease in polymer formation.

The enzyme preparation was diluted 1:2 in either PBS (control), pooled normal rabbit serum (N.R.S.), rabbit anti-GtfB serum, or rabbit anti-chimera serum. The samples were then preincubated at 37°C for 1 h before performing the assay. Total glucan was measured after the reaction proceeded for 18 h at 37°C. All samples were performed in triplicate. Pre-incubation of the enzyme in undiluted antiserum to either the chimera or GtfB resulted in a reduction in total glucan synthesis, relative to the control. Pooled normal rabbit serum (NRS) did not inhibit enzyme activity. In order to assess the kinetics of inhibition, enzyme activity was measured over time. The results demonstrated that the antiserum to GtfB decreased the rate of total glucan synthesis (water soluble + water-insoluble), but did not prevent synthesis of glucans (Fig. 8). This suggested that the inhibition is probably due to steric hinderance rather than disruption of the active site of the enzyme.

The antisera were compared for their level of inhibition by dilution. Antiserum to either GtfB or the chimera was diluted in PBS prior to preincubation with the enzyme. The synthesis of total glucans was measured after the reaction had proceeded for 8 h at 37°C. Sampling was performed in triplicate. Results indicated that the ability of the anti-chimera antiserum to inhibit activity was approximately half that of the anti-GTF antiserum. Again, this was not surprising due to the limited specificity of the antiserum to the chimera. Water-insoluble glucans have been implicated as having a greater role in the accumulation of S. mutans to the tooth surface than water-soluble polymers. In

order to determine if the antisera had any effect on the type of glucan polymer formed, water-insoluble assays were performed (Fig. 9). The results clearly demonstrated a dramatic effect on the synthesis of water-insoluble glucans. Water-soluble glucans comprise most of the total polymer synthesized, and the antiserum to GtfB inhibited this enzyme activity by approximately 50%. However, the formation of water-insoluble glucans was inhibited greater than 90% by the antiserum. The effect of the antiserum on fructan synthesis was determined as well (Fig. 10). The results demonstrated that the antisera were also able to inhibit total fructan synthesis, although much less than for glucans. This result was unexpected, but upon comparison of the sequence data for gtfB.1 and fructosyltransferase, the presence of two tripeptides common to both peptides (phe-asp-asp; ala-trp-asn) was revealed. The antisera to the chimera may partially recognize these sequences, and thus, interfere with the synthesis of fructans. The data has shown that antiserum to peptides of GTF can inhibit glucan synthesis and suggests that these anti-peptide antibodies may impair the ability of S. mutans to colonize the tooth surface.

III. DISCUSSION OF RESULTS

Purification of the V1782 chimera was greatly simplified by two factors. First, by secreting the chimera into the periplasm of E. coli, the contents of the periplasm could be released from the rest of the cell and then separated by centrifugation. This removed the majority of potential contaminants. Second, the high affinity of CTB for GM_1 ganglioside was used to purify the chimera by affinity chromatography. This provided a rapid method of removing the majority of the remaining contaminants from the chimera. As Table III shows, a large amount of protein could be purified by a single elution from the column, which rendered this system amenable to vaccine-scale production of protein. Most of the high molecular weight proteins observed in the eluate are probably oligomeric forms of the chimera that were not separated by reducing SDS-PAGE, because they were reactive in

immunoblots. The V1555 chimera was previously shown to bind to GM_1. Elution of the protein was effective at pH 4.0, which was higher than the pH required to elute native CT from the affinity column (Tayot et al, 1981). It was postulated that fusion of CTB to a relatively large protein altered its affinity for GM_1.

In the V1782 chimera, fusion of a 15 amino acid sequence to CTB did not appear to alter its affinity for GM_1, since it could only be eluted from the column at pH 3.0. Elution at this pH did not appear to degrade the protein. Thus, the GM_1 binding domain of CTB appeared to have been unaltered by fusion of the peptide sequence to CTB. The present inventors have discovered that the use of large peptide fragments may affect the conformation of the GM_1 binding domain of CTB.

The primary structure of the V1782 chimera was verified by sequence and compositional analysis. The amino acid composition of the chimera agreed with that predicted by the nucleotide sequence data. The sequence of the first 25 amino acid residues of the mature form of the chimera was determined, and verified that the ompA leader was cleaved at the site normally recognized by E. coli (Ghrayeb et al, 1984). Cyanogen bromide cleavage provided a convenient method of isolating the C-terminal dipeptide fragment of the chimera and comparing it to that of CTB. As predicted, the C-terminal end of the chimera was no different from that of CTB, thus confirming that translation of the chimera was terminated at the stop codon normally recognized for CTB.

Gel filtration chromatography of the GM_1-purified chimera separated the protein into two fractions. Based on SDS-PAGE and immunoblotting analysis, it was concluded that the chimera was separated into its monomeric and oligomeric forms, since the same immunoreactive proteins appeared in each fraction. Native CTB is composed of five identical subunits, arranged in a non-covalent association (Gill, 1976). Each subunit contains an intramolecular disulfide bond which is crucial for CTB to bind to GM_1 (Ludwig et al,

1985). Assay of the monomer by ELISA, which is dependent on the protein binding to GM_1-coated microtiter wells, demonstrated almost no activity. However, the monomer was still immunoreactive in a Western blot, suggesting that it lacked the disulfide bond required for GM_1 binding. This was verified by reacting samples of the monomer and oligomer with iodoacetamide. Only the monomer was found to contain reduced forms of cysteine. In order for the monomer to co-elute from the affinity column, it must have been able to bind to GM_1. The subunits of CTB are reversibly dissociated by acidification (Hardy et al, 1988), and it may be that a fraction of the chimera was dissociated and reduced during elution from the column. The monomer can be renatured by gradually dialyzing it away from 8 M urea under alkaline conditions. Renaturation resulted in the formation of oligomers and reactivity in ELISA. The disulfide bridge of CTB appears to have a drastic effect on its conformation and function, since the absence of it precludes binding of chimera to GM_1 and forming oligomers. The results reported here with the chimera are consistent with those reported for native CTB and suggest that addition of a peptide to the N-terminal end of CTB has minimal effect on its ability to bind to its ligand or to form oligomers.

Attempts to estimate the molecular weight of the chimera under reducing conditions by SDS-PAGE revealed the presence of two forms of the chimera (16.4 and 17.4 kDal). These forms were both immunoreactive and much larger than predicted (14.4 kDal). Reduction and alkylation of the monomer or oligomer did not affect the pattern observed by SDS-PAGE. At first, these results were thought to demonstrate that the chimera consisted of two different primary structures, with additional amino acids at either the N or C-terminus of the larger species. However, the result from Edman degradation clearly established that only the expected N-terminal sequence was observed. Likewise, the C-terminus was clearly demonstrated to be only that expected from the proposed construction. Therefore, it was concluded

that the appearance on SDS gel must be due to higher level structural effects. This was confirmed, since fractionation of the samples by SDS-PAGE in 8 M urea reduced the pattern observed to a single protein of the expected size. Other species of protein were observed in samples of oligomer, but these were postulated to be undenatured, multimeric forms of the chimera. Thus, the chimera appeared to exist in two preferred conformations under reducing SDS-PAGE which were not observed with CTB. This suggested that the addition of the peptide to the N-terminal end of CTB has changed the way the protein folds in such a way that SDS alone did not completely unfold it under the conditions normally used. However, the addition of a strong chaotropic agent such as urea was able to completely denature the structure of the protein. The ability of the chimera to form oligomers and to bind to GM_1 suggested that the conformational differences observed did not significantly affect the physical properties of the CTB moiety. However, whether these differences had a significant effect on the adjuvant properties of CTB has yet to be determined.

