CONSTRUCTION OF A QUANTITATIVE PCR SYSTEM TO DETERMINE EXPRESSION OF TUMOR ASSOCIATED ANTIGEN

BÜCHLER T.

KONŠTRUKCIA KVANTITATÍVNEHO PCR SYSTÉMU NA DETEKCIU EXPRESIE TUMOROVO-ASOCIOVANÝCH ANTIGÉNOV

Cancer immunotherapy is a strategy for cancer treatment by induction of anti tumor responses. The identification of candidate tumor associated antigens (TAA) suggested their potential use as immunogens for vaccination studies.
Quantification of a TAA expression by cancerous cells is an important factor in determination of induced immune response efficiency against tumors and thus enables us to devise optimal immunotherapy protocol to cure cancer. The quantitative polymerase chain reaction (PCR) enables us to compare the TAA expression in highly metastatic tumor clones with that in less metastatic ones and in normal cells. It allows us to gain more insight into genome rearrangements that occur in malignant transformation as well as broaden our knowledge about tumor cell gene expression regulation.
One of the peptides isolated previously in our lab from a murine lung carcinoma is the mutated Connexin 37 (cx37), a gap-junction protein. Research is underway to determine the expression level of the TAA in various Lewis lung carcinoma cells. This evaluation is achieved by means of quantitative PCR. A quantitative PCR experiment includes preparation of a control template, which is added in known amounts together with the target template in a series of amplification reactions. The control template uses the same primers as the target sequence, yet their PCR products differ in size so as to be distinguishable.
Two methods were used to produce this control template. The first one included specific deletion of a sequence of approximately 100 bp that lay between the two primers, insertion of the new template into a plasmid vector, transformation of competent bacteria, detection of transformed bacterial colonies and isolation of the plasmid DNA in a large quantity. The non-mutated, deleted Connexin 37 cDNA was also isolated from bacteria and used for another experiment aimed at producing deleted, mutated Connexin 37 cDNA by means of primer mutagenesis. *(Fig. 5, Ref. 8.)*
Key words: PCR system, quantitative determine expression, tumor associated antigen.

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Cancer immunotherapy is a strategy for cancer treatment by induction of anti tumor responses. Many murine and human tumors express class I major histocompatibility complex (MHC) associated antigens that are targets for syngeneic cytotoxic T lymphocytes (CTL). CTL are capable of destroying already established tumors as well as preventing the establishment of metastases (Abbas et al., 1994; Mandelboim et al., 1994; Dranoff and Mulligan, 1995). Isolation and characterization of MHC class I associated peptides and utilization of specific CTL enabled us to identify these antigens 3. Recent experiments suggest that peptide vaccination can be an effective tool in fighting metastases. A vaccine containing two such peptides of non- viral origin is currently tried in women with advanced cervical carcinoma in The Netherlands (Celis et al., 1995).

To devise an optimal immunotherapy protocol aimed at destruction of tumor cells expressing particular tumor associated antigen (TAA), it is necessary to obtain information about expression of this TAA in tumor cells and compare it to that in normal cells (Celis et al., 1995). Recently a potential TAA -- the mutated Connexin 37 (cx 37), a gap-junction protein, was isolated in our lab from the murine Lewis lung carcinoma. The mutation of triplet TGT to CAG caused replacement of cysteine by glutamine and changed ability of the peptide derived from the appropriate part of the protein to bind murine class I major histocompatibility complex K^b molecule (Houghton, 1994; Mandelboim et al., 1994; Howard, 1995). Research is underway to determine expression of the mutated and normal Connexin 37 gene on the level of mRNA in various types of cells.

