

ORIGINAL ARTICLE

CONSTRUCTION OF cDNAs FOR BIOLOGICALLY ACTIVE DOMAINS OF FACTOR VIII (A1-A2 AND A3-C1-C2) BY RT-PCR

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Abstract

Background-Construction of complementary DNA (cDNA) is the first step to produce a recombinant protein and reverse transcription of mRNA is the best way to construct cDNA. Construction of cDNAs for biologically active domains of factor VIII has advantages of joining two domains to form a second generation of factor VIII and expression of domains separately for especial purposes.

Method-Total RNA was purified from HepG2 and 293 cell lines, were quantitated and analyzed for presence of factor VIII mRNA by Dot RNA Blotting. One and two step reverse transcription polymerase chain reaction (RT-PCR) were applied. Primers (including nested primers) were designed for both sides of A1-A2 (cDNA1) and A3-C1-C2 (cDNA2) domains with respect to saving open reading frame (ORF) of cDNAs and deleting of B-domain, 3' and 5' untranslated region (UTR). Restriction mapping and sequencing were applied for analysis and confirmation. cDNAs were cloned into the vector and then into the ultracompetent cells by electroporation. Plasmids were purified, restriction analyzed and sequenced.

Results-Five clones of cDNA1 and seven clones of cDNA2 were produced and checked for sequence similarity with factor VIII sequence from database. Some of them had point mutations and were suitable for the assessment of biological activity and yield of production.

Conclusion-The construction of cDNA for A1-A2 and A3- C1-C2 domains and cloning of these two domains, which will be later linked with metal ions, is a way for production of second generation recombinant factor VIII. Another application for recombinant domains would be immune tolerance induction in hemophiliacs with inhibitors against these domains without need for administration of whole molecules.

Keywords • Factor VIII • cDNA • RT-PCR • cloning • sequencing

Introduction

Recombinant factor VIII is the drug of choice in hemophilia¹ and has several advantages including very low risk of blood-born diseases. Its infusion produces a significantly better response and recovery *in vivo* than the infusion of highly purified plasma-derived (pd) factor VIII in children.²

Also, there is no need of a biological stabilizer (potential source for infectious agents). There are four strategies to produce recombinant human factor VIII (rhFVIII) as follows:

1. Production of full-length factor VIII with 2331 amino acids (first generation) which was primarily constructed in 1984.³ The first recombinant factor VIII was also produced in 1984.⁴
2. Deletion of B-domain from the full-length factor VIII molecule. This is the second-generation factor VIII, which has been under

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clinical trial since 1993⁵ and was introduced in 2000. The B-domain is not necessary for biological activities⁶ and is the biggest domain of factor VIII with the most glycosylation.⁶ Deletion of the B-domain can improve production of factor VIII up to six times.⁷

3. Delta B, 3' and 5' UTR factor VIII.⁸ In this method, in addition to deleting the B-domain, 3' and 5' non-translated regions (UTR) are deleted.
4. Construction of cDNA for A1-A2 and A3-C1-C2 domains and cloning of these two domains separately⁹, which will be later linked with metal ions.

In this attempt, two cDNAs were constructed by reverse transcription of purified total RNA and by long range polymerase chain reaction (long range PCR) using gene specific primers (GSP) for A1-A2 and A3-C1-C2 domains.

It was the first time that cDNA for biologically active domains of factor VIII were constructed using RT-PCR. To increase yield, the B-domain, 3' UTR and 5' UTR were deleted ($\Delta 3'$, $5'$, B cDNAs).

Materials and Methods

Cells and reagents for RNA purification

Trizol reagent, which was prepared from Gibco BRL was used for purification of RNA. Total RNAs were isolated from two cell lines of 293 (ATCC Number: CRL-1573, a human kidney cell line) and HepG2 (ATCC Number: HB-8065, a human hepatoma cell line).

Quantitation of mRNA and proving factor VIII mRNA

To assess the quality and integrity of total RNA, purified RNA was electrophoresed under a denaturing condition using formaldehyde as the denaturing agent.