Analysis of the protein by circular dichroism revealed some differences in the conformation of CTB and the oligomeric protein. However, the conformation of the monomer was radically different. The change in structure of the monomer was correlated with the loss of GM_1-binding activity and ability to associate with other subunits. These results demonstrated that the addition of extra amino acids to the N-terminal end of CTB has minimal effect on the conformation of the protein or its function.

Immunoblotting analysis and enzyme inhibition studies have shown that the gtfB.1 peptide was immunogenic, and that antiserum to the peptide could recognize the native protein. The gtfB.1 peptide was derived from the sequence of gtfB enzyme, which catalyzes the formation of primarily water-insoluble glucans from sucrose. It was not known if antiserum to the gtfB.1 peptide would inhibit enzyme activity. However, antiserum to gtfB.1 not only inhibited

the formation of water-insoluble glucans, but also the synthesis of soluble glucans as well. Soluble glucans are produced by the product of the gtfD gene, which has recently been cloned (Hanada and Kuramitsu, 1989), but not yet sequenced. Another enzyme, the product of the gtfC gene, catalyzes the formation of both soluble and insoluble glucans. Comparison of the nucleotide sequence data for gtfB and gtfC demonstrated that the gtfB.1 peptide was located in regions of both gene products that shared significant homology with one another. Although soluble glucan synthesis was somewhat reduced, compared to the control, insoluble glucan synthesis was almost completely eliminated. The reason for this difference is unclear, but it suggests that the antiserum inhibited the gtfB gene product more than the other glucosyltransferases. This may be due to minor differences in the conformation of the peptide between the gene products, which affect antibody binding. It may indicate significant structural differences between the enzymes. The peptide may be more exposed on the surface of GtfB, where it is accessible to antibody, compared to the other enzymes. Alternatively, the peptide may play a greater role in formation of the active site of GtfB, compared to the other enzymes, in which case the antibody affects its structure.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

Table I. Glucosyltransferases of *Streptococcus mutans*.

Gene	Size of Cloned Gene Product (kDal)	Glucan Synthesized	Reference
*gtf*B	150	water-insoluble[1]	Aoki et al, 1986
*gtf*C	140	water-soluble[1] water-insoluble[1]	Hanada et al, 1988
*gtf*D	155	water-soluble[2]	Hanada et al, 1989

[1]Enzyme does not require dextran primer for activity.

[2]Requires dextran primer for enzyme activity.

Table II. Bacterial strains and plasmids.

Bacteria or Plasmid	Genotype[1]	Reference
E. coli		
HB101	*leu*B6*pro*A2*ara*-14*xyl*-5 *gal*K*mtl*-1*lac*Y1*thi* *sup*E44*rec*A13*rps*L20	Boyer and Roulland-Dussoix, 1969
V1555	Ap, Tc	Present Application
V1619	Ap	Present Application
V1782	Ap	Present Application
V1784	Ap	Present Application
S. mutans		
GS-5		Present Application
Plasmid		
pJBK30	Ap, Tc	Kaper et al, 1984
pHK7	Ap	Present Application
pINIII*omp*A2	Ap	Ghrayeb et al, 1984
pVA1542	Ap, Tc	Present Application
pVA1543	Ap, Tc	Present Application
pVA1544	Ap, Tc	Present Application
pVA1555	Ap, Tc	Present Application
pVA1599	Ap, Tc	Present Application
pVA1662	Ap, Tc	Present Application
pVA1782	Ap	Present Application
pVA1784	Ap	Present Application

[1]Concentrations of antibiotics used in all media were: Ap = 50 μg/ml ampicillin; Tc = 20 μg/ml tetracycline.

Table III. Purification table: V1782 chimera.

Sample	Volume (ml)	μg Chimera[3]	mg Total Protein[4]	Specific Protein (μg/mg)[5]	Fold Purification	% Recovery
Shock Fluid[1]	2200	7,136	1084	6.6	1.0	100
GM_1 Column[2]	20	1,210	23	52.6	8.0	17[6]

[1]Expression of the chimera, in *E. coli* V1782, was induced by IPTG. After 2 h, the contents of the periplasm were liberated by osmotic shock.

[2]The shock fluid was loaded onto a GM_1, ganglioside affinity column. After washing the column at pH 6.8, the chimera was eluted from the column by pH 3.0 buffer.

[3]The amount of chimera in the samples was quantitated with a GM_1 ELISA, using native CTB protein as the standard.

[4]Total protein was quantitated using the BCA assay (Pierce).

[5]Specific protein was calculated as the μg chimera per mg protein.

[6]This value represents the amount of chimera recovered from the first passage of the shock fluid through the column.

Table IV. Amino acid composition of the cyanogen bromide peptides.

Amino Acid	Number of Residues[1]			
	CTB		Chimera	
	Peptide A	Peptide B	Peptide A	Peptide B
Asp	0.96 (1)	2.0 (2)	1.1 (1)	2.0 (2)
Thr		1.0 (1)		1.3 (1)
Ser		1.7 (2)		1.4 (2)
Glu		5.7 (5)		5.5 (5)
Pro		N.D. (1)		N.D. (1)
Gly		2.3 (2)		2.2 (2)
Ala	1.0 (1)	2.7 (3)	1.0 (1)	2.9 (3)
Val		1.8 (2)		1.9 (2)
Met		0.0 (1)		0.0 (1)
Ile		3.4 (5)		3.3 (5)
Leu		0.0 (0)		0.0 (0)
Tyr		0.0 (0)		0.0 (0)
Phe		1.7 (2)		1.9 (2)
His		0.9 (1)		1.1 (1)
Lys		2.8 (3)		2.7 (3)
Arg		1.1 (1)		1.1 (1)

[1]The values reported are for 24 h hydrolysates. The value of aspartic acid was assigned as 1.0 and 2.0 for peptides A and B, respectively. All other values were determined relative to this. Values in parentheses are those expected from the known sequence of the protein. Methionine was converted to homoserine by the reaction with cyanogen bromide, and was not determined because homoserine coelutes with glutamic acid. Proline was not determined since it does not react with the o-phthalaldehyde used for detection.

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SUBSTITUTE SHEET

<u>What is claimed is</u>:

1. A chimeric peptide comprising at least a portion of the B subunit of cholera toxin and an epitope region of a desired antigen fused to the N-terminal end of the B subunit of cholera toxin, said epitope region comprising an antigenic determinant of the desired peptide.

2. A chimeric peptide as in claim 1, wherein said epitope region comprises no more than one hundred amino acids.

3. A chimeric peptide as in claim 2, wherein said epitope region comprises about 15 to 20 amino acids.

4. A vaccine for administration to a subject to elicit an immune response in the subject to a desired antigen, comprising a chimeric peptide having at least a portion of the B subunit of cholera toxin and an epitope region of a desired antigen fused to the N-terminal end of the B subunit of cholera toxin, said epitope region comprising an antigenic determinant of the desired peptide, and a pharmaceutically acceptable carrier.

5. A vaccine as in claim 4, wherein said epitope region comprises no more than one hundred amino acids.

6. A vaccine as in claim 5, wherein said epitope region comprises about 15 to 20 amino acids.

7. A vaccine as in claim 4, wherein the B subunit of cholera toxin enhances the immune response of said epitope region when administered orally.

8. A vaccine as in claim 4, wherein said vaccine is administered to help prevent dental caries and said epitope region includes a segment of the glucosyltransferase B protein no greater than one hundred amino acids long.

9. A vaccine as in claim 8, wherein said epitope comprises the following amino acid sequence:
Ser-Ala-Trp-Asn-Ser-Asp-Ser-Glu-Lys-Pro-Phe-Asp-Asp-His-Leu.