This goal is achieved by the quantitative polymerase chain reaction (PCR). A quantitative PCR experiment includes preparation of a control template, which is added in known amounts to-

Supervisor: Prof. Lea Eisenbach
Mentor: Erez Bar-Haim
Department of Immunology, Weizmann Institute of Science, Rehovot, Israel

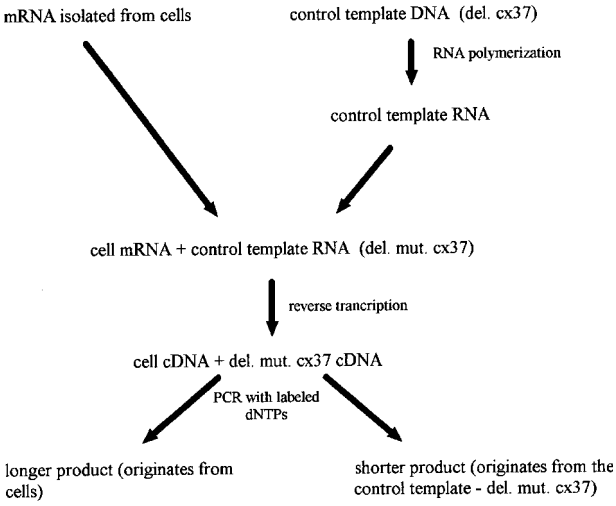

Fig. 1. The quantitative polymerase chain reaction.

gether with the target template in a series of amplification reactions. The control template uses the same primers as the target sequence, yet its PCR product is about 100 bp shorter in order to be distinguishable. First, the control template RNA is synthesized by RNA polymerase. The product is added to the RNA isolated from cells and reverse transcription is performed. The resulting mixture of cDNA is used for PCR with radiolabeled deoxyribonucleotides (dNTPs). The relative amounts of the two amplified products determined by radioactivity measurement reflect the relative concentrations of the control and target sequences in the original mixture (fig. 1) (Sambrook et al., 1989).

In this work we attempted to construct the control template for quantification of mutated Connexin 37 expression using two methods. The first method included synthesis of two non-overlapping fragments using the mutated, non-deleted Connexin 37 cDNA as a template, external and internal primers followed by their hybridization. We produced deletion at the DNA sequence that had already been mutated. The other method was that of primer mutagenesis, where the polymerase chain reaction mixture included non-mutated, deleted Connexin 37 cDNA as a template, specific internal primers containing point mutation and external primers. In this procedure, we produced mutation of the previously deleted DNA sequence.

Materials and methods

Polymerase chain reaction

Following PCR protocol was used: Step 1: 5 min 94 ˚C Step 2: 1 min 92 ˚C Step 3: 1 min annealing temperature Step 4: 1 min 72 ˚C Step 5: Repeat steps 2--4 40x Step 6: 10 min 72 ˚C, Step 7: Maintain 4 ˚C Step 8: End

Annealing temperature (in. ˚C) was calculated using following formula: 4 x (number of G and C bases in the sequence of the primer) + 2 x (number of A and T bases in the sequence of the primer) - 4. When using two primers with different annealing temperatures we set the annealing temperature to the lower value of the two.

Primers

1. External primers that bind to the pGEM vector:

T7 - 5'- AAT ACG ACT CAC TAT AG - 3'

SP6 - 5' - GAT TTA GGT GAC ACT ATA G - 3'

2. Internal primers, i.e. primers specific for the Connexin 37 cDNA

con 11 - binds to bases 191-208, strand 5'

con 17 - binds to bases 884-900, strand 3'

mut 1 - binds to bases 224-247, 5', contains mutation in one triplet

anti mut 1 - binds to bases 197-220, 3', contains mutation in one triplet

The 290 and 410 primers have two parts: the first one binds to the Connexin 37 cDNA starting with the base pair 290 and 410, respectively. The other parts of the primers are complementary to each other and make possible hybridization of the fragments that were synthesized using these primers.

290 - 5' Γ Γ CAA TAT TAG | GTA TCG GAT GTC GGA G 3'

410 - 5' CCT AAT ATT GCC CC | CCA TCC AAG GAC CTA 3'

Vector

The pGEM Vector System was used. It contains T7 and SP6 promoters. Their sequence is complementary to the that of the T7 primer on the 5' end of the cloning site and to the SP6 primer on the 3' end of the cloning site. Furthermore it contains the ampicillin resistance gene and lac-Z gene (at the cloning site) to make the screening for positive clones easier.

Bacteria

We used the Escherichia coli bacteria, strain JM 109.

