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Interferon alpha 5 and human serum albumin fused protein: strategies for cloning and display of genetic instability

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A prolonged action interferon alpha-5 has a potential to become an effective pharmaceutical preparation for hepatitis C treatment. The family of interferon alpha proteins has the greatest antiviral activities against hepC infections in all the interferon families, and IFNa5 protein is the only subtype expressed in healthy liver. The *in vivo* circulation time of interferon alpha 5 can be prolonged by fusing this protein with human serum albumin on genetic level. Different cloning strategies were performed for insertion of fused protein into various expression strains and several problems of genetic instability were encountered.

Key words: IFNa5, Alb, *E. coli*, *K. lactis*, *S. cerevisiae*, PCR

INTRODUCTION

The treatment of hepatitis C virus infected patients currently is performed with preparations of interferon-alpha. Chronic hepatitis C is a widely spread hepatic disease which affects 3% of world population, i. e. approximately 170 million of people. Additionally, approximately 30% HIV-infected patients suffer from hepatitis C viral infection. There is an intensive search of various therapic means for treatment of viral infection with hepatitis C, the means that could effectively inhibit the replication of viral RNA, including the use other subtypes of IFN-alpha. It is considered that a great potential is presented by recombinant form of IFN-alpha 5, which could be used for patients with chronic hepatitis C immune to other IFN-alpha forms. Currently, the use of recombinant IFN-alpha 5 for hepatitis C treatment is in Phase II clinical trials.

Meanwhile, a lot of interferonic therapies are extended to the use of prolonged forms. This strategy enables to extend a period of in vivo circulation which is crucially important reducing the frequency of drug administration. Among other widely used pegylated IFN-alpha preparations (PEG-Intron, Pegasys), a lot of alternative forms of prolonged proteins are created. The most advanced strategy is Albufuse® technology, developed by Novozyme (Denmark). The fundamental base of this technology consists of fusing human

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serum albumin to target protein on the basis of DNA level. Generated fused DNA is subsequently cloned into *S. cerevisiae* yeast or cell culture. Novozyme proceeded this technology to GMP level and had created several effective expression systems in this framework. In this study, an attempt to produce an expression strain with albumin-fused IFN-alpha 5 was performed.

This study is performed after extended comparison with INF-alpha 2b, a high-homology protein to IFN-alpha 5. IFN-alpha 2b has been prolonged with Albufuse technology, and its molecular, physicochemical and biological characterization has shown that the functional part of the fused protein maintains the functions of unmodified protein. Albufuse version of IFN-alpha 2b has successfully passed preclinical and Phases I–II of clinical trials. As seen in Fig. 1, several preparations of IFN-alpha 2 are compared – native, pegylated and albumin-fused. Even though the antiviral activity of albumin-IFN fused construction is lower (EC_{50} of 3.813 fmol vs 476 fmol), it is almost equivalent to that of pegylated preparation of IFN-alpha 2a (3.813 fmol vs. 4.401 fmol). Furthermore, albumin-fused interferon shows a higher anti-HCV activity, which counterweighs its slower distribution. Slow distribution in plasma is also an advantage of prolonged action and avoiding acute side effects.

Considering all the above mentioned phenomena and including a high homology and similar pattern of action of IFN-alpha 5, it can be assumed that genetic fusion of this protein to human serum albumin can provide the anticipated effect of prolonged action while retaining a biological function.

EXPERIMENTS AND DISCUSSION

Genetic characteristics of strains and plasmids are presented in Table 1. The polymerase used for PCR reactions was recombinant Pfu DNA polymerase (UAB Fermentas), all the enzymes and their corre-