10. A substantially pure DNA sequence coding for a chimeric peptide comprising at least a portion of the B subunit of cholera toxin and an epitope of a desired peptide fused to the N-terminal end of the B subunit of cholera toxin, said epitope region comprising an antigenic determinant of the desired peptide.

11. A substantially pure DNA sequence as in claim 10, wherein said epitope region comprises a 15-20 amino acid sequence of the glucosyltransferase B protein.

12. A vector containing the DNA sequence of claim 10, said vector being capable of expression in a host organism.

13. A vector containing the DNA sequence of claim 11, said vector being capable of expression in a host organism.

14. A vector comprising pVA1782.

15. A vector comprising one of pVA1542, pVA1543 and pVA1544.

16. A substantially pure epitope region of the glucosyltransferase B protein comprising the following amino acid sequence:
Ser-Ala-Trp-Asn-Ser-Asp-Ser-Glu-Lys-Pro-Phe-Asp-Asp-His-Leu.

17. A host organism transfected with the vector of claim 10.

18. A method of vaccinating a subject comprising administering orally to the subject a composition in a dosage sufficient to elicit an antibody response thereby raising antibodies in the patient directed against a given peptide, said composition including a chimeric peptide comprising at least a portion of the B subunit of cholera toxin and an epitope region of the given peptide to which an antibody response is desired fused to the N-terminal end of the B subunit of cholera toxin, said epitope region being an antigenic determinant of the peptide to which an antibody response is desired, and a pharmaceutically acceptable carrier.

19. A recombinant-DNA mediated method for the production of a chimeric peptide comprising:

(a) preparation of at least one portable DNA sequence encoding at least a portion of a B subunit of cholera toxin and an epitope capable of eliciting an antibody response in a patient, the DNA encoding said epitope being at the 5′ end of the DNA portion encoding the B subunit of cholera toxin;

(b) cloning the portable DNA sequence into at least one vector capable of being transferred into and replicated in a host microorganism, said vectors containing

elements for the expression of the chimeric peptide encoded by the portable DNA sequences;

(c) transferring the vectors containing the portable DNA sequences into a host microorganism capable of producing at least one chimeric peptide under the direction of the vector;

(d) culturing the host microorganism under conditions appropriate for maintenance of the vectors and synthesis of the chimeric peptide; and

(e) harvesting the chimeric peptide.

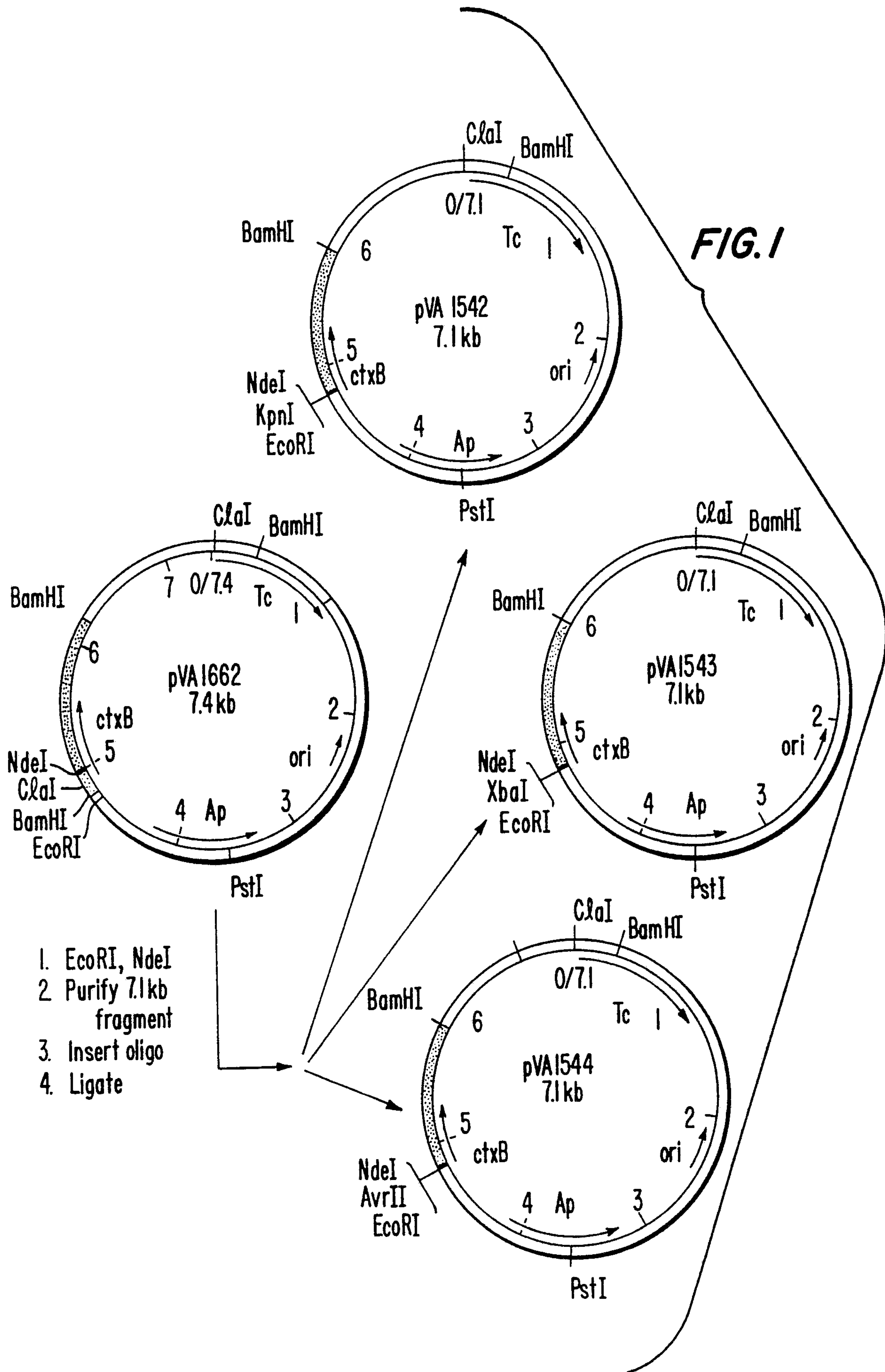


FIG. 1
ClaI
BamHI
0/7.1
Tc
1
BamHI
6
pVA 1542
7.1 kb
2
ori
5
ctxB
NdeI
KpnI
EcoRI
4
Ap
3
PstI
ClaI
BamHI
7
0/7.4
Tc
1
BamHI
6
pVA1662
7.4kb
ctxB
2
NdeI
5
ori
ClaI
BamHI
4
Ap
3
EcoRI
PstI
ClaI
BamHI
0/7.1
BamHI
Tc
1
6
pVA1543
7.1kb
2
5
ctxB
ori
NdeI
XbaI
EcoRI
4
Ap
3
PstI
1. EcoRI, NdeI
2. Purify 7.1kb fragment
3. Insert oligo
4. Ligate
ClaI
BamHI
0/7.1
BamHI
Tc
1
6
pVA1544
7.1kb
5
2
ctxB
ori
NdeI
AvrII
EcoRI
4
Ap
3
PstI

