대한의생명과학회지: 제6권 제1호 2000
Korean J. Biomed. Lab. Sci.
Vol. 6, No. 1, p.73~82, March, 2000

Brief Communication

PCR-based Determination of the Correct Orientation of Subcloned DNA Fragments, and its Application in the Rapid Cloning and Recombinant Expression of Rat Urocortin in Eukaryotic Cells

Jung-Hyun Park, Yun-Jung Lee, Shin-Young Na and Kil Lyong Kim†

Protein Engineering Research Group, Korea Research Institute of Bioscience and Biotechnology, Yusong, P.O. Box. 115, Taejon 305-600, Korea

Abstract: Blunt-end DNA fragments can be inserted in two different orientations. Conventionally, their directions are determined by restriction enzyme digestion or by DNA sequencing, however, these methods are often limited in their use due to the lack of appropriate enzyme sites or large sample numbers, respectively. In the present study, a novel strategy and the corresponding protocol for the simple determination of insert orientation is introduced. Using conventional sequencing primers and PCR primers that have been used for amplification of the insert, single clones, which have inserted the fragment in the desired orientation, were easily identified by this PCR-based method. The fidelity of this system was confirmed by cloning of a rat urocortin cDNA, which is a recently discovered neuropeptide. Recombinant clones identified by this method were further shown to be fully functional, and using these, for the first time, urocortin was recombinantly expressed in eukaryotic cells.

Key Words: PCR; sequencing primers; Ligation; Urocortin; Transfection

The subcloning of short DNA fragments into plasmid vectors has become one of the most principal techniques in molecular biology. Using site-specific DNA endonucleases, covalently closed plasmid DNA can be cleaved and foreign DNA fragments with compatible ends are inserted and ligated into these vector. In the case of subcloning using two different restriction enzyme sites, the orientation of the insert is prior determined, since the compatibility of the vector and insert DNA ends allows only one configuration to be happen. However, DNA fragments with blunt ends or those generated by a single restriction enzyme digestion have the possibility of two different orientations by which the insert can be introduced into the vector. This is also true in the case of PCR-amplified DNA fragments, which usually have a single 3'-adenine overhang on each end attached by the terminal transferase activity of the *Taq* polymerase[2]. In many cases, it is of no relevance in whether direction the insert is cloned. Obtaining the genetic information for further subcloning or sequencing is largely fulfilled by the vector-insert ligation itself, and the orientation of the insert makes no difference. However in some cases, not only the successful

*Received : January 5, 2000
Accepted after revision : February 16, 2000
†Corresponding author: Kil Lyong Kim, Protein Engineering Research Group, Korea Research Institute of Bioscience and Biotechnology, Yusong, P.O. Box. 115, Taejon 305-600, Korea
Tel: +82-42-860-4163 Fax: +82-42-860-4593
e-mail: kimkl@mail.kribb.re.kr

insertion but also the correct orientation of the subcloned insert within the vector is of interest. For example, to express a specific cDNA sequence as a recombinant protein, this DNA fragment must be placed in the correct orientation downstream to the promoter region. Also for *in vitro* transcriptions, the DNA sequence has to be correctly orientated in regard to the promoter region to produce sense-strand mRNA transcripts[8,16].

The conventional strategies for determining the orientation of such directional inserts are either the use of asymetrically placed restriction enzyme sites or the direct sequencing of the inserts using vector-encoded sequencing primers. As for the former case, usually two different restriction enzyme sites are selected, one of which is located in the vector and the other in the insert. The asymetrically positioned restriction site within the insert results in the generation of either a long or short fragment depending on the orientation of the insert, and in this way, the direction of the subcloned fragment can be indirectly determined. While this method is quite simple in its procedure, there is the limitation that not always restriction enzyme sites are available, which can distinguish the orientation of the insert. Direct sequencing of the intervening region of vector to insert is in such pitfalls the other alternative method, but in the case of large sample numbers or the necessity of a rapid determination, nucleotide sequencing will not satisfy these needs. In the present study, a PCR-based method is introduced, which can overcome the problems and inconveniences of the methods as described above, and a working protocol for this method is described in details.

In its essence, this method enables the determination of the orientation of PCR products by a PCR-based strategy involving the use of conventional sequencing primers and some insert-specific oligonucleotide primers. It is good documented that some thermophilic DNA polymerases without proofreading activities, such as the *Taq* DNA polymerase, have a terminal transferase activity that adds frequently a single adenine nucleotide to the 3' end of PCR products[1]. While this property largely prevents the direct ligation of PCR products into blunt-end opened vectors, such PCR products are compatible with insertion sites which have single 3'-thymidine overhangs[7]. Nowadays, a large collection of vectors are available that have single dT-overhang sites for the cloning of PCR products generated by amplification using *Taq* DNA polymerases[9,11]. One of the commercial versions of such T-vectors is the pGEM®-T vector (Promega, Madison, WI), which has been prepared from the pGEM®-5Z (Promega) vector by blunt-end digestion with the restriction enzyme *Eco*RV and the subsequent addition of single 3'-thymidines. While the screening for the successful ligation of PCR fragments is facilitated by blue/white discrimination, further confirmations on the successful insertion and about the orientation of the insert require additional steps such as restriction enzyme digestions or nucleotide sequencing of the recombinant plasmid. In the former case, the selection of appropriate enzyme sites is impossible when the amplified sequence is unknown, and even in the case of a published sequence, the selection of optimal enzyme sites is a not always assured. In the case of DNA sequencing, higher costs and delayed processing time are the major considerations which prevent the routine use of this method in the detection of successfully and also in the desired direction cloned inserts among a large number of colonies.

