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A monoclonal antibody generated against a recombinant peptide fragment of the B3 domain of carcinoembryonic antigen reacts with intact carcinoembryonic antigen

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Abstract

The chemical synthesis of a gene coding for a polypeptide of 77 amino acid residues (designated ceaB3) representing a fragment of the CEA-B3 domain of carcinoembryonic antigen (CEA) was achieved. The ceaB3 fragment was cloned into the plasmid pLZPWB1 at the C-terminus of a derivative lacZMF of the lacZ gene, devoid of methionine and cysteine amino acid residues. The fusion protein lacZMF-ceaB3 represented approx. 30% of total proteins expressed after induction. The fusion protein was formed as inclusion bodies. Simple washing steps led to an insoluble fusion protein which was of approx. 80% purity. Another fusion gene was generated by inserting ceaB3 between the malE gene encoding maltose binding protein (mbp) and lacZα of the pmal-c2 vector. Expression of the resulting pmal-c2-ceaB3-lacZα yielded the fusion protein mbp-ceaB3-lacZα with a molecular mass of 57.94 kDa, which was obtained as a soluble protein in almost homogenous form after affinity chromatography employing amylopectin. Polyclonal sheep anti-CEA antiserum specifically reacted with fusion proteins lacZMF-ceaB3 and mbp-ceaB3-lacZα. A monoclonal antibody CEA/HK2 was generated employing lacZMF-ceaB3 for immunization and CEA for screening purposes. The mAB CEA/HK2 specifically recognized CEA in immunoblots. The described experimental strategy should be generally applicable for generation of fusion proteins. These fusion proteins are suitable for epitope characterization of existing antibodies, production of regiospecific polyclonal or monoclonal antibodies.

Keywords: Carcinoembryonic antigen; Recombinant peptide fragment; Monoclonal antibody

1. Introduction

Carcinoembryonic antigen (CEA) was independently discovered by Gold and Freedman [1] and von Kleist and Burtin [2] to be present in colonic tumors as well as in fetal gut. Although CEA was also later found in normal body fluids and colonic mucosa of adults, CEA concentrations in tumor cases were on average significantly higher. A further complication is the existence of a large number of structurally related crossreacting antigens. However, CEA is still a widely employed parameter for monitoring cancer patients following treatment. A number of CEA-related genes have now been identified by molecular cloning. The human CEA gene family is classified into two subgroups, the CEA- and the pregnancy-specific glycoprotein (PSG) subgroup. For an excellent overview, see Thomson et al. [3]. Proteins of the CEA subgroup are membrane proteins, anchored by a glycosyl phosphatidyl inositol moiety. The release of CEA-related proteins might be controlled by hydrolysis involving phospholipases [3].

CEA, like the CEA-related proteins, is built in a domain-like structure [3]. Within a subgroup members reveal a high sequence conservation, whereas between subgroups the sequence homologies are significantly lower. Due to the high sequence similarity of CEA-related proteins and the limited number of accessible epitopes, it is evident that the number of monoclonal antibodies specific for CEA must also be limited. Other investigations concerned the localization of natural epitopes in CEA. Despite extensive glycosylation of CEA, most monoclonal antibodies recognize polypeptide epitopes [4]. Classification of CEA-specific epitopes made use of the expression of fusion-proteins with β-galactosidase to characterize the epitope specificity of monoclonal antibodies [5]. Hass et al. [6]

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expressed the four principal domains of CEA in *Escherichia coli* and employed the polypeptides to characterize the specificity of CEA-specific monoclonal antibodies.

The problem of measuring the serum concentrations of CEA are two-fold: (1) specificity of monoclonal antibodies with the potential to discriminate between CEA and CEA-related proteins and (2) the calibration of different