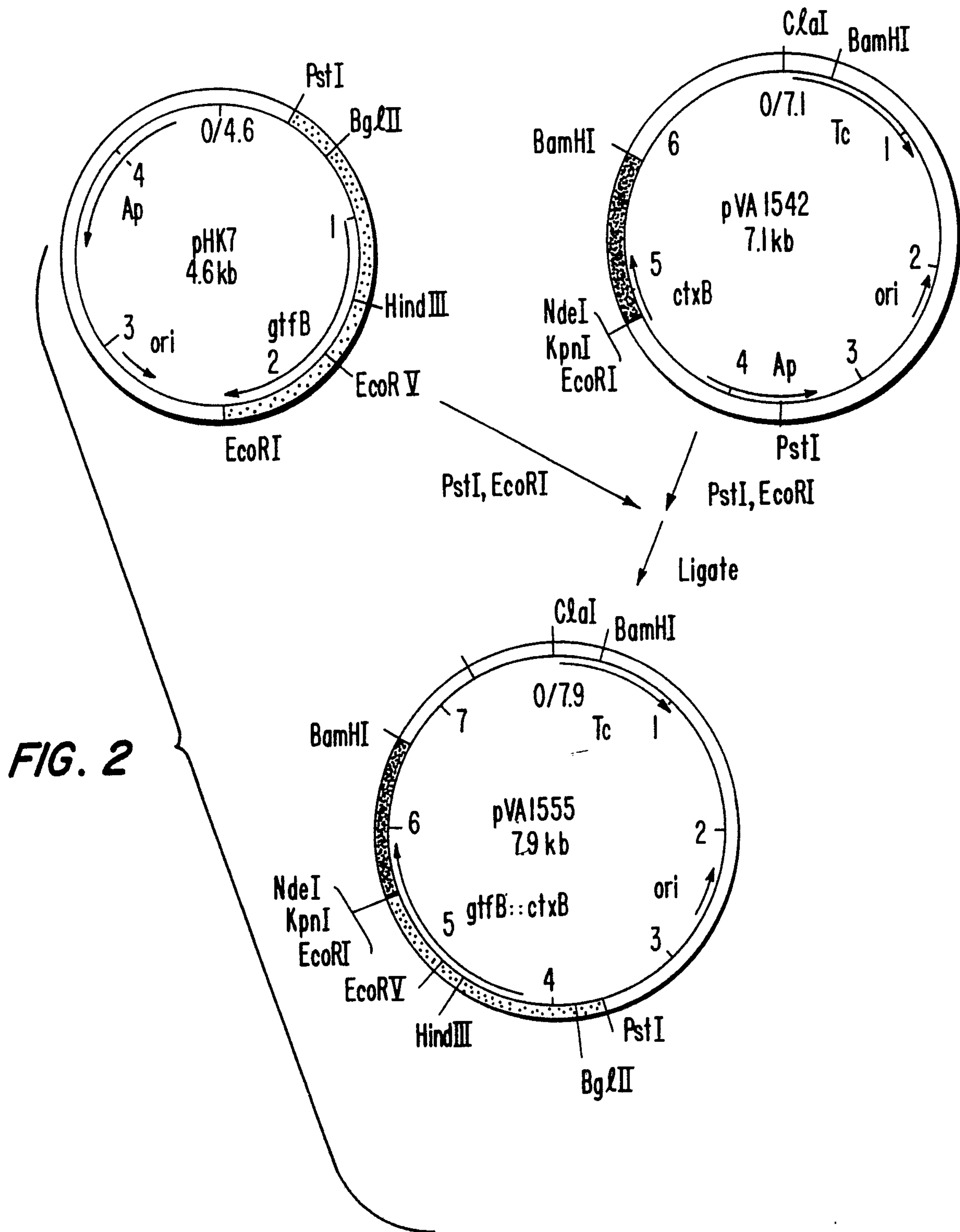


FIG. 2

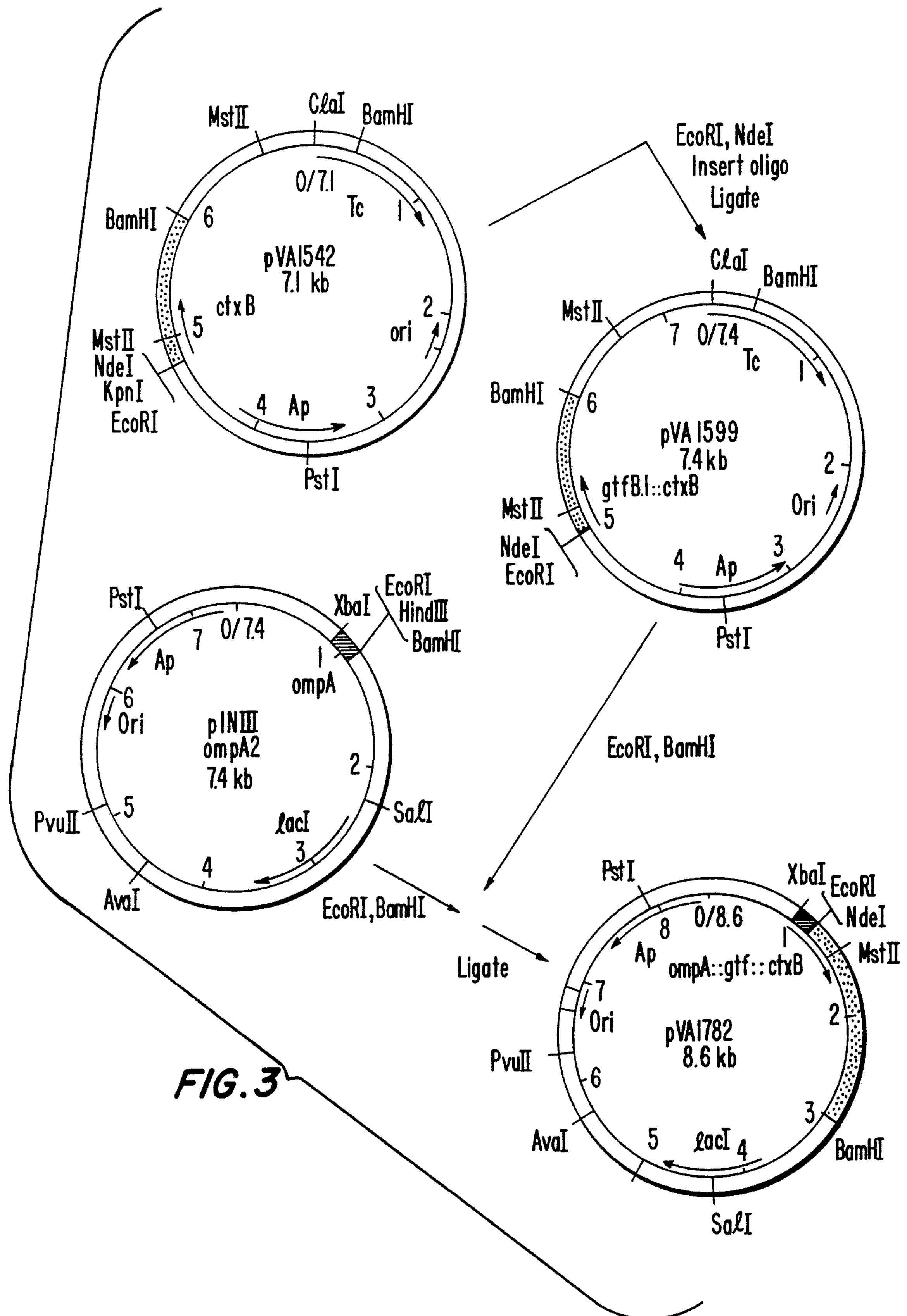


FIG. 3

FIG. 4

pVA1542 (20 bases):

EcoRI, KpnI, NdeI

gtfB---GAATTCGGTACCGATCCTTCATATG---ctxB

Glu Phe Gly Thr Asp Pro Ser Tyr

pVA1543 (21 bases):

EcoRI, XbaI, NdeI

gtfB---GAATTCGGATCCTTCTAGAGCATATG----ctxB

Asn Ser Asp Pro Ser Arg Ala Tyr

pVA1544 (19 bases):