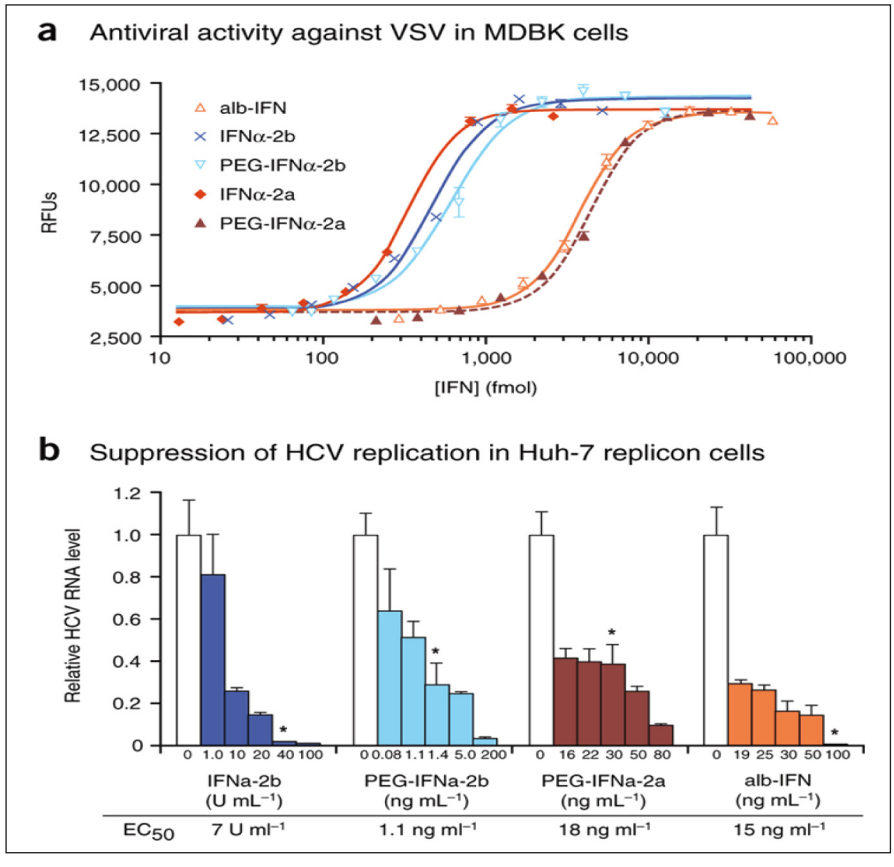

Fig. 1. The *in vitro* antiviral activity of IFN-α preparations. (a) antiviral activity against vesicular stomatitis virus (VSV) in Madin-Darby (MDBK) cells. (b) suppression of HCV replication in liver cell Huh7 (Subramanian et al., 2007)

Table. Combinations of primers used for different cloning strategies

Primer title and sequence (5'→3')	Generated product	Destination
IFNA5-FOR-KEX TTTCTCGAG*AAAAGA*TGTGATCTGC-CGCAGACC	527 bp length *XhoI-Kex*-IFNa5-(-stop)	IFNa5 gene dedicated for fusing via blunt end. The product has XhoI target for cloning into pKLAC2 plasmid and Kex protease recognition sequence
IFNA5-REV-(-STOP) TTCCTTACGACGTAAACGTTCTTGC		
ALB-FOR-(-START) GATGCACATAAAAGCGAAGTTGCC	1 704 bp length (-start)-Alb-*NotI*	Albumin gene dedicated for fusing via blunt end. The product has NotI target for cloning into pKLAC2 plasmid
ALB-REV-NOT AAAGCGGCCGCTTACTACAGA		
IFNA5-FOR-KEX TTTCTCGAG*AAAAGA*TGTGATCTGC-CGCAGACC	547 bp length *XhoI-Kex*-IFNa5-(-stop)-20 bp Alb	IFNa5 gene dedicated for overlapping PCR strategy. Has additional 20 bp of albumin 5' end, as well as XhoI and Kex elements for cloning and secretion
IFNA5-REV-A+ AACTTCGCTTTTATGTGCATCTTCCT-TACGACGTAA ACG TTC TT		
ALB-FOR-(-START) GATGCACATAAAAGCGAAGTTGCC	1 704 bp length (-start)-Alb-*NotI*	Albumin gene dedicated for overlapping PCR strategy. The product has NotI target for cloning into pKLAC2 plasmid
ALB-REV-NOT AAAGCGGCCGCTTACTACAGA		

sponding buffer solutions for cloning procedures were also purchased from Fermentas and used according to manufacturers recommendations. Primers were synthesized in MWG Eurofins DNA and were obtained in HPSF purity class.

For fusion, both genes were amplified with primers carrying required targets for restriction endonucleases. First, IFNa5 gene was amplified using a pair of primers:

IFNA5-FOR-NDE (underlined sequence is *NdeI* target): 5'-TACATATGTGTGATCTGCCG-CAGA-3'

IFNA5-REV-(-STOP):5'-TTCCTTACGACG-TAAACGTTCTTGC-3'

Forward primer introduces a target for NdeI restriction endonuclease for cloning procedure, reverse primer removes stop codon from the open reading frame. After condition optimization, a 512 bp length DNA fragment is amplified.

Human serum albumin sequence was fully codon-optimized for bacterial expression and codes for 3-599 aminoacid natural preproalbumin sequence (identification No in Expasy database NP000468). An optimized sequence is inserted in commercial plasmid pQE-T7 (Qiagen). To amplify the sequence and prepare it for cloning procedures, PCR was performed with this pair of primers:

ALB-FOR-(-START): 5'-AACTTCGCTTT-TATGTGCATCT-3'

ALB-REV-XHO (XhoI target is underlined): 5'-GGTGCTCGAGTTACTACAGACCCAGT-3'

Forward primer removes ATG(Met) start codon, and reverse primer introduces a XhoI target for cloning. After amplification, a 1 761 bp length PCR product is obtained and purified.