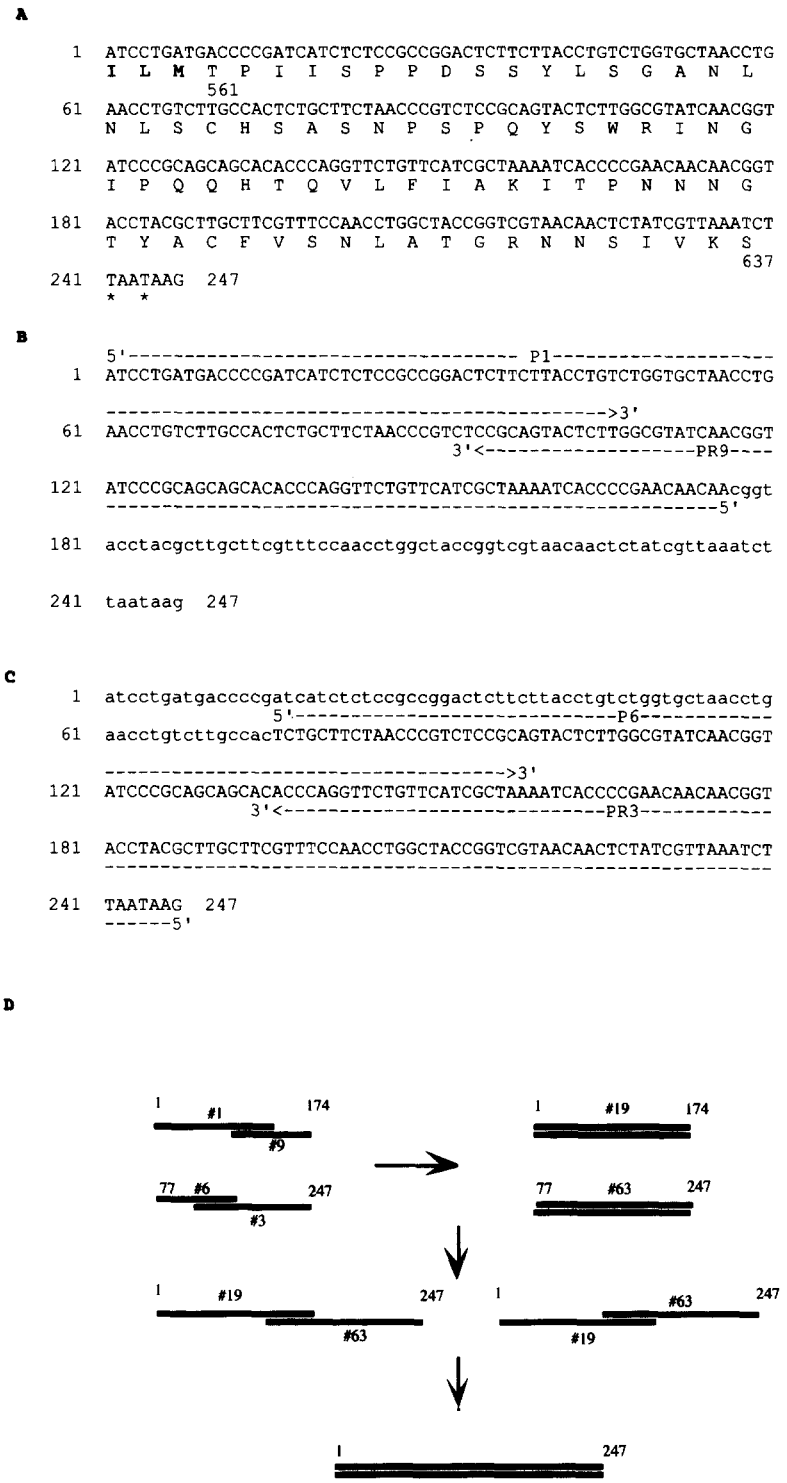

Fig. 1. Synthesis of the DNA fragment encoding ceaB3. (A) DNA sequence encoding the peptide region comprising residues 561–637 of CEA. The N-terminal residues represented by italics are not part of the CEA sequence. (B) Sequence and location of primers P1 and PR9 employed in the reactions outlined in scheme D. (C) Sequence and locations of primers P6 and PR3 employed in the reactions outlined in scheme D. (D) Schematic representations of the steps leading to the synthesis of ceaB3 DNA.

immunological test systems due to the lack of a characterized CEA preparation. The latter is difficult to achieve because a homogenous large scale preparation of CEA from either tissue or cell cultures seems to be impossible. We describe in this investigation an approach which addresses both problems. We identified by sequence comparison a 77 amino acid residue long fragment (designated ceaB3) of the B3 domain of CEA, which differed most significantly from other CEA-like proteins, especially nonspecific crossreactive antigen (NCA) (see [3] for reference). A synthetic DNA gene encoding ceaB3 was generated and expressed as a fusion protein. The fusion protein containing ceaB3 was employed both for the generation of monoclonal antibodies and for a CEA-specific antigen.

2. Materials and methods

2.1. General

Transformation of *E. coli*/XL-1 and *E. coli*/HB101 was performed as published [7]. The yield of transformation was generally about $2 \cdot 10^6$ transformants/μg DNA. Recombinants were screened on replica nitrocellulose filters (Millipore HATF) with a ^{32}P-labeled hybridization probe (specific activity $2 \cdot 10^8$ cpm/μg DNA). Prehybridization and hybridization were performed according to Hanahan and Meselson [8] at 58°C. Positive clones were characterized by sequencing. Double strand DNA sequencing was carried out by the dideoxy chain-termination method of Sanger et al. [9]. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer model 381A. A polyclonal antiserum against CEA was generously provided by Prof. Dr. Müller of Medizinische Akademie Dresden.

2.2. Bacterial strains and plasmids

E. coli XL-1/placZMFceaB3, *E. coli* XL-1/pmal-c2-ceaB3, *E. coli* HB101/pmal-c2-ceaB3-lacZα and *E. coli* XL-1/pUC18ceaB3 were grown in either LB-medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl/l) or 2 × TY-medium (16 g Bacto-tryptone, 10 g yeast extract and 5 g NaCl) containing 100 μg/ml ampicillin.

2.3. Synthesis of ceaB3-DNA

Oligonucleotides P1 (10 μg) and PR9 (10 μg) were annealed at 37°C and the formed duplex subjected to a 'fill in' reaction using 10 units of DNA polymerase Klenow fragment and the following standard reaction mixture: 0.2 mM dNTP in 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$ in a total volume of 100 μl. Incubation was carried out for 1 h at 37°C. A similar experiment was performed using the oligonucleotides P6 and PR3. The sequences of the oligonucleotides employed in these reactions were depicted in Fig. 1. The formed fragments #19 and #63 were purified by polyacrylamide gel electrophoresis in 5% gels under standard conditions. Fragments #19 and #63 (2 μg each) were mixed in the same buffer as above, heated to 90°C, cooled quickly to 37°C and subjected to 'fill in' reaction under conditions as described above. The reaction mixture was analyzed by agarose gel electrophoresis; a fragment of the appropriate size of 250 bp was purified from the agarose gel and ligated into a pUC18 vector restricted with *Sma*I, employing the following standard ligation reaction (mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 25 μg/ml BSA, 4 units T4-DNA ligase and incubation at 15°C for 15 h). Competent *E. coli* XL-1 were transformed with the ligation mixture and the cells plated on LB-agar containing 100 μg/ml ampicillin. Positive recombinant clones were detected by colony hybridization. Plasmid was prepared from 9 positive clones and subjected to double stranded sequencing. Two positive clones harboured plasmids which contained ceaB3-DNA with the correct sequence. The plasmid construct was named pUC18ceaB3.

2.4. Construction of placZMFceaB3

The vector pUC18ceaB3 was cut with *Eco*RI/*Bam*HI, the resulting ceaB3-fragment was purified by agarose gel electrophoresis and blunt ended by 'fill in' reaction under standard conditions. pLZPWB1 [10] was restricted with *Bam*HI/*Hin*dIII and sticky ends were blunt ended by 'fill in' reaction. The cut vector pLZPWB1 and the blunt ended ceaB3-DNA fragment were ligated in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 25 μg/ml BSA and 4 units T4-DNA ligase at 15°C for 15 h. This ligation mixture was directly employed to transform *E. coli* XL-1 using standard conditions [11,12]. Recombinant cells harbouring plasmids with ceaB3-DNA inserts were detected by colony hybridization. From positive recombinant cells, plasmids were isolated and subjected to double stranded sequencing. The plasmid containing the ceaB3 DNA fragment of correct sequence and in the proper reading frame was designated placZM-FceaB3.