EcoRI, AvrII, NdeI

gtfB---GAATTCTGGATCCTAGGTCATATG---ctxB

Ile Leu Asp Pro Arg Ser Tyr

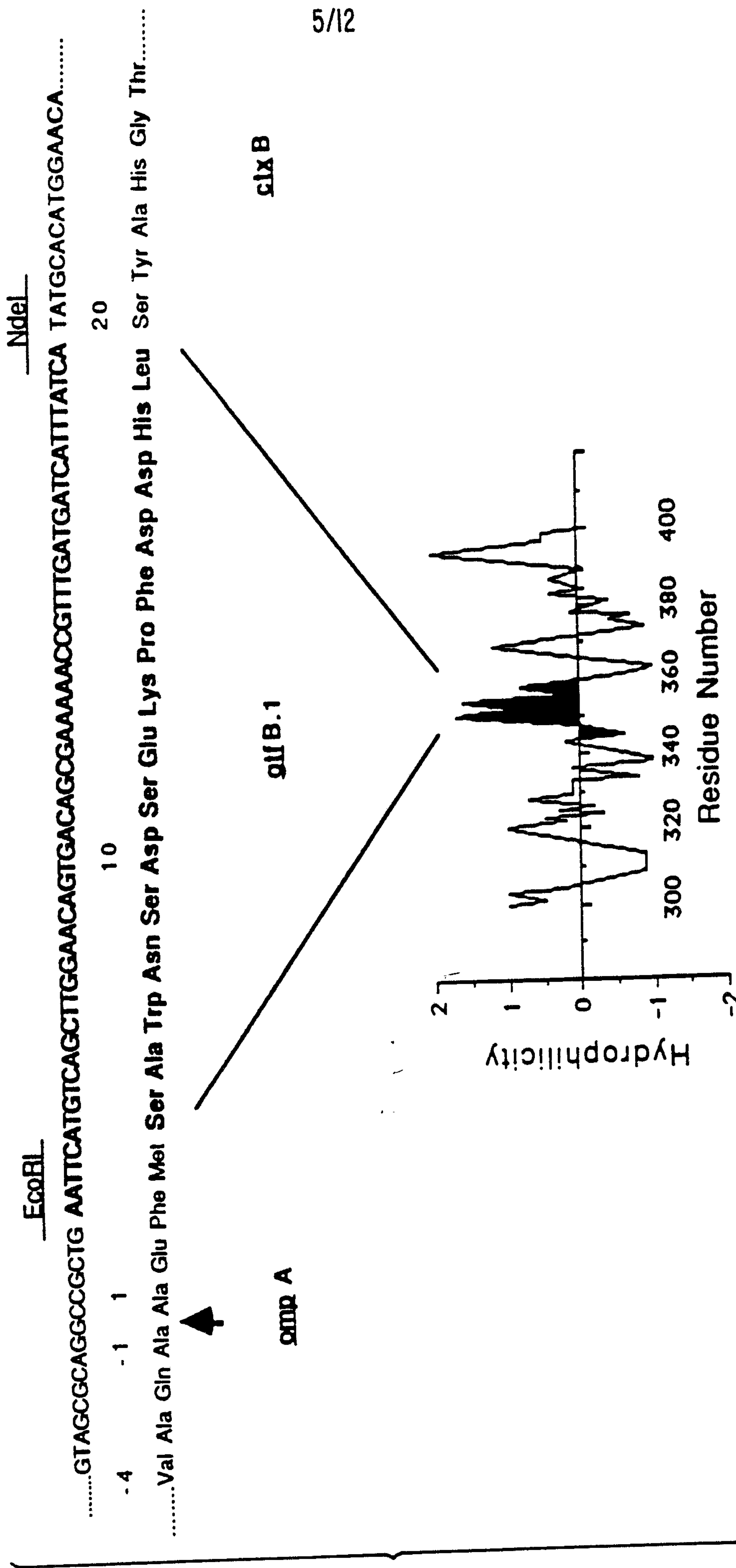


FIG. 5
EcoRI
NdeI
........GTAGCGCAGGCGCTG AATTCATGTCAGCTTGGAACAGTGACAGCGAAAAACCGTTTGATGATCATTTATCA TATGCACATGGAACA........
-4 -1 1 10 20
........Val Ala Gln Ala Ala Glu Phe Met Ser Ala Trp Asn Ser Ser Asp Ser Glu Lys Pro Phe Asp Asp His Leu Ser Tyr Ala His Gly Thr........
omp A
glf B.1
clx B
Hydrophilicity
2
1
0
-1
-2
300
320
340
360
380
400
Residue Number