Purified PCR products are ligated in equimolar ratios via blunt ends; a fused genetic fragment is purified from gel and reamplified with external pair of primers and Pfu polymerase for bigger DNA yields. The fused DNA product is double digested with NdeI and XhoI restriction endonucleases and inserted into accordingly digested and purified pET21a+ plasmid (Invitrogen). After gene-to-plasmid ligation, the construct is transformed into transient strain *E. coli* JM109 (Stratagene) for positive clone selection and *in vivo* amplification of plasmid DNA. The positive construct, corresponding restriction pattern (see Fig. 2, the restriction map of IFNa5-Alb/pET21a+ construct)

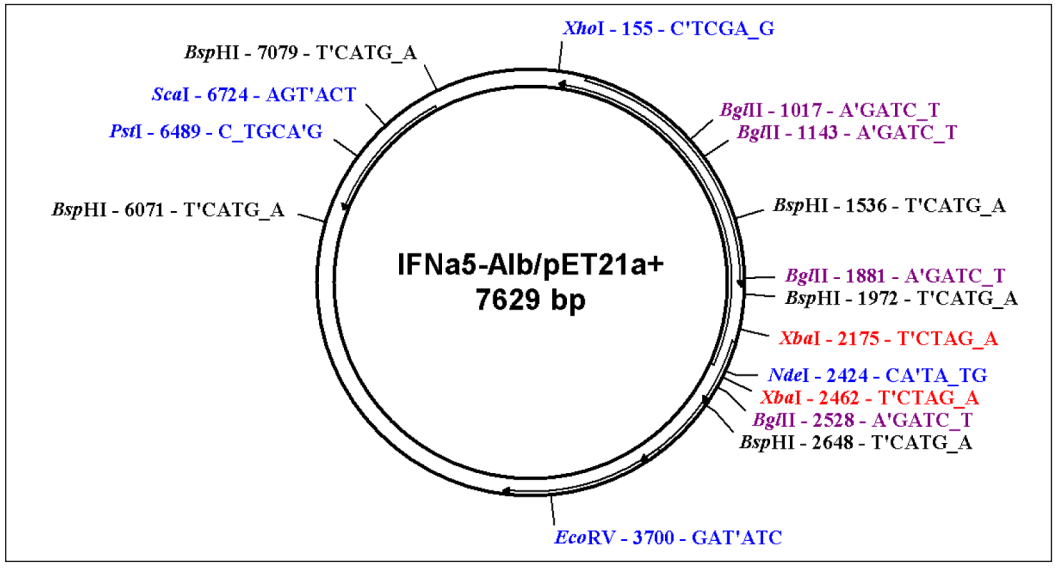

Fig. 2. The restriction map of IFNa5-Alb/pET21a+ construction. Indicated are restriction endonucleases used for routine positive clone selection and structure analysis

was amplified, purified and retransformed into expression *E. coli* strain BL21(DE3) (Stratagene).

The DNA of selected positive clone was sequenced and confirmed full identity to theoretical sequence. However, the overall background of genetic stability was less than satisfactory. Clones were viable for several days only, and gene expression was low. One clone was selected for best performance but reached only 1.2 A. U. and 54% of plasmid stability after 1.5 hours of cultivation in LB medium at +37 °C. Two and more hours were crucial and all plasmid was lost, no gene expression achieved and cell viability deterred. The research cell bank was prepared from viable cells after 1 hour of cultivation and concentrated to manageable cell densities by centrifugation. This cell bank was investigated and optimized for better gene expression and cell viability. Conditions for optimization included medium change (LB, TB, M9, M9m, mineral medium with trace elements), temperature shift and IPTG concentration. However, none of the applied optimization variants ensured any improvement on growth curves, viabilities or plasmid stabilities. Gene expression was not observed in SDS-PAGE and only faint signal was detected after Western blotting operation.

Considering all these results, the most convincing experimental pathway was to change genetic construction to yeast expression system which is much more disposed towards the expression of higher molecular weight proteins. Glycosylation pattern for interferons does not interfere with their biological activity. New England Biolabs commercial *Kluyveromyces lactis* strain GG799 was chosen for expression (see characteristics of the strain and accompanying vector pKLAC2 in Appendices 1 and 2). *K. lactis* strain is adapted to use with vectors of pKLAC group which integrates into yeast genome. pKLAC2 plasmid used for further experiments has a multiple cloning site for gene insertion, and three genes indispensable for yeast positive clone selection and maintenance: *acds* – a gene, coding for fungal acetamidase, α*MF* – alpha-mating factor gene and *bla*, gene of beta lactamase. The expression and integration of recombinant gene is ensured by $P_{LAC4\text{-}PBI}$ promoter and LAC4 locus for homologous recombination. For bacterial intermediate steps and manipulation, an ori sequence is also inserted in plasmid (for normal replication of plasmid in bacterial cells). Fungal acetamidase is dedicated for selection of yeast transformants in non-nitrogenous media with sole nitrogen source as acetamide. Acetamide is utilized as nitrogen source only by clones carrying a copy of acetamidase gene, even though non-transformed cells have a basal level of native acetamidase. Transformated clones have an advantageous growth in acetamide medium. Alpha-mating factor is fused with N-terminal part of recombinant gene during the cloning process and is required for secretion guidance of recombinant protein towards medium via Golgi appara-