2.5. Preparation of lacZMF-ceaB3

An overnight culture (20 ml) of *E. coli* XL-1/placZMFceaB3 in LB/amp was used to inoculate 2 l of LB/amp. After growth at 37°C to an OD (600 nm) of 0.5 the expression of the fusion protein was induced by addition of IPTG to a concentration of 1 mM. After 3 h at 37°C, the cells were harvested by centrifugation. The wet weight of the cell pellet was 7.2 g. The cell pellet was resuspended in 30 ml of 20 mM sodium phosphate buffer, pH 6.5 and the cells were then disrupted by sonication for two times 1 min at full power under cooling with ice. The

suspension was centrifuged for 20 min at 12000 rpm in a Beckman centrifuge employing a JA20 rotor. The pellet was suspended by sonication in 20 mM phosphate buffer, pH 6.5 containing 0.5% Triton X-100 and 1 mM EDTA. The suspension was centrifuged as described above and the procedure of resuspension repeated. The pellet was then dissolved in 8 M urea containing 10 mM DTT employing sonication. Insoluble particles were removed by centrifugation as described above. The supernatant was removed and the fusion protein was precipitated from the solution by diluting with 100 mM phosphate buffer, pH 6.0 to 3 M urea. The precipitate was centrifuged off as described and the remaining pellet washed successively with 20 mM phosphate buffer, pH 6.0 containing 3 M urea and finally with water. Sodium azide was added to the suspension up to a concentration of 0.1%. Lyophilization yielded 120 mg of fusion protein.

2.6. Cleavage of lacZMF-ceaB3 with cyanogen bromide

lacZMF-ceaB3 fusion protein (50 mg) was solubilized in 70% trifluoroacetic acid (TFA) (5 ml). A solution of cyanogen bromide (300 mg) in 70% TFA (1 ml) was added to the fusion protein. The solution was stirred at room temperature in the dark for 24 h. The proteins were precipitated from the solution by 10 volumes of ether, centrifuged and dried. The pellet was solubilized in a buffer of 50 mM Tris-HCl, pH 8.3 and 8 M urea. The cleaved lacZMF part of the fusion protein was precipitated from the solution by dilution to 2 M urea with 100 mM Tris-HCl pH 5.7 and centrifuged. The supernatant, containing the ceaB3 portion of the fusion protein was dialyzed against 10 mM Tris-HCl pH 7.5, 50 mM NaCl.

2.7. Construction of pmal-c2-ceaB3 and pmal-c2-ceaB3-lacZα

The ceaB3 DNA fragments for both constructions were prepared from placZMFceaB3 by polymerase chain reaction (PCR). PCR was carried out in the following manner: 3 min 94°C (start); 2 min 94°C, 3 min 50°C, 1 s 72°C (40 cycles); 10 min 72°C (end). The reaction mixture contained: 0.2 pmol placZMFceaB3, 100 pmol each of forward and reverse primer (forward primer for both constructions was the same, only the reverse primer differed), 25 nmol dNTPs, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 1 mg/ml gelatine and 2.5 U *Taq* polymerase. The reaction volumes were for both constructions 100 μl. The amplified ceaB3 fragments were restricted by *Hin*cII and *Hin*dIII. Vector pmal-c2 was restricted by *Xmn*I and *Hin*dIII. The cut pmal-c2 and each of the ceaB3 fragments were ligated, employing conditions as described above for the construction of placZMFceaB3, and the resulting mixture was used to transform competent *E. coli* strains. The *E. coli* strain XL-1 was used in both constructions, the strain HB101 only in construction of pmal-c2-ceaB3-lacZα. Recombinant cells harbouring plasmids with inserts were detected by colony hybridization. From positive recombinant cells, plasmids were isolated and subjected to double strand sequencing. The plasmid containing the ceaB3 fragment with the stop codon at the end of the ceaB3 sequence in the proper reading frame and correct sequence was designated pmal-c2-ceaB3. The other plasmid containing the correct sequence of the ceaB3 fragment without a stop codon and in the proper reading frame for the following lacZα gene was designated pmal-c2-ceaB3-lacZα.

2.8. Preparation of mbp-ceaB3

1 l of LB/amp was inoculated with an overnight culture (20 ml) of *E. coli* XL-1/pmal-c2-ceaB3. After growth at 37°C up to a cell density of OD (600 nm) 0.5 the expression of the fusion protein was induced by addition of IPTG to a concentration of 0.3 mM. The culture was incubated for 3 h at 37°C and then harvested by centrifugation. The cell pellet (6.6 g) was resuspended in 50 ml lysis buffer containing 10 mM sodium phosphate pH 7.0, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA and 1 mM PMSF. The suspension was sonicated for two times 30 s. After centrifugation for 30 min at 10000 rpm, 4°C in a Beckman centrifuge using a JA20 rotor, the supernatant was adjusted to 500 mM NaCl and diluted five times with affinity column buffer (10 mM Tris-HCl, pH 7.2, 500 mM NaCl). The diluted supernatant was run to an affinity chromatography column of amylose resin. The column was washed with 10 column volumes of affinity column buffer. The fusion protein was eluted with a buffer containing 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM maltose, 1 mM EDTA and then dialyzed against 10 mM Tris-HCl pH 8.0, 100 mM NaCl. The yield was 30.5 mg of mbp-ceaB3 fusion protein.