The ratio of 28S to 18S eukaryotic ribosomal RNAs should be approximately 2:1 by ethidium bromide staining, indicating that gross degradation of RNA has not occurred. In the degraded RNA samples, this ratio will be reversed since the 28S ribosomal RNA is characteristically degraded to an 18S-like species. The smear between two bands (18S and 28S) is intact mRNA and intense smear after 18S RNA reveals mRNA degradation. RNA and all types of DNAs (genomic, cDNA, and plasmid) were quantitated by scanning spectrophotometry (range: 190-350 nm). For proving factor VIII mRNA, exon 14 was chosen as template for construction of DIG labeled probe due to the fact that exon 14 is the most specific part (highly conserved part) of factor VIII molecule. To increase sensitivity enhanced chemiluminescence (ECL) technique was applied.

Primer design

The primers were designed with the Oligo 4.0 software for the construction of cDNA1 for A1-A2 domain (region of 172-2361 in mRNA) and cDNA2 for A3-C1-C2 domain (region of 5310-7206 in mRNA) (Table 1).

The first set of designed primers was not efficient, so a second set was designed with special criteria. Some unusual criteria were applied for primer design according to our previous experiments and requirements. For prevention of unspecific binding, primers with 30 mers, high annealing temperature (T_a) and melting temperature (T_m) were designed. To increase specificity, non-homologous and highly conserved parts of factor VIII were used to design the primers. Finally, to check the specificity, primers were controlled with basic local alignment search tools for nucleotide (BLASTn) at the National Center for Biotechnology Information (NCBI). Only primers with no sequence similarity or with

Table 1. Primers specifications.

Primer	Position in mRNA	mer	Tm
F1	133	30	62.6
R1	2501	30	63.3
F2	5057	30	62.1
R2	7446	30	60
F1-1	34	30	64.8
F2-1	5058	30	64.4
F2-2	4971	30	64.8
R1-1	2585	30	65.1
R2-1	7293	30	68.7
F1-2	75	30	65.1

Table 2. Dimers combinations for One Tube RT-PCR.

Primer combinations	Ta (-0.4° c/cycle)	Primer concentration (µM)
F1+R1-1	68	R2=20
F1+R1	68	F1-1=10
F1-1+R1	68	R2-1=10
F1-1+R1-1	68	F1-2=10
F1-2+R1	68	Others=15
F1-2+R1-1	68	
F2+R2	65	
F2+R2-1	68	
F2-1+R2	65	
F2-1+R2-1	68	
F2-2+R2	65	
F2-2+R2-1	68	
F1-2+R2	68	
F1+R2	65	
F1+R2-1	68	
F1-1+R2-1	68	
F1-2+R2-1	68	

very low sequence similarity were chosen. For primers with some sequence similarity, very high Ta were applied. Numbers 1 and 2 denote cDNA1 and cDNA2, respectively. Primer specifications are shown in Table 1.

RT-PCR and PCR

Titan One Tube RT-PCR, Expanded High Fidelity System (Roche Diagnostics, Germany) and Superscript II Reverse Transcriptase, Platinum High Fidelity Taq Polymerase (Gibco BRL, USA) were used for reverse transcription, second strand synthesis, nested PCR and amplification of cDNA. Gold Taq Polymerase (Perkin Elmer, USA) and Platinum Taq Polymerase (Gibco BRL, USA) were used for screening purposes. Hot start kits or systems were chosen.

To overcome secondary structures in mRNA several extension temperatures of 45, 50, 55 and 60°C were applied for reverse transcription. Optimal extension temperature was found at 50°C.

Table 3. Specification of Nested primers.

RT-PCR primers	Position on mRNA F;R	Nested primers	Position on mRNA F;R
1) F1-1+R1-1	34; 2585	F1+R1	133; 2501
2) F1-1+R1-1	34; 2585	F1-2+R1	75; 2501
3) F2-2+R2	4791; 7446	F2+R2-1	5057; 7293
4) F2-2+R2	4791; 7446	F2-1+R2-1	5058; 7293

Due to different Tm of primers, different concentrations of primers were used for second strand synthesis and amplification of cDNA, thus, primers with higher Tm were used in lower concentrations (Table 2). To increase specificity and overcome extensive secondary structures in the cDNAs and primers, High Ta was used (depending on primers Tm). To increase yield, the Touch-down PCR (-0.4° C/cycle decrement) was used for the amplification of cDNA.

After many attempts, no results were obtained and therefore, nested PCR was done using more internal primers (nested primers) and with combinations shown in Table 3.