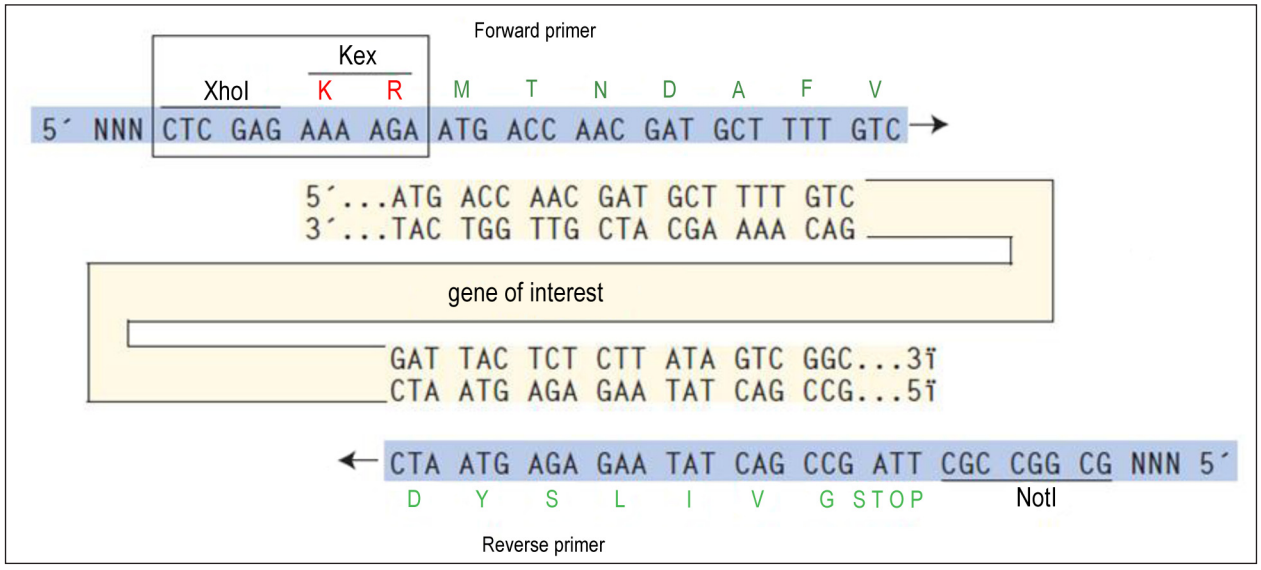

Fig. 3. The formation of targets for pKLAC2 plasmid cloning. In the case of fused IFNa5-Alb construct, forward primer is used for gene of interferon-alpha 5 amplification, including the XhoI target and Kex protease recognition sequence paired with reverse primer dedicated for STOP codon removal (see Table). Reverse primer in this figure, with STOP codon and NotI target is used on albumin gene amplification paired with according to forward primer which removes the START codon

tus. Several steps are required, and, finally, fused protein is directed into endoplasmic reticulum and cleaved there with signal peptidase; then additional two aminoacids are removed by Kex protease in Golgi apparatus. This way, a native folded protein is expressed by inducing with lactose via P_{LAC} promoter and secreted into growth media employing αMF and Kex sequences.

Cloning into pKLAC2 / *K. lactis*

The cloning strategy consisted of repeated amplification of both fused genes. The targets for restriction endonucleases, START and STOP codon removal, the Kex protease recognition sequence must be added using primers (see Fig. 3). As with bacterial construction cloning strategy, an intensive optimization experiment onf PCR conditions was performed. Temperature gradient was employed, 16 different combinations of $MgSO_4$ and primers concentrations were tested. As before, Pfu DNA polymerase was used for proof reading activity and blunt end generation.

Two strategies were used for fusing two genes in yeast cloning. Unique restriction endonuclease recognition site was not found in both genes for sticky end ligation, so fusion process was performed by overlapping PCR and blunt end ligation.

As it had been mentioned above, both genes were amplified in PCR reaction. Depending on further ligation / fusion strategy, several sets of primers were used (see Table). For all these combinations, extensive optimization of conditions was performed.

Overlapping PCR strategy is defined as the cloning event where one or both to-be-fused genes are amplified in such a manner that approximately 20 bp of other gene are synthesized into the future product. This allows to generate an overlapping identical DNA fragment which can be annealed in the second round of primerless PCR (see Fig. 4).

This experiment also requires an extensive condition optimization considering Mg ions concentration and annealing temperatures. Primers are used only in the first step of PCR, the second PCR round is performed only with two genes. The typical PCR product, as visualized in agarose gel electrophoresis, is presented in Fig. 5. Side products are also generated, especially in the region of 500–800 bp length (the size of IFNa5 gene) and non-specific products contaminating the main fused product of 2 200 bp length.