2.9. Preparation of mbp-ceaB3-lacZα

1 l of LB/amp was inoculated with 20 ml of an overnight culture of *E. coli* HB101/pmal-c2-ceaB3-lacZα and incubated at 37°C until a cell density of OD (600 nm) 0.5 was achieved. The expression of the fusion protein was then induced by addition of IPTG to a concentration of 0.3 mM. Incubation was continued for 3 h at 37°C and then cells were harvested by centrifugation. The cell pellet (2.3 g) was resuspended in 40 ml of a buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol and 1 mM EDTA. The suspension was sonicated for two times 30 s. The mixture was centrifuged 30 min at 4°C and 10000 rpm in a Beckman centrifuge using a JA14 rotor. The supernatant was removed and 3 g amylopectin (A-7780, Sigma) was added to it under vigorous shaking. The mixture was kept on ice for 30 min. The amylopectin was separated by centrifugation at 10000 rpm for 30 min

in at 4°C. The supernatant was discarded and the pellet washed with 40 ml of a buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Tween 20 and two times in the same buffer without Tween 20 under suspension. After centrifugation as described above, the pellet was eluted with 10 ml of a solution containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA and 0.5 M maltose. The yield was 20 mg of mbp-ceaB3-lacZα.

2.10. Generation of the monoclonal antibody HK2

Female Balb/C mice were immunized with a suspension of lacZMF-ceaB3 employing standard protocols. Mouse spleen cells were fused with the mouse myeloma cell line P3-X63Ag8 by standard techniques. Hybridomas were cloned by limited dilution. Screening was performed employing a direct ELISA employing CEA as antigen. A positive clone CEA/HK2 was characterized as $IgG_1(\kappa)$; a culture supernatant with an IgG concentration of 15 μg/ml had a titer of $1:10^4$ in the direct ELISA. The CEA-specific mAB CEA/HK2 reacted in Western blots with lacZMF-ceaB3, mbp-ceaB3-lacZα as well as with CEA. The monoclonal antibody reacted with CEA in a direct ELISA employing concentrations as low as 0.6 ng/ml.

2.11. Gel electrophoresis and immuno-blotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [13] and immuno-blotting after SDS-PAGE followed a published protocol [14]. For immunodetection of sheep IgGs or mouse IgGs, the blots were incubated with either peroxidase-conjugated goat anti-sheep antibodies or sheep anti-mouse antibodies (Amersham).

2.12. Sequence data analysis

DNA and protein sequence analysis were performed using the computer program of the University of Wisconsin genetics computer group [15].

3. Results

3.1. Synthesis of ceaB3 DNA

The polypeptide sequence of 77 residues from the CEA-B3 domain was chosen because sequence comparison indicated that it showed the least identity to the other sequence domains in CEA. The sequence of the polypeptide repre-

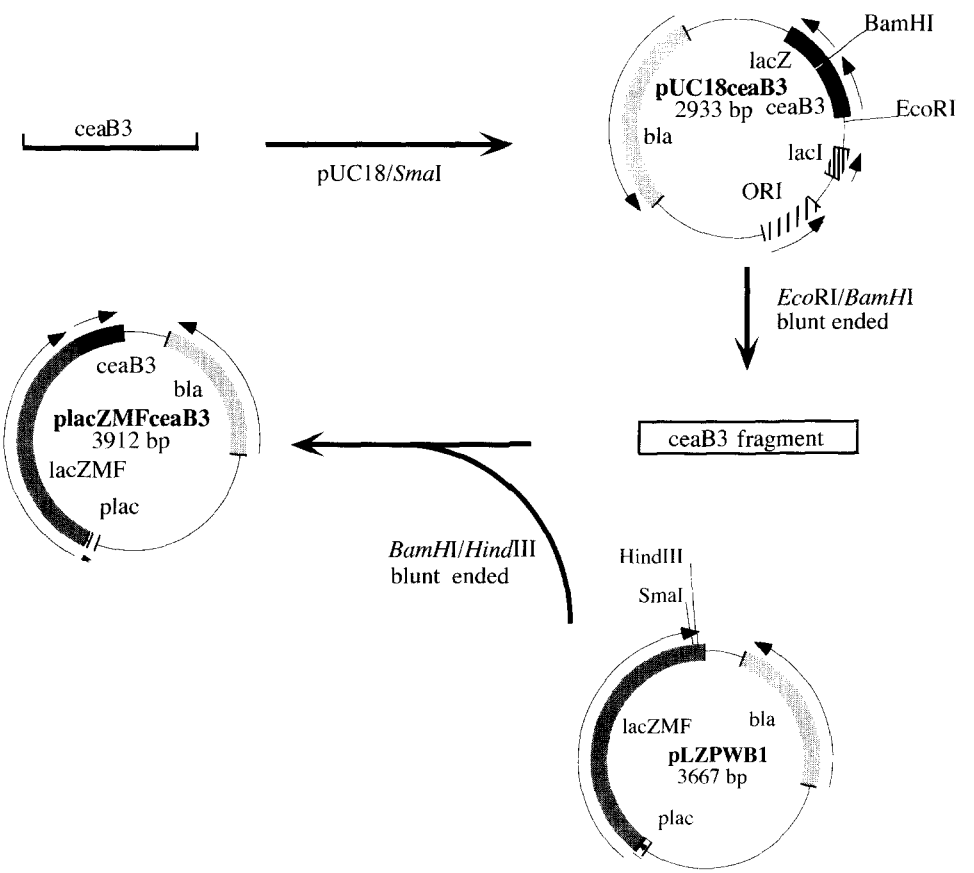

Fig. 2. Schematic representation of the steps leading to the construction of placZMFceaB3. The DNA as well as the deduced amino acid sequence of the C-terminal region of the lacZMFceaB3 is shown in Fig. 6.

senting a fragment of the CEA-B3 domain ranging from residue 561–637 (designated ceaB3) is shown in Fig. 1A. The experimental strategy to generate a fusion protein comprising the ceaB3 involved the chemical synthesis of a gene coding for ceaB3 and the integration of the synthetic DNA fragment into the plasmid pLZPWB1 at the C-terminus of a gene coding for a special protein, devoid of methionine and cysteine amino acid residues. This gene was derived from the lacZ gene, in which all methionine as well as cysteine residues have been substituted by mutation [10]. This strategy appeared to be attractive for the following reasons: expression of the fusion gene would be inducible and the fusion gene lacZMF-ceaB3 would be insoluble and easy to purify.