Bacterial media

For screening, plates with the LB medium containing ampicillin were used. Prior to plating the bacteria we mixed them with X-gal and IPTG. For small and large scale plasmid preparation we grew the bacteria in liquid LB medium containing 50 mg/l ampicillin.

Ligation

Ligation reaction was performed using the Rapid DNA Ligation Kit (Boehringer Mannheim, Germany).

Transformation

Transformation of the competent E. coli JM 109 by pGEM plasmid containing the target fragment was performed by heat shock.

Small and large scale plasmid DNA isolation

We incubated bacteria overnight in LB/ampicillin medium. In small scale preparation we used 1.5 ml, in the large scale plasmid preparation 500 ml of the LB/ampicillin medium. Plasmid DNA was isolated after alkali lysis of the bacteria.

Detection of the positive bacterial colonies

metabolize lactose. Two methods were used for definitive screening: PCR with primers specific for the Connexin37 cDNA, and

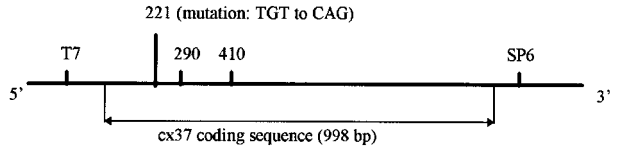

Fig. 2. The mutated, non-deleted cx37 cDNA. Primer binding sites and mutation site are shown.

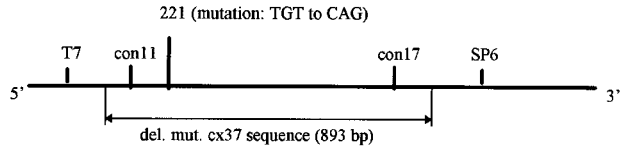

Fig. 3. The mutated deleted cx37. Primer binding sites and mutation site are shown.

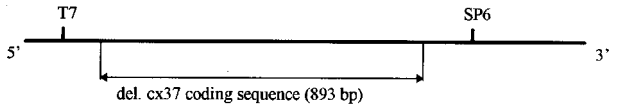

Fig. 4. The non-mutated, deleted cx37 cDNA. Primer binding sites and mutation site are shown.

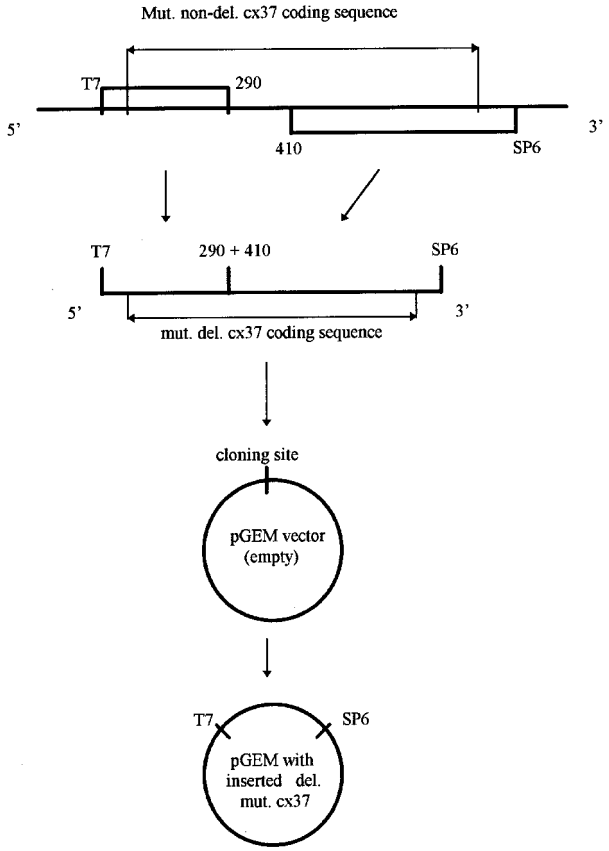

Fig. 5. The construction of the deleted cx37 cDNA, method 1. Primer binding sites are shown.

cleavage with the Apa I restriction enzyme, which cuts the pGEM plasmid at For rough screening, the white and blue colonies test was used which is based on bacterial ability to the cloning site.