The obtained fused gene is extracted from agarose gel removing all side products and is subjected to either of the following operations:

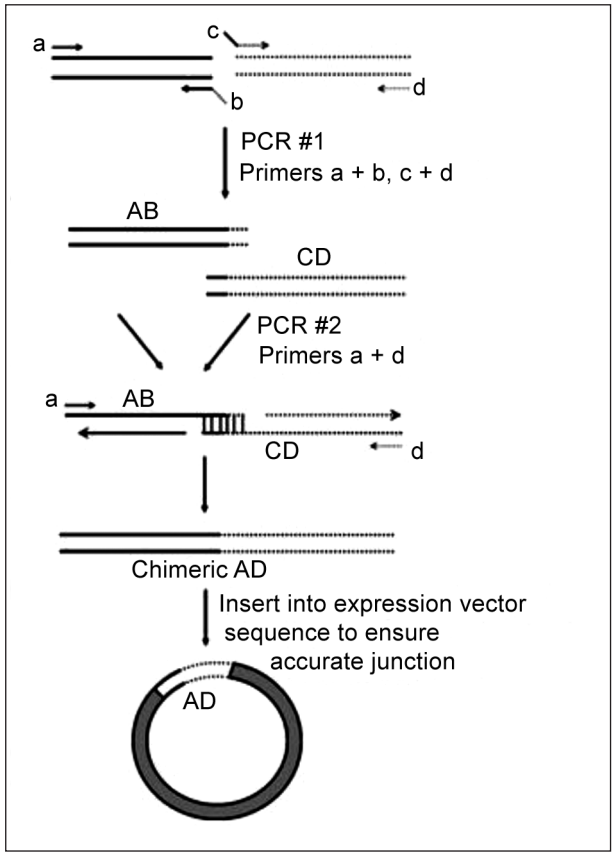

Fig. 4. The schematical workflow of overlapping PCR for chimeric gene generation

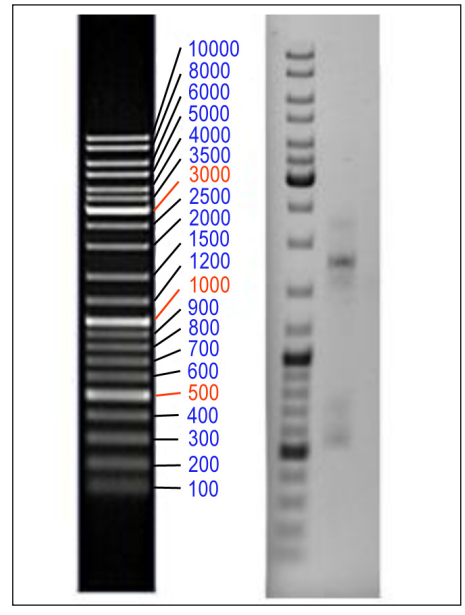

Fig. 5. The fused IFNa5-Alb gene, as generated in overlapping PCR reaction. Gel electrophoresis in 1% of agarose gel, run in TAE buffer solution and stained in ethidium bromide solution. Molecular weight marker – DNA Ladder Mix, Fermentas

- Reamplified with Pfu DNA polymerase for increased concentration and ligated into transient bacterial vector pJET1.2;
- Reamplified with Taq DNA polymerase thus introducing dA nucleotides on the ends of fused gene and ligated into transient bacterial vectors pTZ57R/T, pSURE, polyApTZ;
- Digested with XhoI and NotI restriction endonucleases and directly ligated into prepared pKLAC2 vector.

The first two variants have one additional cloning into *E. coli* JM109 strain, positive clone selection and recloning into pKLAC2 expression vector. After all the cloning, clones are selected for right size insert, amplified and sequenced.

The second strategy, ligating via blunt ends, was also successful. This strategy requires the removal of START and STOP codons, Kex protease recognition site introduction and insertion of XhoI/NotI targets (see Table). After an extensive optimization, generated gene products are digested with according to restriction endonucleases (IFNa5 gene is digested with XhoI, Alb gene – with NotI), their blunt ends are phosphorylated using T4 polynucleotide kinase and ligation reaction is set up with T4 DNA ligase and equimolar ratio of genes. The generated 2 200 bp gene product is subjected to agarose gel electrophoresis (see Fig. 6).