For reverse transcription in two steps RT-PCR and prevention of RNase H activity, Superscript II H⁻ Reverse Transcriptase (recombinant MMLV Reverse Transcriptase) was used at the maximum temperature allowed (i.e. 42° C). Combinations of primers applied for the synthesis of the second strand and for the amplification of the double stranded cDNA1 is shown in Table 4.

Electrophoresis

Chemicals, apparatus and accessories were prepared from BIO-RAD Laboratories, Biozyme, and Merck.

DNA purification

Invitrogen Gel Purification Kit with SNAP columns was used for the purification of cDNA from Agarose. QIAGEN Mini, Midi and Maxiprep Kits were applied for the purification of plasmid from cloned bacteria. Extension products of sequencing reaction were purified with size exclusion-permission chromatography as follows:

- a) Sephadex G-50 based: Centri-sepTM Spin Columns (Princeton Separation)
- b) Polyacrylamide based: Micro Bio Spin P30(BIO-RAD)

Restriction analysis

Not I, Mlu I and Xmn I Restriction Enzymes

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Table 4. Primers specifications for two steps RT-PCR.

RT primer (position)	2 nd synthesis primer (position)	Amplification primers (position)
R1-1 (2585)	a F1-1 (34)	F1(133)+R1(2501)
	b F1-2 (75)	F1+R1
	c F1 (133)	F1+R1
R1 (2501)	d F1-1 (34)	F1+R1
	e F1-2 (75)	F1+R1
	f F1 (133)	F1+R1

were prepared in New England BioLabs. Apa I was obtained from MBI Fermentas. Simple and Combination Restriction Mapping were applied for cDNA and plasmid, respectively. For restriction analysis of cDNA2 and its plasmid, a combination of 3 restriction enzymes were used to cut both sides of insert in plasmid (Mlu I and Not I) and one had an additional restriction site inside the insert (Apa I). Not I, Mlu I and Xmn I Restriction Enzymes were applied for restriction analysis of cDNA 1.

Cloning

The Topo T-A cloning method was used for cloning cDNA. Topo XL PCR cloning kits were prepared from Invitrogen. The vector of this kit contains ccdB gene (codes for a DNA Gyrase Inhibitor) for the positive selection of clones and therefore, the blue and white screening was not required. Electroporation was applied for the transformation of electrocompetent Topo 10 bacteria. LB agar and broth were used for bacterial culture at 30° C and amplification (at 30° C and 300 rpm), respectively. Grown clones are positive clones containing insert (positive selection). Plasmids were purified from grown clones and analyzed with PCR and restriction mapping. Due to the large size of cDNA, it was necessary to decrease the transcription rate to prevent physiological disturbance of bacteria. To achieve this, the plasmid copy number was kept as low as possible by culturing bacteria at 30 ° C.

Sequencing and sequence analysis

Big Dye Terminator Ready Reaction Mix (Perkin Elmer) was used for Cycle Sequencing. Sequence detection was done by the ABI PRISM 373 Sequencer with 12 h run time (overnight).

Polyacrylamide/Bisacrylamide 19/1 pre-prepared solution, TEMED, TBE pre-prepared powder and Ammonium Persulfate (BIO-RAD

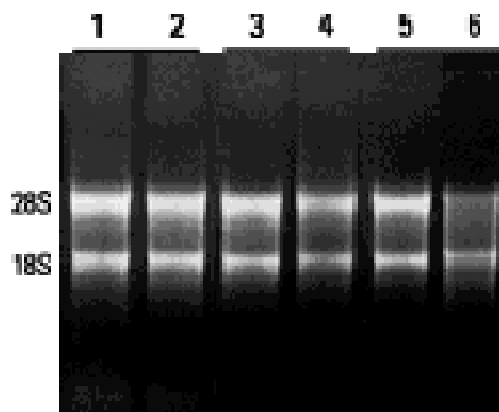


Figure 1. RNA electrophoresis .Different amounts of sample were applied in wells. 30, 25, 20, 15, 10 and 5µ l were applied in wells number 1, 2, 3, 4, 5 and 6 respectively.

Laboratories) were used for sequencing. Sequence analysis and alignment was done with Sequence Analysis (Perkin Elmer) and Sequencher 3.1 (GeneCodes) softwares, respectively.

Results

Electrophoresis of RNA under denaturing condition showed a more intense band in 28s than 18s without degradation of mRNA (smear between 2 bands of ribosomal RNA) which means good quality of purified total RNA (Figure 1).