FIG. 6

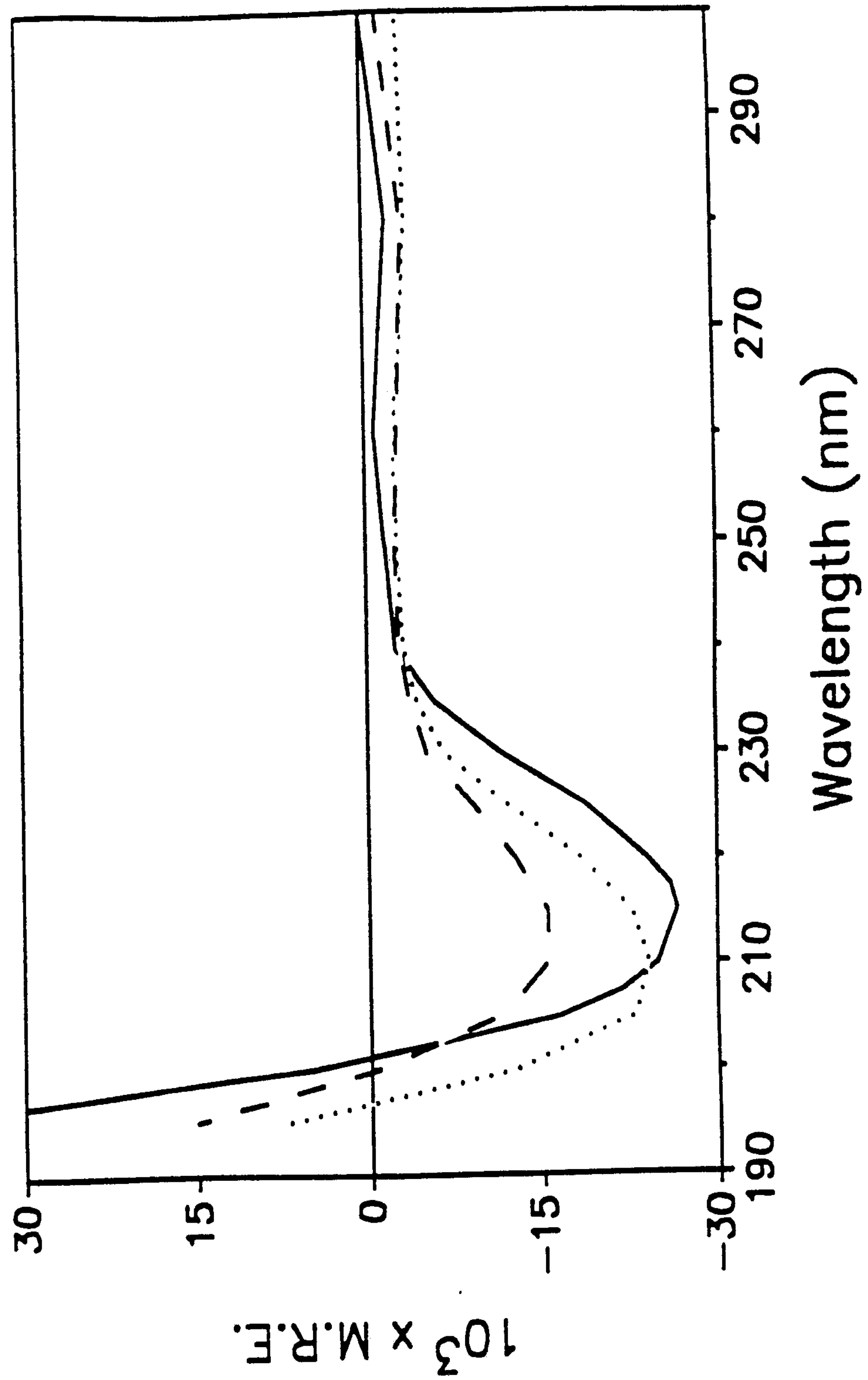
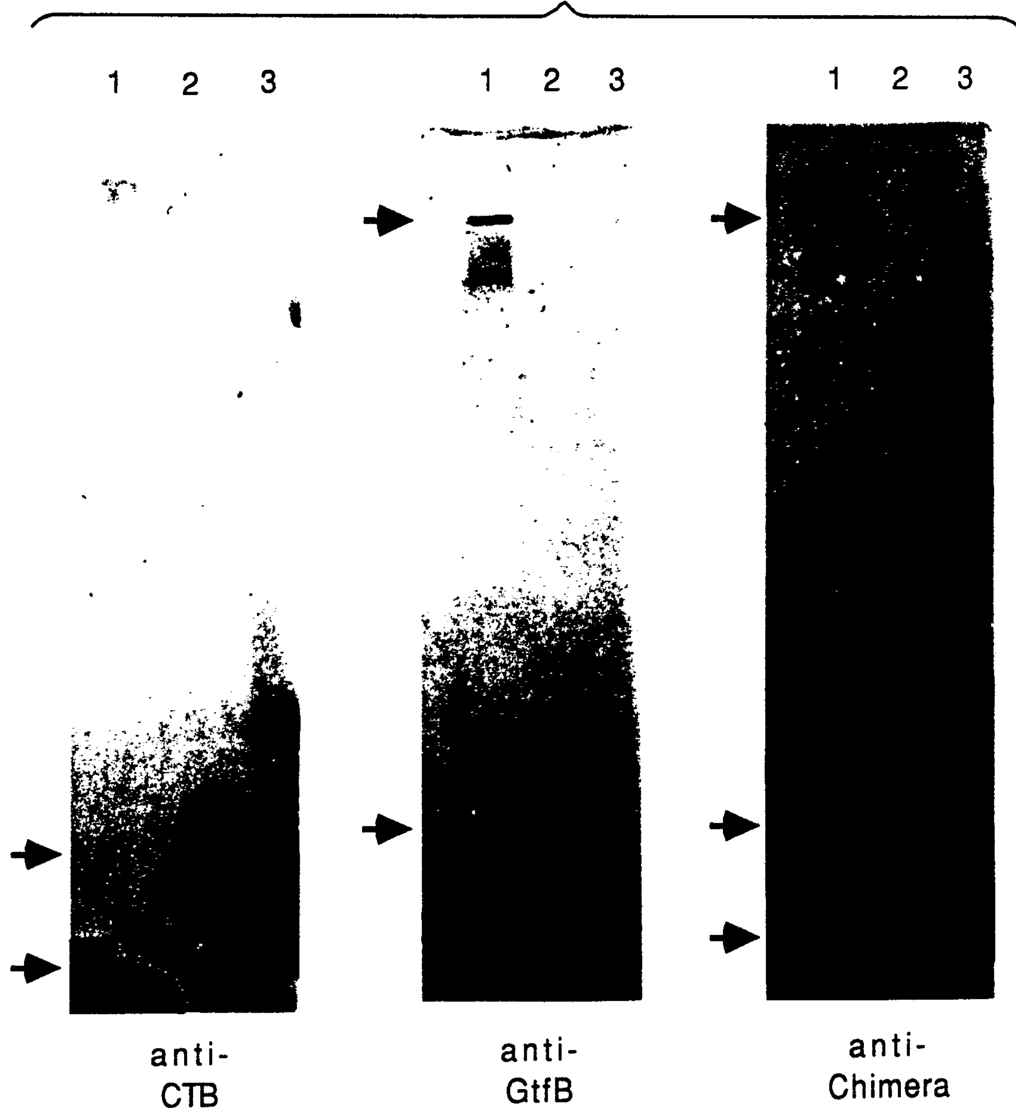


FIG. 7



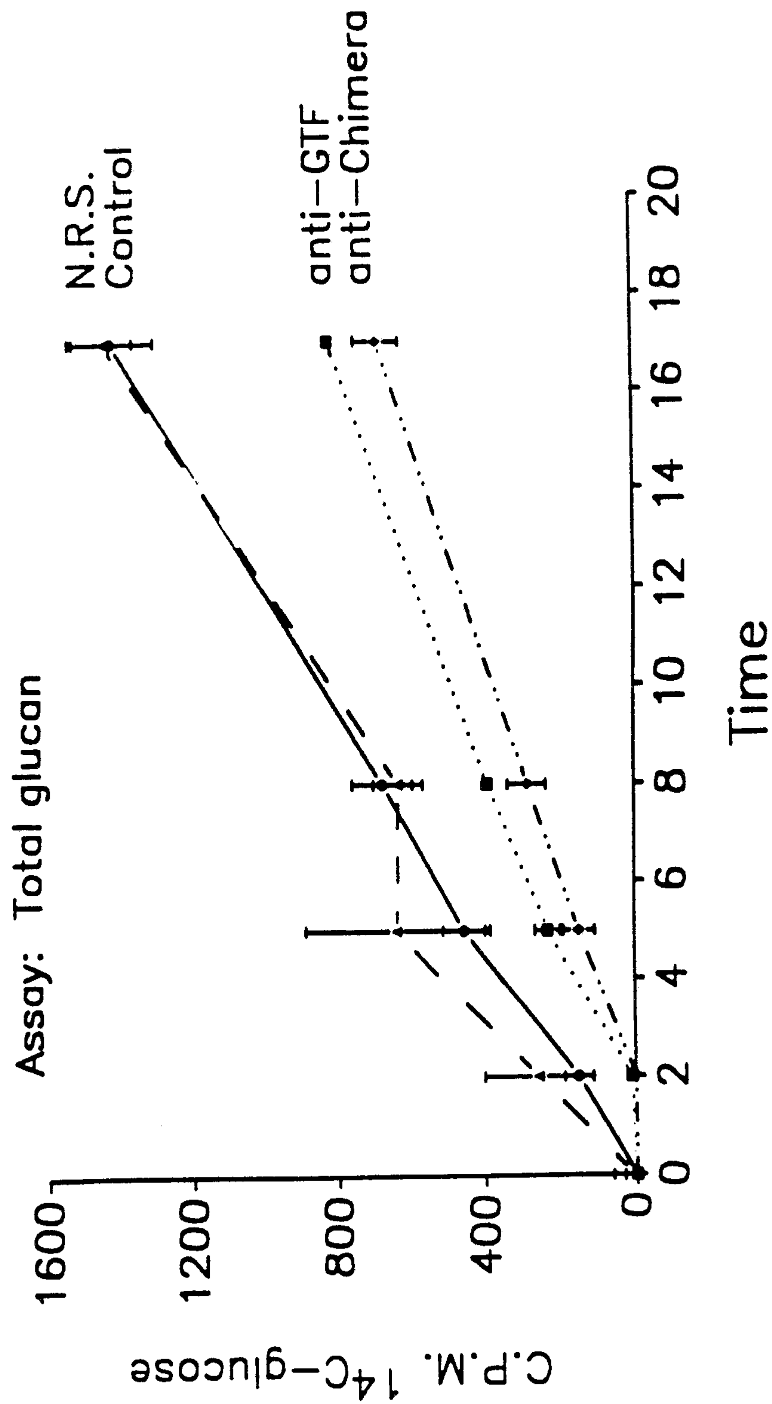


FIG. 8
Assay: Total glucan
N.R.S. Control
anti-GTF
anti-Chimera
C.P.M. 14C-glucose
1600
1200
800
400
0
0
2
4
6
8
10
12
14
16
18
20
Time