The sequence of the synthetic DNA was devised employing *E. coli* codon usage. The synthesis of ceaB3 DNA was achieved as follows. Four synthetic oligonucleotides #1, #6, #9 and #3 representing parts of the sequence of ceaB3 DNA (see Fig. 1) were hybridized, and the single stranded gaps which formed were filled by primer extension employing the DNA polymerase Klenow fragment to yield the blunt ended double stranded fragments #19 and #63 (see Fig. 1D). The two fragments #19 and #63, which comprised the ceaB3 DNA from position 1–177 and 77–250 respectively, were denatured in a second step, rehybridized and the resulting hybrids filled in with Klenow fragment to yield the complete ceaB3 DNA (see Fig. 1D). The ceaB3 DNA fragment was ligated into pUC18 linearized by *Sma*I treatment and the reaction mixture employed to transform *E. coli*. Recombinant *E. coli* cells harbouring pUC18ceaB3 were detected by colony hybridization. From 9 positive clones only two were shown by sequencing to contain the complete sequence of ceaB3 DNA.

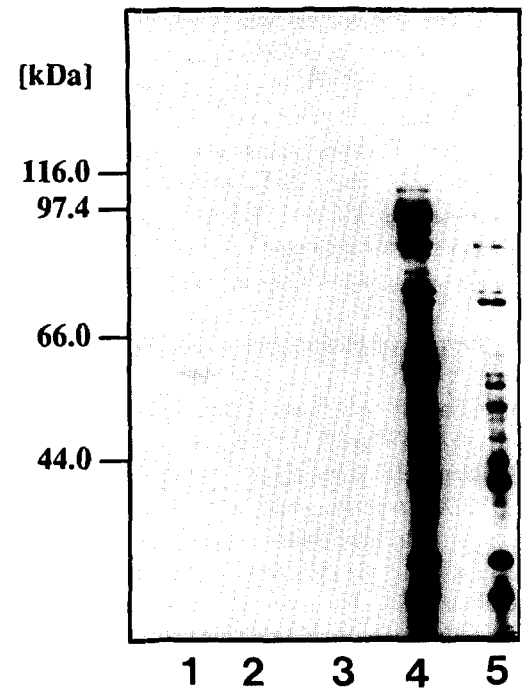

Fig. 4. Immunoreactivity of the fusion protein lacZMFceaB3 with anti-CEA antiserum. Total protein mixtures obtained from *E. coli* XL-1/placZMFceaB3 were separated by SDS-PAGE using a 12% gel and electrophoretically transferred onto a membrane. The transferred proteins were subjected to immunoreaction employing sheep anti-CEA antiserum as described in Materials and methods. Lanes 4 and 5 of the membrane were stained for proteins by ColAurion. Lane 1, induced; lane 2, uninduced; lane 3, CEA; lane 4, induced; lane 5, uninduced.

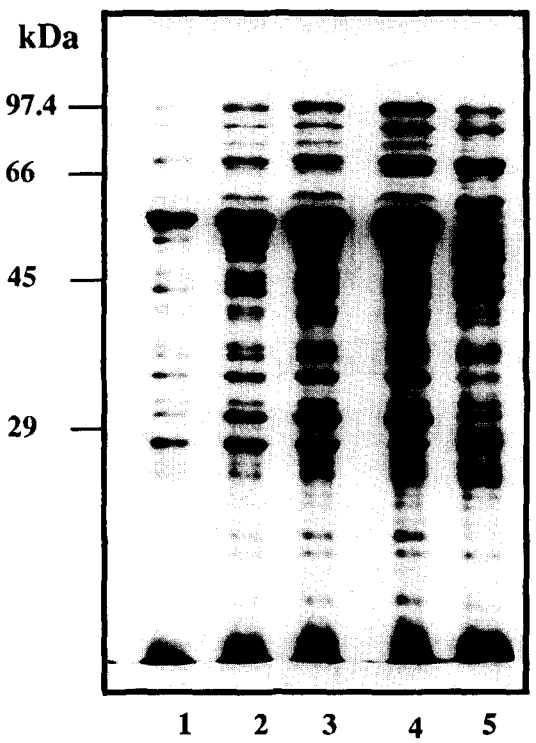

Fig. 3. Expression of placZMFceaB3. Total protein mixtures of *E. coli* XL-1/placZMFceaB3 after various times of induction by IPTG were subjected to SDS-PAGE. Lane 1, 30 min; lane 2, 60 min; lane 3, 120 min; lane 4, 180 min; lane 5, uninduced.

3.2. Construction and expression of the fusion gene lacZMFceaB3

The construct pUC18ceaB3 was the basis for the further construction of a fusion gene in plasmid pLZPWB1. The experimental steps leading to construct placZMFceaB3 are represented schematically in Fig. 2:

(a) Restriction of pLZPWB1 with *BamH*I/*Hin*dIII and blunt-end formation by 'fill-in' using Klenow fragment;
(b) restriction of pUC18ceaB3 with *Eco*RI/*Bam*HI and isolation of the *Eco*RI-*Bam*HI fragment containing the ceaB3 DNA;
(c) the ceaB3 DNA *Eco*RI-*Bam*HI fragment was blunt-ended by fill-in with Klenow fragment;
(d) ligation of the blunt end ceaB3 DNA *Eco*RI-*Bam*HI fragment into blunt-ended pLZPWB1 and transformation of *E. coli* strain XL-1 with the ligation mixture.

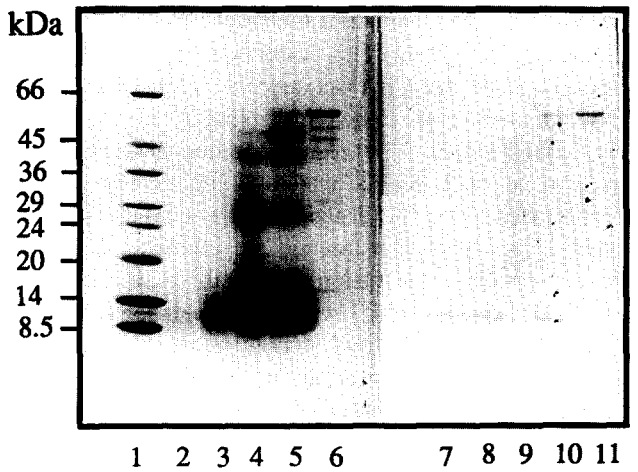

Fig. 5. Fragmentation of the fusion protein lacZMF-ceaB3 by CNBr: Analysis by SDS-PAGE and immunoblot. Protein mixtures were separated by SDS-PAGE and electrophoretically transferred onto a membrane. Lanes 1 and 3–6, were stained for proteins with India ink. The proteins in lanes 8–11 subjected to immunoreaction with sheep anti-CEA antiserum. Lane 1, marker proteins; lane 3 and 8, CNBr-reaction, soluble proteins; lane 4 and 9, CNBr-reaction, insoluble protein fraction; lane 5 and 10, CNBr-reaction, total reaction mixture; lane 6 and 11, fusion protein lacZMF-ceaB3.