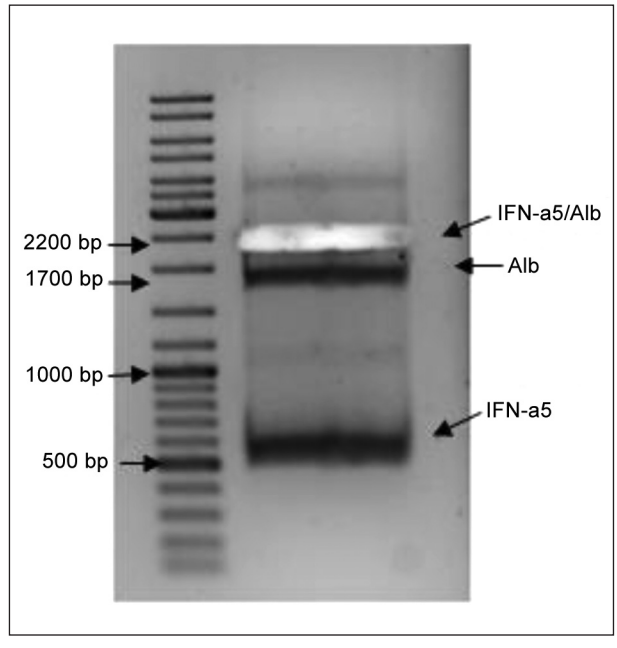

Fig. 6. IFN-a5 and Alb gene ligation electrophoretic analysis

As it is seen, unligated genes remained in the mixture, accompanied by side products of gene dimers IFNa5-IFNa5 (1 100 bp) and Alb-Alb (3 400 bp). A required 2 200 bp fragment is extracted from agarose gel and ligated into dephosphorylated pKLAC2 plasmid. The construct is transformed into *E. coli* JM109 strain and clones are verified for right size inserts.

pKLAC2 plasmid for all the variations of cloning was prepared uniformly, i. e. digested with NotI and XhoI restriction endonucleases (in positions 304 and 257, respectively), generating a 47 bp small fragment and a 9 060 bp length backbone. The small fragment is effectively removed using PCR purification kit (Fermentas), and the backbone is dephosphorylated using CIAP (calf intestine alkaline phosphatase, Fermentas). After the reaction, CIAP is inactivated thermally and removed by purification on membrane. A generated IFNa5-Alb fuse with digested ends from any of the above-mentioned strategies was ligated in pKLAC2 plasmid backbone and transformed into *E. coli* JM109 strain. Ampicillin resistant clones were subjected to restriction endonucleases digestion and pattern verification. Fig. 7 presents a typical clone verification electrophoresis. Nine clones were cultivated in a small volume of LB medium, plasmid DNA extracted and digested with XhoI-NotI. 7 clones proved to be self-ligated pKLAC2 plasmid, clone No. 3 shows unusual pattern and clone No. 5 is correct, with a 9 000 bp backbone and a 2 200 bp insert.

Instability of clones

Selected positive clones were cultivated in 150 ml of LB medium for maxi prep purification of plasmid. Larger volume was required for amplification of plasmid, so the transformation into yeast cell could be performed and full restriction map recorded. However, a genetic instability of produced plasmids was observed. 24–48 hours after transformation into *E. coli* JM109 strain, the positive clones showed normal restriction patterns and overall size of plasmid. After 3 days following the transformation, flasks inoculated from the same Petri dishes produced significantly smaller plasmids. A typical 3rd day restriction pattern of positive clones (XhoI-NotI) is presented in Fig. 8. As seen in clones No. 1, 3, 4 and 8, an insert of 2 200 bp is still observed but the remaining backbone is only 3 600 bp length.

Exploring this phenomenon more accurately, sequencing around expression cassette was performed with adjacent primers. The results showed a repeated plasmid sequence inserted instead of a great part of Alb gene. This sequence inside of XhoI-NotI insert with false positive size of 2 200 bp was identified as a sequence for homologous recombination from LAC4 locus. It is assumed that albumin gene has generated

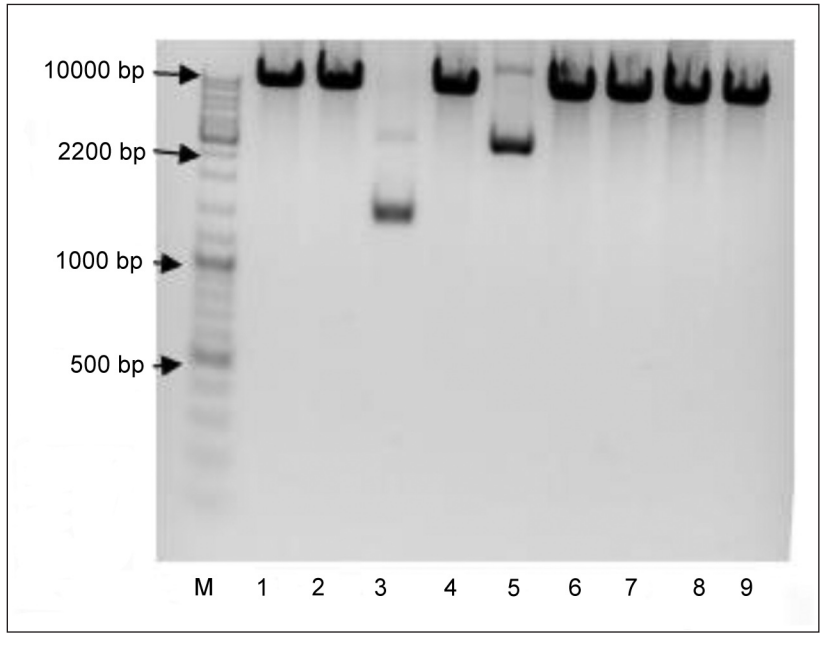

Fig. 7. Typical agarose electrophoresis of IFNa5-Alb / pKLAC2/ *E. coli* JM109 clones