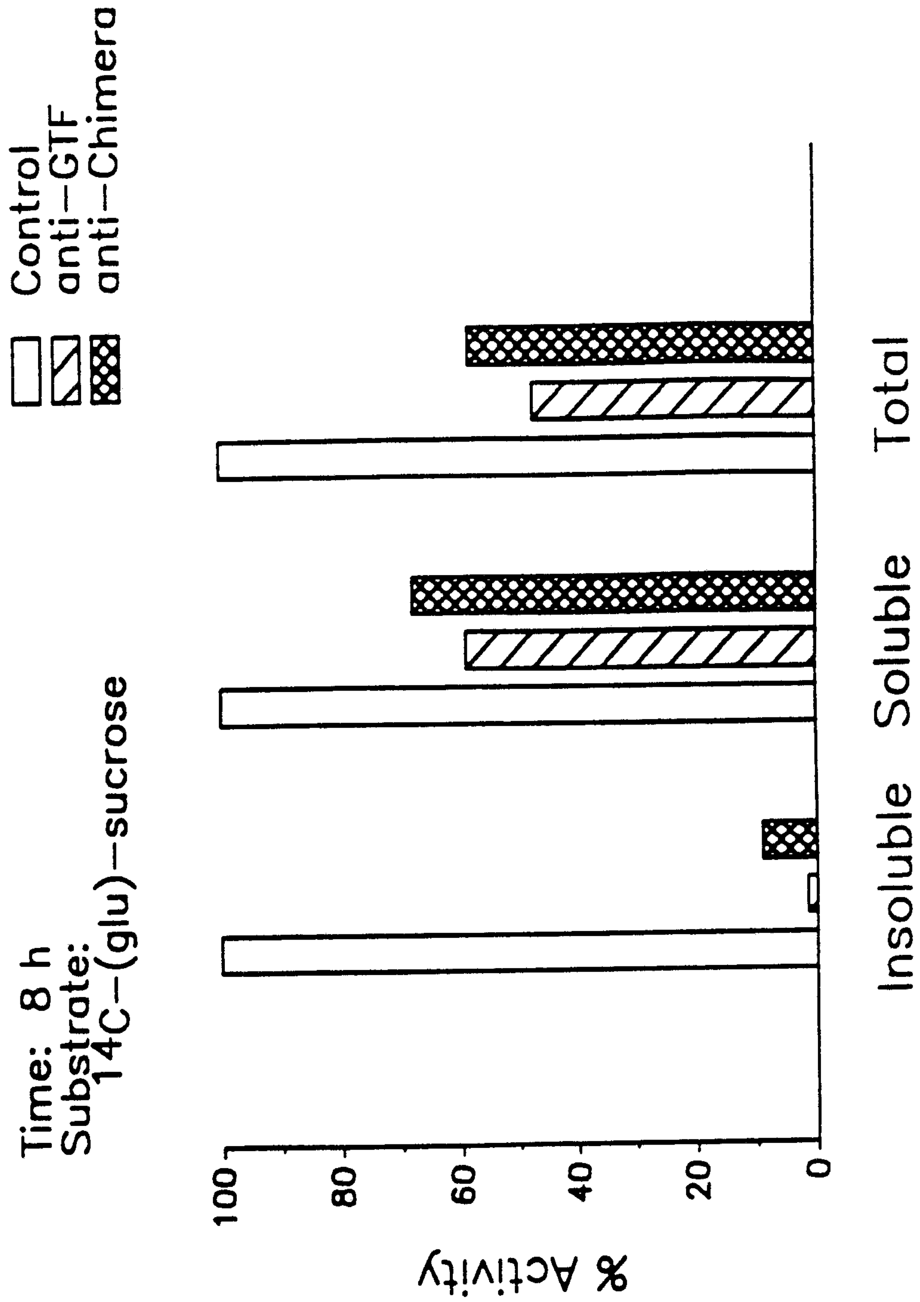


FIG. 9

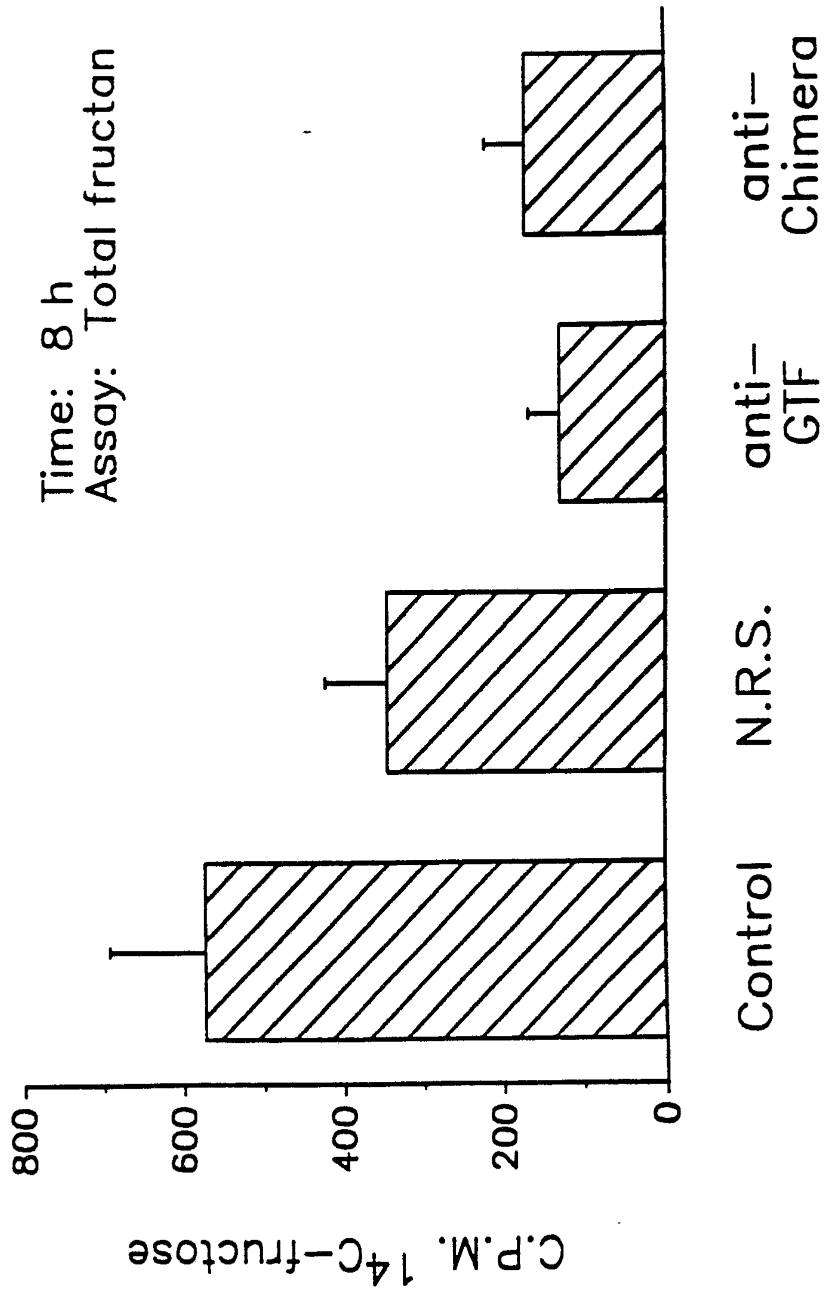


FIG. 10

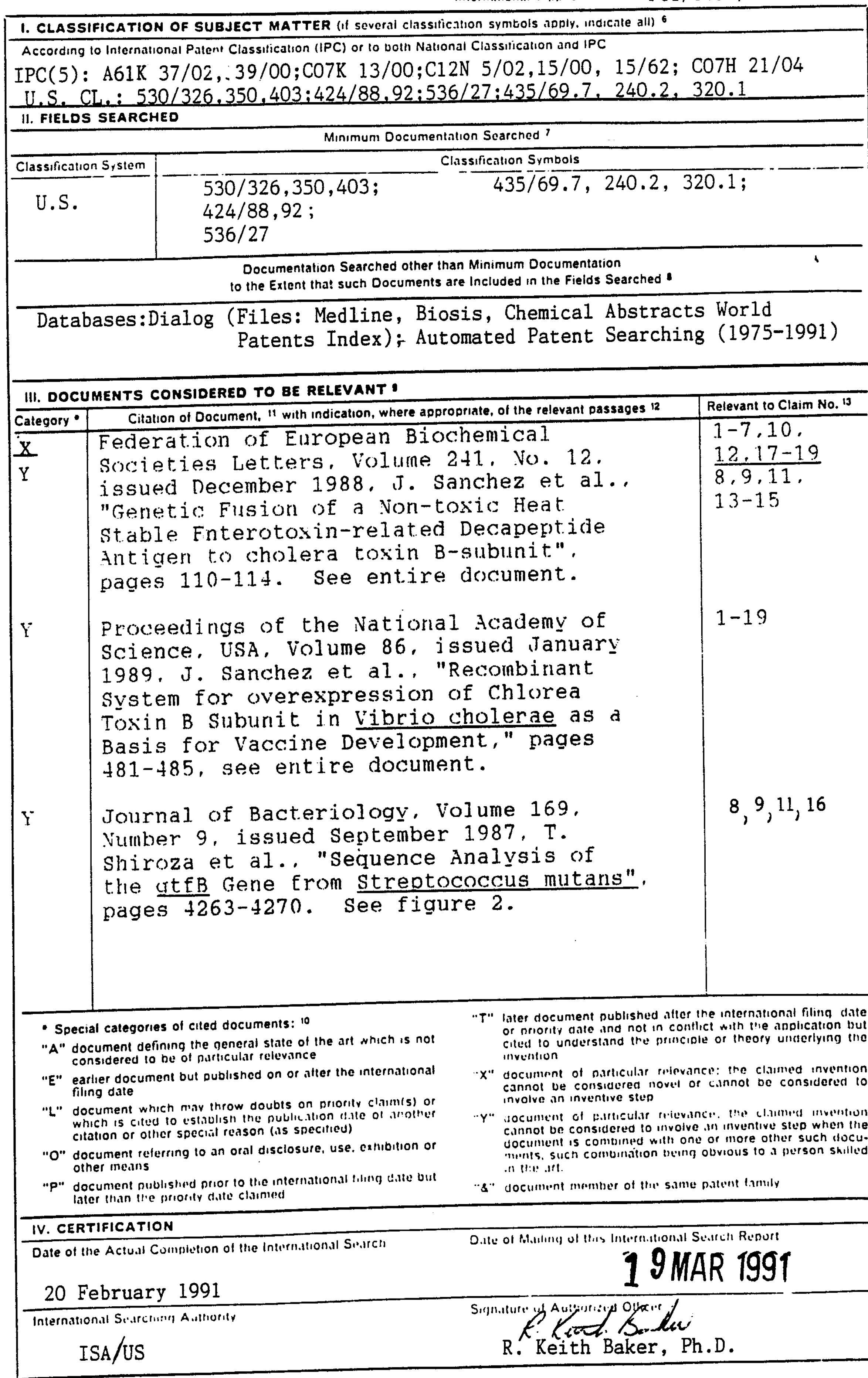
FIG. 11A

```
        10         20         30         40         50         60
GAATTCATGT CAGCTTGGAA CAGTGACAGC GAAAAACCGT TTGATGATCA TTTATCATAT

        70         80         90        100        110        120
GCACATGGAA CACCTCAAAA TATTACTGAT TTGTGTGCAG AATACCACAA CACACAAATA

       130        140        150        160        170        180
TATACGCTAA ATGATAAGAT ATTTTCGTAT ACAGAATCTC TAGCTGGAAA AAGAGAGATG

       190        200        210        220        230        240
GCTATCATTA CTTTTAAGAA TGGTGCAATT TTTCAAGTAG AAGTACCAGG TAGTCAACAT

       250        260        270        280        290        300
ATAGATTCAC AAAAAAAAGC GATTGAAAGG ATGAAGGATA CCCTGAGGAT TGCATATCTT

       310        320        330        340        350        360
ACTGAAGCTA AAGTCGAAAA GTTATGTGTA TGGAATAATA AAACGCCTCA TGCGATTGCC

       370        380
GCAATTAGTA TGGCAAATTA A
```

FIG. 11B

```
                                       10                                      20
Ala Glu Phe Met Ser Ala Trp Asn Ser Asp Ser Glu Lys Pro Phe Asp Asp His Leu Ser
                                       30                                      40
Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
                                       50                                      60
Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala Gly Lys Arg Glu
                                       70                                      80
Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Ile Phe Gln Val Glu Val Pro Gly Ser Gln
                                       90                                     100
His Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr
                                      110                                     120
Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile

Ala Ala Ile Ser Met Ala Asn End
```

SUBSTITUTE SHEET



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06811