Results

In the first step two non-overlapping fragments of DNA were obtained in two PCR reactions using the cDNA for mutated, non-deleted Connexin 37 (998 bp long coding sequence) (fig. 2). This template had been synthesized previously by reverse transcription and cloned into the pGEM bacterial vector as a template. In the reactions, a shorter fragment of about 300 bp was produced using the T7 and 290 primers and the fragment of approx. 700 bp was obtained in the presence of SP6 and 410 primers. A 120 bp long sequence long was left out. After electrophoresis, both fragments were cut out of the gel and cleaned.

In the second step both fragments were added to reaction mixture together with the SP6 and T7 external primers. A hybrid of approximately 1000 bp with the coding sequence of 893 bp (fig. 3) was produced and cleaned. We used primers with parts that were complementary to each other. The final product was 105 bp shorter than the non-deleted Connexin 37 cDNA.

This hybrid was inserted into the pGEM bacterial vector (fig. 5) and used to transform the competent E. coli. The bacteria were put on a plate containing LB/ampicillin. The resistant (plasmid containing) bacteria gave growth to numerous (>30) blue and white colonies. Knock-out of the lac-Z gene at the plasmid cloning site in the white colonies enabled us to selectively screen only the colonies where ligation with the target DNA probably occurred. Analysis of the bacterial plasmid DNA by PCR using specific primers and restriction at the cloning site showed positive results in one colony.

The deleted non-mutated Connexin 37 cDNA (fig. 4) necessary for the quantitative PCR was produced as well. Bacteria transformed previously with the pGEM vector ligated with the deleted, non-mutated Connexin 37 cDNA were grown and the plasmid DNA was isolated in large amount.

Finally, another approach to obtain the deleted, mutated Connexin 37 cDNA was tried using the method of primer mutagenesis. We attempted to synthesize two fragments using the SP6 — mut1 and T7 — anti mut1 pairs of primers and the deleted non-mutated Connexin37 cDNA as the template. The resulting two fragments with complementary ends containing the mutated triplet were hybridized with external SP6 and T7 primers in a way similar to the first described procedure. The yield of this procedure was substantially lower.

Discussion

Quantification of the normal and mutated Connexin 37 expression in normal cells and in cells of the murine Lewis lung

carcinoma is an important factor that determines efficiency of therapeutically induced immune response against this antigen. It allows us to gain more insight into genome rearrangements occurring in malignant transformation and broaden our knowledge about tumor cells gene expression regulation.

If results of the quantitative PCR are encouraging, the mutated Connexin 37 cDNA can be used in gene transfer mediated immunotherapy experiment to induce immune response against established tumor and/or micro metastases (Bar-Haim, 1995).

Specific deletion of approx. 100 bp from the mutated Connexin 37 cDNA enables us to perform the quantitative PCR using the newly synthesized fragment as a control template. To achieve this deletion, we used two approaches. The first one was to produce the deletion of 105 bp by hybridization of two non-overlapping fragments of the Connexin 37 cDNA. The product, the Connexin 37 cDNA which was both deleted and mutated was then ligated into a plasmid and incorporated into E. coli bacteria. In the other approach we tried to produce mutation on the already deleted Connexin 37 cDNA by primer mutagenesis.

It is worth noting that the fragments obtained by the described methods are not similar. The fragment constructed by the first of the described methods contains insert of 15 bp which was formed in the hybridization step and includes oligonucleotide sequence which enables the two primers to hybridize with each other. Therefore it is only 105 bp shorter than the target sequence of the quantitative PCR. Nevertheless, both types of deleted, mutated Connexin 37 cDNA can be used for the quantitative PCR.

The Lewis murine lung carcinoma is a good model for research of solid human malignant tumors because it arose spontaneously and was not induced. However, application of immunotherapy with gene transfer into autologous cells to cure cancer still remains experimental although the number of known human TAA is increasing. It is due to the immunologic and genetic heterogeneity of the tumors as well as to high cost of such procedures 4,5.

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