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3541
ggattggtggcgacgactcctggagcccgtcagtatcggcggaattcgagctcgcccggg
  I  G  G  D  D  S  W  S  P  S  V  S  A  E  F  E  L  A  R  G
        CCATCgTcgacACCCCGATCATCTCTCCGC--->3'
gatcgatccccATCCTGATGACCCCGATCATCTCTCCGCCGGACTCTTCTTACCTGTCTG
  S  I  P  I  L  D  T  P  I  I  S  P  P  D  S  S  Y  L  S  G

GTGCTAACCTGAACCTGTCTTGCCACTCTGCTTCTAACCCGTCTCCGCAGTACTCTTGGC
  A  N  L  N  L  S  C  H  S  A  S  N  P  S  P  Q  Y  S  W  R

GTATCAACGGTATCCCGCAGCAGCACACCCAGGTTCTGTTCATCGCTAAAATCACCCCGA
  I  N  G  I  P  Q  Q  H  T  Q  V  L  F  I  A  K  I  T  P  N
                                                 3'<---AGAT
ACAACAACGGTACCTACGCTTGCTTCGTTTCCAACCTGGCTACCGGTCGTAACAACTCTA
  N  N  G  T  Y  A  C  F  V  S  N  L  A  T  G  R  N  N  S  I
AGCAATTTAGAATTATTCgaaATGGC
TCGTTAAATCTTAATAAGgggtaccgagctcgaattagcttcgcgatgcatcagatctac
  V  K  S

catggcatgccaattc 3916
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Fig. 6. Generation of the ceaB3 DNA fragment for the construction of pmal-c2-ceaB3. The sequence represents part of the fusion gene of placZMFceaB3. The nucleotides in lower print are from the original pLZPWB1 sequence, those in lower print and italics are derived from pUC18. Employing the two primers of indicated location, a PCR fragment was amplified. The fragment contained a *Hin*cII site at 5′ and a *Hin*dIII site at the 3′ end. These two restriction sites are underlined in the primer sequences. The generated fragment represents a modified ceaB3 fragment with an aspartate residue (underlined, bold print) at the N-terminus instead of a methionine residue.

Positive recombinants harbouring plasmid constructs placZMFceaB3 were detected by colony hybridization; 19 out of 24 colonies were positive.

The correct sequence of placZMFceaB3 was verified by sequencing. The obtained sequence showed that a fusion gene was generated which contained the ceaB3 DNA fragment in a reading frame with the C-terminus of the modified lacZMF gene.

The growth curve of placZMceaB3 was apparently normal; no indication was given of an inhibition of growth by expression of the fusion gene (data not shown). The fusion protein lacZMF-ceaB3 displayed an apparent molecular mass of approx. 60 kDa, in accordance with the theoretical molecular mass as calculated from the amino acid sequence. The lacZMF-ceaB3 represented approx. 30% of the protein species expressed after induction (Fig. 3). Employing a polyclonal sheep anti-CEA antiserum, it could be shown that lacZMF-ceaB3 was the only immunoreactive protein present (Fig. 4). The fusion protein, as expected, was formed as insoluble inclusion bodies. Simple washing steps led to an insoluble fusion protein which on SDS-PAGE was of approx. 80% purity (Fig. 5). The

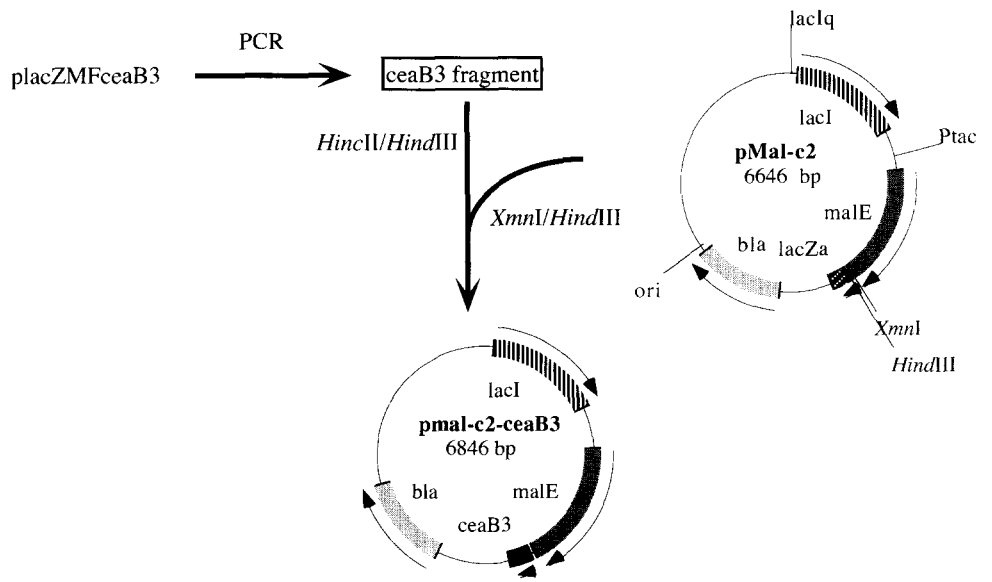

Fig. 7. Schematic representation of the steps leading to construction of pmal-c2-ceaB3. The sequence of the *Hin*cII/*Hin*dIII ceaB3 fragment is shown in Fig. 6.