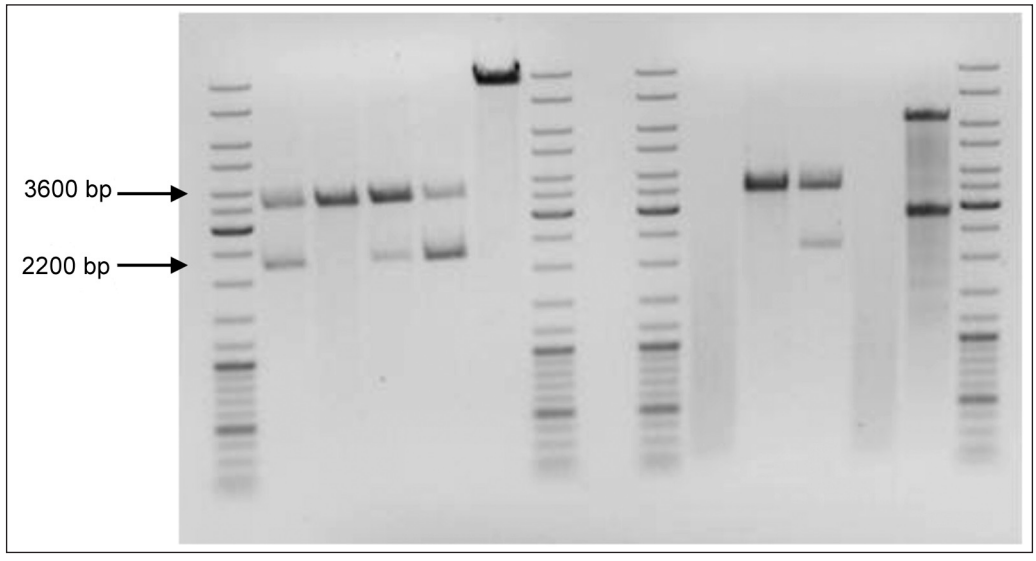

Fig. 8. A verification of ten IFNa5-Alb / pKLAC2/ *E. coli* JM109 clones with XhoI-NotI restriction endonucleases on the day 3 after transformation

a possibility of internal plasmid recombination due to its highly AT-rich sequences.

Selection of yeast strain to avoid genetic instability

After generation of instable pKLAC2 clones, another yeast expression system was probed. The vectors of pESC series are also dedicated for eukaryotic gene expression, functional analysis and addition of epitopic markers in *S. cerevisiae* strains. These vectors have GAL1 and GAL10 yeast promoters that are situated in opposition to each other. There are two multiple cloning sites and auxotrophic selection marker (in the pESC-His plasmid, a histidin auxotrophy). pESC vectors are yeast episomal plasmids constructed on the base of phagmid pBluescript II SK(+). The following steps were performed in this plasmid:

1. A BamHI recognition sequence was inserted into IFNa5 gene in 5' end, and STOP codon removed from the 3' end.

2. A XhoI recognition sequence added to albumin gene at its 3' end, START codon is removed and 20 bp of interferon gene sequence is added for overlapping PCR strategy.

As described above with pKLAC plasmid, all the experiments were thoroughly optimized, genes were fused either via blunt end ligation or overlapping PCR. After the digestion of fused gene with BamHI and XhoI restriction endonucleases, genes are recloned into:

- pJET1.2 blunt plasmid;
- after reamplification with Taq DNA polymerase – into pTZ57R/T plasmid;
- directly into pESC-His plasmid.

Direct insertion into pESC-His plasmid was ineffective with a very low transfation ratio. Recloning from smaller plasmids pJET1.2 and pTZ57R resulted in the same phenomenon of reduction in size by 3 000 bp. A loss of genetic material due to high internal homology of albumin gene is observed again.

CONCLUSIONS

After several different cloning strategies tested in this experiment, a fused IFNa5-Alb gene was generated but none of the constructed strains demonstrated stability and gene expression. In bacterial strain, a vector remained structurally stable but effectively lost plasmid during cultivation and showed a very low level of expression. After recloning the fused gene into two different yeast plasmids, a genetic recombination and the loss of fused gene fragment were observed, stipulated by recombinant sequences in plasmids and high AT content in albumin sequence. After an accurate research of the cloning process, several conclusions are suggested. A thorough codon optimization of both interferon and albumin sequences could be the possibility to solve this problem. Plasmid sequences can be optimized, too. The most probable

solution could also be a truncation of albumin gene. There are reports where albumin fused proteins are fused with only several of four albumin domains. A smaller construct without a problematic fragment could be possible to maintain in reported strains.