Seventeen reactions were performed for Titan One Tube RT-PCR but the results were negative. Nested primers were efficient in combination shown in Table 3. Results of nested PCR are shown in Figure 2. From 17 nested PCR reactions, only four were positive with correct size.

PCR products were purified from gel and

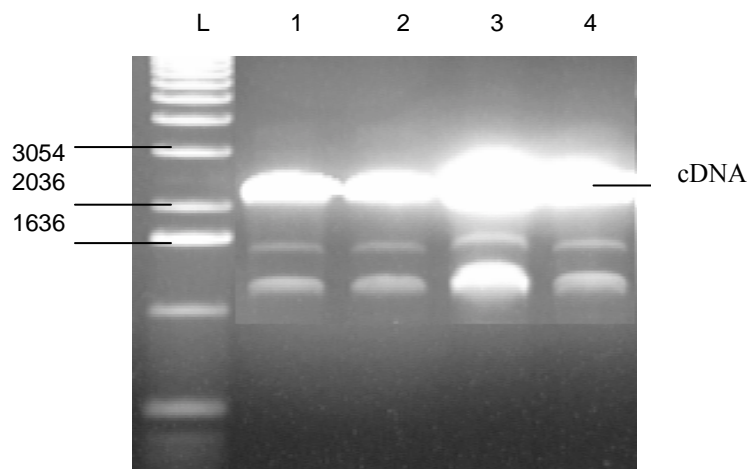


Figure 2. Nested PCR result. L , 1kb ladder; 1, 2, 3 and 4 are the same as 1,2,3 and 4 of Table 3.

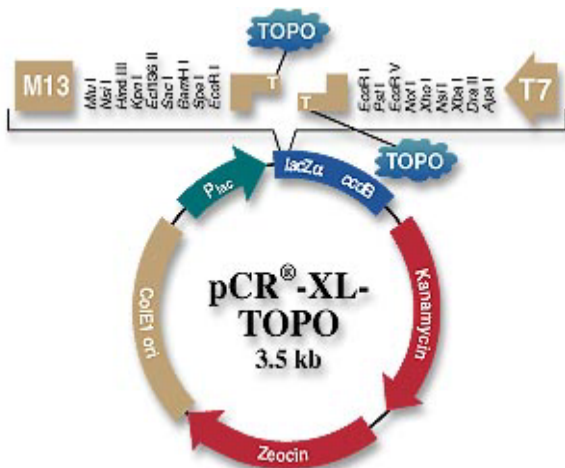


Figure 3. Map of Topo XL Vector.

cloned into the Topo XL Vector and then into the Ultracompetent *E. coli* by electroporation. Purified plasmids from grown clones were analyzed with F1+R1 and F2-1 + R2-1 GSPs for cDNA 1 and cDNA 2, respectively. Results of PCR analysis are shown in Figure 4. Most plasmids are positive with PCR but it was necessary to confirm results with another method, which requires restriction analysis and sequencing.

A combination of 3 restriction enzymes of Not I, Mlu I and Apa I for combined restriction analysis of plasmid 2, resulted in three fragments: linearized plasmids (3.5 kb) and two cDNA fragments (1312 and 953 bp). Only Apa I was used for PCR products of cDNA2 and the resulting fragments were similar to the plasmid (1312, 953bp) (Figure 5). All results were compatible with theoretical restriction map from databases (Figure 5).