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3541
ggattggtggcgacgactcctggagcccgtcagtatcggcggaattcgagctcgcccggg
  I  G  G  D  D  S  W  S  P  S  V  S  A  E  F  E  L  A  R  G
         CCATCgTcgacACCCCGATCATCTCTCCGC--->3'
gatcgatccccATCCTGATGACCCCGATCATCTCTCCGCCGGACTCTTCTTACCTGTCTG
  S  I  P  I  S  D  T  P  I  I  S  P  P  D  S  S  Y  L  S  G

GTGCTAACCTGAACCTGTCTTGCCACTCTGCTTCTAACCCGTCTCCGCAGTACTCTTGGC
 A  N  L  N  L  S  C  H  S  A  S  N  P  S  P  Q  Y  S  W  R

GTATCAACGGTATCCCGCAGCAGCACACCCAGGTTCTGTTCATCGCTAAAATCACCCCGA
  I  N  G  I  P  Q  Q  H  T  Q  V  L  F  I  A  K  I  T  P  N
                                                3'<---CATTGTTGAGAT
ACAACAACGGTACCTACGCTTGCTTCGTTTCCAACCTGGCTACCGGTCGTAACAACTCTA
  N  N  G  T  Y  A  C  F  V  S  N  L  A  T  G  R  N  N  S  I
AGCAATTTtaccgTtcgaaCgccc
TCGTTAAATCTTAATAAGgggtaccgagctcgaattagcttcgcgatgcatcagatctac
  V  K  M

catggcatgccaattc  3916
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Fig. 8. Generation of the ceaB3* DNA fragment for the construction of pmal-c2-ceaB3-lacZα. The sequence represents part of the fusion gene of placZMFceaB3. The nucleotides in lower print are from the original pLZPWB1 sequence, those in lower print and italics are derived from pUC18. Employing the two primers of indicated location, a PCR fragment was amplified. The fragment contained a *Hin*cII site at 5′ and a *Hin*dIII site at the 3′ end. These two restriction sites are underlined in the primer sequences. The generated fragment represents a modified ceaB3* fragment with an aspartate residue (underlined, bold print) at the N-terminus instead of the methionine residue and a C-terminal methionine (underlined, bold print) instead of a serine residue.

design of the lacZMF-ceaB3 allowed the cleavage of the ceaB3 fragment from the fusion protein by CNBr treatment (Fig. 5). This procedure led to the disappearance of the immunoreactive intact lacZMF-ceaB3 and to the formation of an insoluble core protein of approx. 52 kDa which had lost the immunoreactivity towards anti-CEA antiserum. Soluble fractions retained the immunoreactivity as had been predicted.

3.3. Construction and expression of the fusion gene malE-ceaB3

Using placZMFceaB3 and the two primers located as shown in a partial sequence comprising ceaB3 (Fig. 6), a DNA fragment was amplified by PCR which possessed a *Hin*cII site at the 5′ and a *Hin*dIII site at the 3′ end. The use of modified primers led to the amplification of a DNA fragment with an aspartate residue at the N-terminal region of the ceaB3 fragment. This additional amino acid residue corresponds to the CEA sequence. The obtained ceaB3 DNA fragment was cloned into pmal-c2 restricted with *Xmn*I and *Hin*dIII. This resulted in the generation of a fusion gene malE-ceaB3, encoding the maltose binding protein fused to ceaB3 (designated mbp-ceaB3). The individual steps of this construction are illustrated in Fig. 7. The expression of the fusion gene malE-ceaB3 led to the fusion protein mbp-ceaB3 with a molecular mass of 50.8 kDa. Unfortunately, a lot of smaller immunoreactive fragments were observed in an immunoblot with anti-ceaB3 mAB CEA/HK2. By the pattern of this fragments and by the possibility of purification by affinity chromatography on amylose resin it can be hypothesized that this is proteolysis of ceaB3 part of the fusion protein mbp-ceaB3. A soluble fusion protein with a protected ceaB3 part was therefore decided upon.

3.4. Construction and expression of pmal-c2-ceaB3-lacZα

Using placZMFceaB3 and two primers located as shown in the partial sequence in Fig. 8 a DNA fragment was amplified by PCR, which possessed a *Hin*cII site at the 5′ and a *Hin*dIII site at the 3′ end. The modified primers led to the amplification of a DNA fragment, in which besides restriction sites the C-terminal amino acid sequence was modified by changing serine into methionine and by removing the stop codons. The obtained modified ceaB3 fragment (designated ceaB3*) was cloned into pmal-c2 restricted with *Xmn*I and *Hin*dIII. The individual steps of construction of pmalE-ceaB3-lacZα are detailed in Fig. 9. Expression of pmal-c2-ceaB3-lacZα in *E. coli* XL-1 led to the formation of an insoluble protein of approx. molecular mass 60 kDa as the major species. The theoretical molecular mass of the fusion protein mbp-ceaB3-lacZα is 57.94 kDa. This major protein was found to be soluble when the expression was done in *E. coli* HB101, which lack the potential to express lacZα. The fusion protein

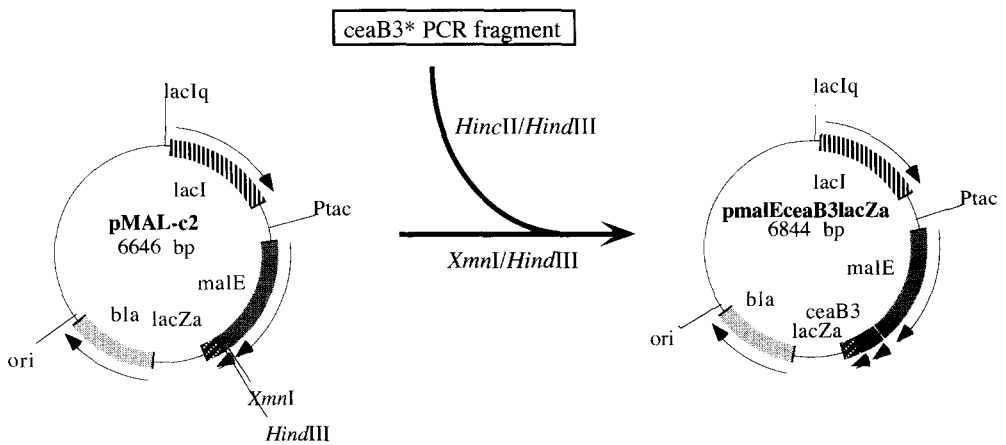

Fig. 9. Schematic representation of the construction of pmal-c2-ceaB3-lacZα.