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02, 39/00; C07K 13/00; C12N 5/02, 15/00, 15/62; C07H 21/04

U.S. CL.: 530/326, 350, 403; 424/88, 92; 536/27; 435/69.7, 240.2, 320.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/326,350,403; 424/88,92; 536/27; 435/69.7, 240.2, 320.1;

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (Files: Medline, Biosis, Chemical Abstracts World Patents Index); Automated Patent Searching (1975-1991)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Federation of European Biochemical Societies Letters, Volume 241, No. 12, issued December 1988, J. Sanchez et al., "Genetic Fusion of a Non-toxic Heat Stable Fnterotoxin-related Decapeptide Antigen to cholera toxin B-subunit", pages 110-114. See entire document.	1-7,10, 12,17-19 8,9,11, 13-15
Y	Proceedings of the National Academy of Science, USA, Volume 86, issued January 1989, J. Sanchez et al., "Recombinant System for overexpression of Chlorea Toxin B Subunit in *Vibrio cholerae* as a Basis for Vaccine Development," pages 481-485, see entire document.	1-19
Y	Journal of Bacteriology, Volume 169, Number 9, issued September 1987, T. Shiroza et al., "Sequence Analysis of the *gtfB* Gene from *Streptococcus mutans*", pages 4263-4270. See figure 2.	8,9,11,16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

20 February 1991

Date of Mailing of this International Search Report

19 MAR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

R. Keith Baker, Ph.D.

International Application No. PCT/US90/06811

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Proceedings of the National Academy of Science, USA, Volume 83, issued September 1986, V. Mehra et al., "Efficient Mapping of Protein Antigenic Determinants", pages 7013-7017. See entire document.	8,9,11, 16
A	Vaccine, Volume 6, issued October 1988, S. Tamura et al., "Protection Against Influenza Virus Infection by Vaccine Inoculated Intranasally with Cholena Toxin B Subunit", pages 409-413. See entire document.	1-19