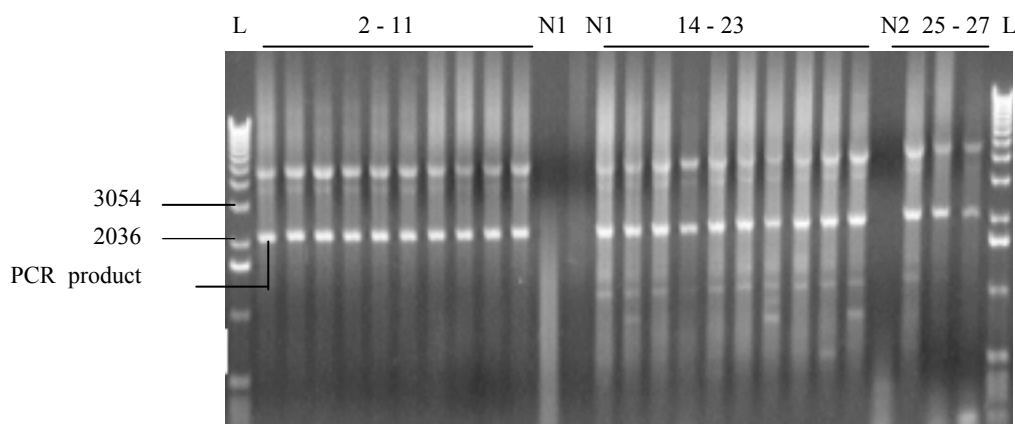


Figure 4. PCR analysis of cloned plasmids. L: 1kb ladder ; 2-11 : plasmid 1 from electroporated cells ; N1 negative control for cDNA 1; 14-23 plasmid 2 from electroporated cells; N2 negative control for cDNA2; 25-27 : positive control for cDNA2. Plasmid 1 and plasmid 2 denotes to plasmids containing cDNA1 and 2, respectively.

cDNA1 was not positive with either restriction analysis or sequencing, therefore the cell line and procedures were changed to the HepG2 and the two-step RT-PCR. The results are shown in Figure 6.

Reactions of a,b and c showed positive results with correct size. Reverse primers of reverse transcription reaction of d,e and f are the same as reverse primers of amplification reaction but for a,b and c reactions more internal primers were applied for amplification reactions that were efficient. For checking relevancy of cDNA, restriction analysis was applied. cDNA1 was cut into two fragments of size 1420 and 980 bp by Xmn I restriction enzyme and the results are shown in Figure 7.

The cloned plasmids containing cDNA1 were checked with restriction analysis using two enzymes of Not I and Mlu I for cutting both sides of the insert. Linearized plasmid (3.4 kb) and intact insert (2492 bp) resulted which were compatible with information of databases (Figure 8).

Cycle sequencing with Big dye terminator ready reaction mix were applied for cDNAs sequencing. Sequencer 3.1 software was used for sequence analysis and alignment. All sequences were checked with BLASTn at NCBI and all were compatible with factor VIII cDNA (Gene Bank Code No.M 14113).

Discussion

On the whole, 5 clones of cDNA1 and 7 clones of cDNA2 were produced, some of them had point mutations and were suitable for the assessment of biological activity and production yield.

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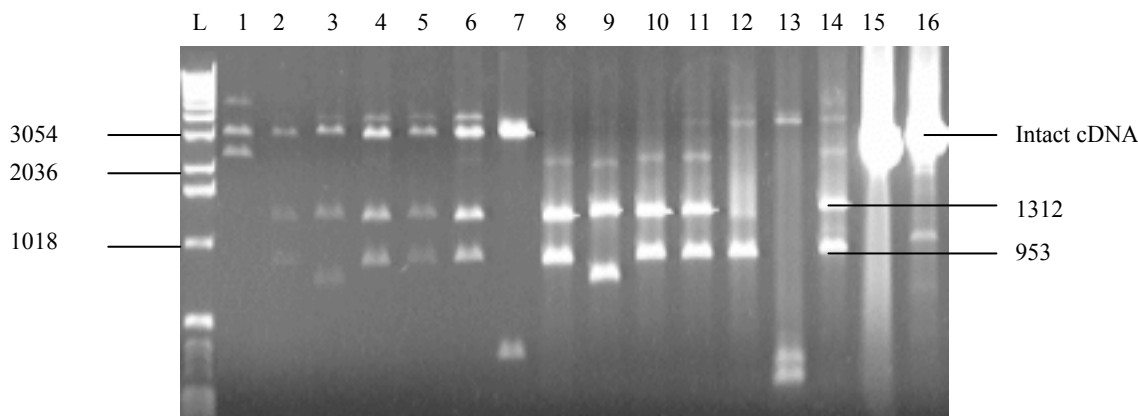


Figure 5. Restriction map of cDNAs 2 and plasmid 2. L : 1 kb ladder ; 1: plasmid cut by Mlu I and Not I ; 2-7 plasmid cut by Mlu I, Not I and Apa I; 8-13 PCR products cut by the same enzymes; 15 and 16 intact cDNA 1 & 2, respectively.