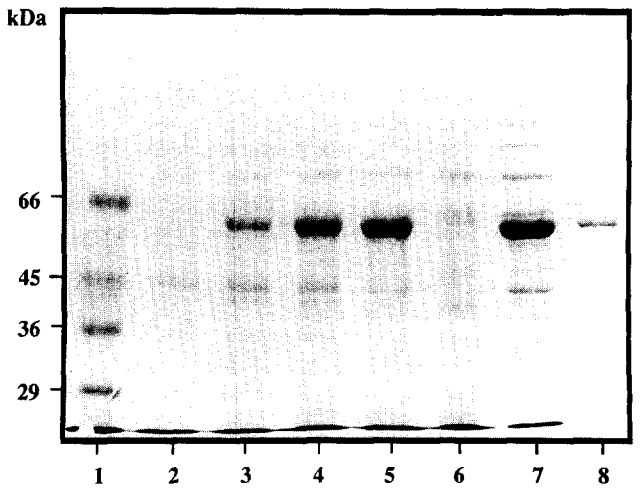

Fig. 10. Gene expression of pmal-c2-ceaB3-lacZα: Analysis by SDS-PAGE. Proteins isolated from cultures of *E. coli* HB101/pmalEceaB3lacZα induced by IPTG, were analyzed by SDS-PAGE. Lane 1, molecular weight standards; lane 2 to 5, total proteins 0, 60, 120 and 180 min after induction by IPTG; lane 6, insoluble proteins; lane 7, soluble proteins; lane 8, mbp-ceaB3-lacZα fusion protein purified by affinity chromatography.

could be isolated by affinity chromatography employing amylopectin in almost homogeneous form (Fig. 10). The fusion protein turned out to be reactive with anti-ceaB3 mAB CEA/HK2 when probed in an immunoblot (Fig. 11).

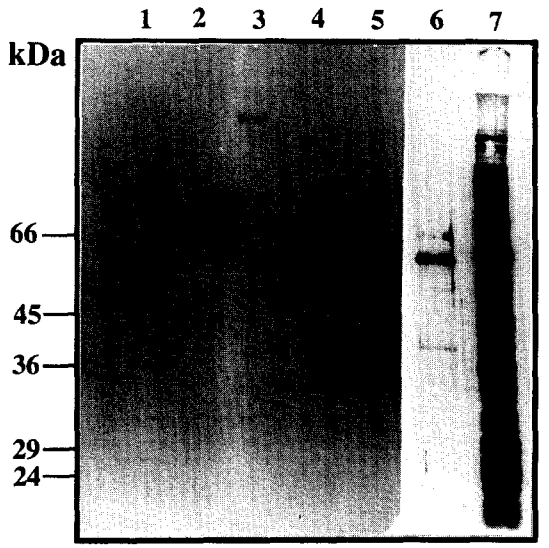

Fig. 11. Immunoreactivity of the anti-ceaB3 mAB CEA/HK2. Proteins were separated by SDS-gel electrophoresis and electrophoretically transferred onto a membrane. Lanes 1 to 5, immuno blot; lanes 6, 7, proteins stained with ColAurion; lane 1, mbp; lane 2, lacZMF; lane 3, CEA; lane 4, mbp-ceaB3-lacZα; lane 5, lacZMF-ceaB3; lane 6, mbp-ceaB3-lacZα; lane 7, total protein mixture of *E. coli* XL-1/placZMFceaB3 after induction.

3.5. Monoclonal mouse anti-ceaB3 antibody CEA / HK2

Specificity of the mouse anti-ceaB3 mAB CEA/HK2 was investigated by immunoblots. As shown in Fig. 11, mAB CEA/HK2 reacted with CEA, lacZMF-ceaB3 and mbp-ceaB3-lacZα. The mAB CEA/HK2 did not react with either lacZMF or mbp-lacZα.

4. Discussion

The synthetic gene ceaB3 was successfully cloned in various vectors yielding constructs with fusion genes. Construct placZMF-ceaB3 led to expression of insoluble fusion protein in the form of inclusion bodies. The easily purified lacZMF-ceaB3 was employed for the generation of monoclonal antibodies and it could be shown that latter reacted with the intact CEA molecule. Attempts to express a soluble fusion protein mbp-ceaB3 turned out to be unsuccessful, because of the susceptibility of the fusion product against proteolytic degradation. Only the construction of a fusion gene embedding the ceaB3 between mbp and lacZα leading to expression of the fusion protein mbp-ceaB3-lacZα provided a high yield of soluble protein. A general advantage is that proteolytic degradation of the desired antigen within the fusion protein is unlikely. Therefore, sophisticated proteinchemical sequence verifications of fusion proteins are in most cases unnecessary. It appears that this strategy for the design and expression of soluble fusion proteins might be generally applicable.

The described mouse anti-ceaB3 mAB is the first directed against a short peptide region of the CEA protein which also displayed immunoreactivity against the whole CEA protein. In this context, it is necessary to recall the amino acid sequence of ceaB3 which comprises a loop structure formed by a disulfide bridge. Furthermore, it contains three putative *N*-glycosylation sites. That an mAB directed against this 77 residue fragment also reacted with epitopes from this region in the whole CEA confirms the notion that the majority of epitopes of the heavily glycosylated CEA molecule are of peptidic nature.

Fusion proteins containing relatively large protein regions of the CEA molecule have already been employed to characterize the epitope specificity of CEA-specific mABs [3]. The approach described above aimed at the use of fusion proteins containing the ceaB3 region to achieve the generation of CEA-specific mABs. This goal could be achieved. It has still to be shown whether these antibodies can be employed in immunological test systems such as enzyme linked immunosorbent assays or immunoradiometric assays.

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