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IFNa5 IR ŽMOGAUS SERUMO ALBUMINO SULIETAS BALTYMAS: KLONAVIMO STRATEGIJOS IR GENETINIO NESTABILUMO PASIREIŠKIMAS

Santrauka

Naudojant šiuolaikinės molekulinės biologijos ir biotechnologijos laimėjimus buvo bandoma sukurti prailginto veikimo interferoną alfa-5, galintį tapti efektyviu vaistu gydant hepatitą C. IFNa šeima pasižymi didžiausiu antivirusiniu aktyvumu hepatito C infekcijos atveju, palyginti su kitomis interferonų šeimomis, o IFNa5 yra vienintelis IFNa šeimos potipis, sintetinamas sveiko žmogaus kepenų ląstelėse. IFNa5 biosintezės lygis labai sumažėja chroniško hepatito C atveju. Interferono alfa-5 cirkuliacijos *in vivo* laiką buvo siekiama prailginti jį genetiškai suliejant su žmogaus serumo albuminu. Buvo išbandytos įvairios klonavimo strategijos, kai sulietas konstruktas įkeliamas į įvairius raiškos kamienus, ir identifikuotos įvairios genetinio nestabilumo problemos.

Raktažodžiai: IFNa5, Alb, *E. coli, K. lactis, S. cerevisiae*, PGR

Appendix 1. Strains used for cloning

No.	Name	Genotype	Description	Compatible plasmid
1	*E. coli* JM109	**endA1** glnV44 thi-1 relA1 gyrA96 **recA1** mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ **lacI^q** lacZΔM15] hsdR17($r_K^-m_K^+$)	Strain is suitable for high homology DNA fragment cloning (RecA$^-$ mutation)	All plasmids with ori sequence, a perfect transient strain for intermediate cloning operations
2	*E. coli* BL21(DE3)	F$^-$ **ompT** gal dcm **lon** hsdS_B(r_B^- m_B^-) λ(DE3 [lacI **lacUV5-T7 gene 1** ind1 sam7 nin5])	Strain deficient with proteases ompT and lon, dedicated for recombinant protein expression systems with T7 DNA polymerase system and lac operon repression	pET21 type or other plasmids employing lac repressor system. Expression strain
3	*K. lactis* GG799	Haploid (α) wild type industrial strain without any genetic markers.	While being a wild type strain is valued for achievable high densities of cultures and recombinant protein secretion	pKLAC series
4	*S. cerevisiae* YPH499	ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1–Δ63 **his3–Δ200** leu2–Δ1	Intracellular fused protein synthesis	pESC-His
5	*S. cerevisiae* cdc25h(alpha)	Matα ura3-52 **his3-200** ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal+	Intracellular fused protein synthesis	pESC-His

Appendix 2. Plasmids used for cloning

No.	Plasmid	Marker of resistance	Promoter	Targets for insert	Compatible strain	Notes
1	pET21a+ (Novagen)	Amp	T7	NdeI-HindIII NdeI-XhoI	*E. coli* BL21(DE3)	Cloning of fused gene into bacterial systems with strong bacteriophage T7 promoter
2	pJET1.2 blunt (Fermentas)	Amp	T7**	Any type of blunt ends	*E. coli* JM109	Vector for intermediate PCR products after amplification with Pfu DNA polymerase. Vector is used for PCR product amplification if the reaction yield is low. T7 promoter present on vector is suitable for *in vitro* transcription verification only
3	pTZ57R/T (Fermentas)	Amp	T7**	dT ends	*E. coli* JM109	Vector for intermediate PCR products after amplification with Taq DNR polymerase. T7 promoter dedicated to lacZ gene expression, with possible blue / white colony assay with X-Gal
4	pKLAC2 (NEB)	Amp*, acm	LAC4	XhoI-NotI	*E. coli* JM109*, *K. lactis* GG799, *K. lactis* YPH 499	Vector where gene is inserted via NotI-XhoI targets in one open reading frame with Kex protease recognition site and fusion with α-MF sequence. Clone selection is executed through integration of ammonia ions using acetamidase gene. Plasmid is integrated into yeast genome using homologous recombination via lac4 locus with genomic *K. lactis* sequences. Only expression cassette is integrated, and the expression of the recombinant gene is induced by addition of lactose
5	pESC-His (Agilent)	Amp*, his	GAL1	BamHI-XhoI	*E. coli* JM109* *S. cerevisiae* YPH499	Yeast vector for intracellular high molecular weight recombinant protein expression in *S. cerevisiae* cells. There is a possibility to add MYC and FLAG anchor sequences in front of recombinant genes. Plasmid selection is resolved through the expression of auxothropic histidin marker. The plasmid is maintained as YEP, and gene expression is induced by the addition of galactose

* the resistance for ampicillin in pKLAC2 and pESC-His plasmids is required only in intermediate cloning when recombinant DNA is transformed into *E. coli* JM109 strain. These experiments are performed for positive clone selection prior to transformation into yeast cells

** T7 promoter in pJET1.2 and pTZ57R plasmids are dedicated to *in vitro* transcription and blue / white assay with X-Gal. The promoter is not used for *in vivo* gene expression