Further studies will focus on expression of cDNAs in eukaryotic or prokaryotic cells and on the assessment of production. Biological activities and half-life are evaluated in the next step, thereby finding a super-molecule for factor VIII with a higher half-life and biological activity, which reduces the required dose of factor VIII. A big dilemma in hemophilia is inhibitory antibody against factor VIII. Treatment of hemophilia with the inhibitor (factor VIII antibody) is difficult and very expensive. Immune tolerance induction is the definitive treatment of patients with the inhibitor. In this procedure a very high amount of FVIII is administrated in association with immunosuppressive drugs. Plasma-derived FVIII (pdFVIII) is used for administration (rFVIII is very expensive). Some points should be noted in this regard:

1-Plasma-derived FVIII is associated with vWF. Therefore, pdFVIII can be used for treatment of vWB disease and administration of pdFVIII for immune tolerance induction is waste of vWF.

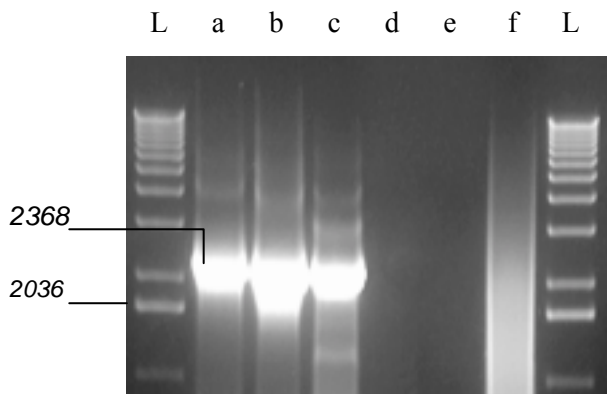


Figure 6. Two-step RT-PCR for cDNA1. L: ladder and a, b, c, d, e, f are as a, b, c, d, e, f of Table 4.

2- Any plasma-derived products have potential risk of blood borne diseases and are not as safe as recombinant products.

3- Ani-FVIII antibodies are against A1-A2 and A3-C1-C2 domains only.¹⁰⁻¹⁴ Total molecular weight of these two parts is around half of whole molecule. Therefore, administration of the whole molecule seems unnecessary. On the other hand, usually patients with inhibitor have antibody to one of the domains and appropriate domain can be applied for immune tolerance induction.

The best solution to these problems is the production of domains separately and by recombinant DNA technology.

Finally, production of biologically active fragments of FVIII separately will have two applications: A-Production of second generation FVIII by joining cDNAs before expression or joining expressed protein by ion metal. B-Immune tolerance induction in hemophilia by more specific, safer and smaller size products.

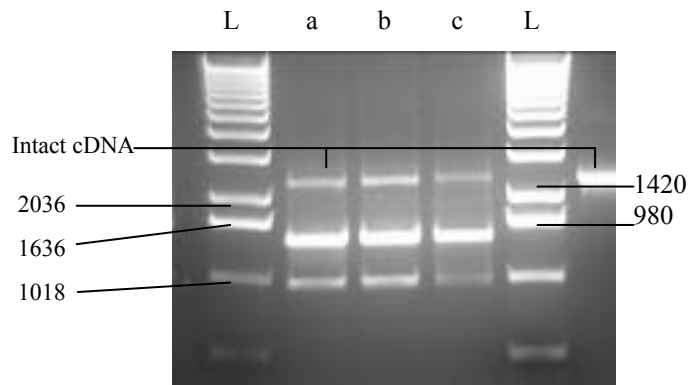


Figure 7. Restriction analysis of cDNA1 with Xmn I. L: 1kb ladder and abc as Table 4.

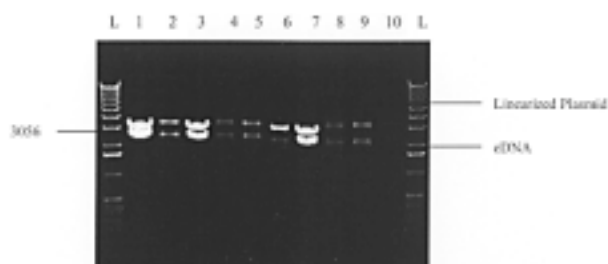


Fig 8. Restriction analysis of cloned plasmids containing cDNA1. Two enzymes of Not I and Mlu I were applied for cutting both sides of the insert; L: kb Ladder ; 1-10:plasmids with appropriate inserts.

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