In Fig. 1. is shown a schematic presentation of the present PCR-based method, which simultaneously confirms the successful insertion of the PCR product and also determines the direction of the cloned insert. A merit of this protocol is its almost universal applicability, which is only restricted by the presence of a sequencing primer binding site in the flanking region of the multiple cloning sites. The principle of this method is quite simple. A conventional

PCR is designed which uses a vector-binding primer as the upstream primer and an insert-binding PCR primer as a downstream primer.

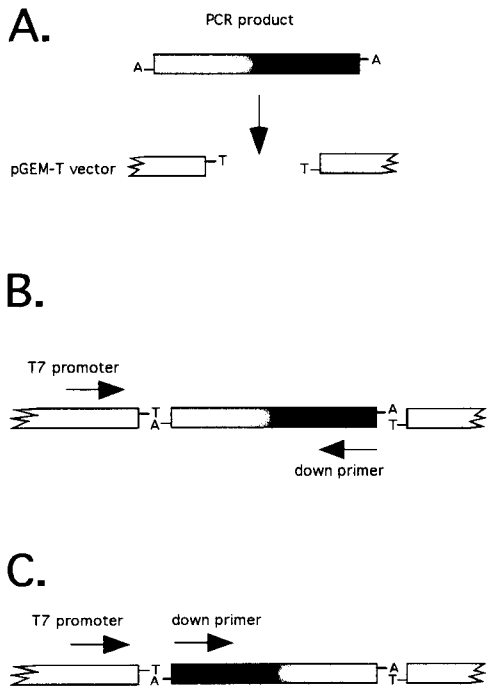

Fig. 1. Experimental design of PCR-based determination of the insert orientation.
Schematic representation of the principle of the PCR-based determination of the insert orientation in regard of an anchored, vector-primed sequencing primer region. **A.** Insertion of an PCR product into the vector T-overhang site. **B.** Successful PCR amplification in the case of correctly inserted DNA fragment. **C.** Failed PCR amplification in the case of DNA fragment insertion in the reverse orientation.

In the case of correctly cloned insert, the PCR will be successful, and in the case of either no insertion or the subcloning into the opposite direction, the PCR will fail. Depending on the selection of the primer pair, the direction of the cloned insert can also be determined by using vector-compatible primers as downstream- and insert binding primers as upstream-primers. For example, when using the pGEM®-T vector, in which the multiple cloning site is placed between the T7 and SP6 promoter, the successful insertion and the direction of the insert can be determined by either SP6 or T7 promoter primers (Table 1), which are conventional sequencing primers. In practice, the PCR will be performed using one of the sequencing primers at one end, and a PCR primer, which binds to the opposite end of the DNA insert, to the other end.

To prove its applicability, and to show the convenience and efficiency of this method, a cDNA of the rat urocortin was amplified by RT-PCR and this PCR product was cloned into single dT-overhang vectors. Urocortin is a recently described neuropeptide[22] that has been implicated in the regulation of stress-mediated biological responses, such as the release of ACTH[15], as well as the induction of behavioral changes like anxiety, appetite-suppressing effects and so on[13,20]. Mid-brain tissues from young adult Lewis rats were used as source for isolation of rat urocortin cDNA. Lewis rats were obtained from the Animal Laboratory

Table 1. The nucleotide sequences of commonly used sequencing primers and their suggested Tm values for using as PCR primers

Name	Sequence	Tm value
SP6 promoter primer	5'-gatttaggtgacactatag-3'	52℃
T7 promoter primer (Stratagene)	5'-gtaatacgactctatagggc-3'	58℃
T7 promoter primer (Promega)	5'-taatacgactcactataggg-3'	56℃
M13-20 primer	5'-gtaaaacgacggccagt-3'	52℃

The Tm values for each of the primers were estimated by the *Primers* program (http://www.williamstone.com/primers/calculator)

Division of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon. Isolation and synthesis of the cDNA from total RNA was essentially performed as described in a previous study[18]. In brief, total RNA was isolated from the mid-brain region after a modified protocol as originally described by Chomczynski and Sacchi[1], and the intactness and purity were determined in a denaturing formaldehyde agarose gel and spectrophotometry, respectively. The cDNA was synthesized using oligo dT_{15} primers (Promega) and the Super-Script II reverse transcriptase, following the protocol as provided by the supplier.

The complete coding region of the urocortin cDNA was then amplified using the primer set Uro-up (5'-tatacatctggcaccatgaggcagagggga-3') and Uro-down (5'-cgcgaattcggatcacttgcccaccga-atg-3') by PCR using the following protocol: denaturation at 96℃ for 30 sec, annealing at 54℃ for 1 min, and elongation at 72℃ for 1 min. The PCR was run for 36 cycles. The oligonucleotide primers used in this study were purchased from GenoTech Inc. (Taejon, Korea), and all of the reagents for RNA extraction and cDNA synthesis were obtained from Sigma (St. Louis, MO), if not else indicated. SuperScript II reverse transcriptase was purchased from Life Technologies (Gaithersburg, MD), and the *Taq* polymerase was a product from Promega. PCR was performed with a GeneAmp® PCR system 2400 thermal cycler (Perkin-Elmer, Foster city, CA). After purification of the urocortin-PCR product over a 1.5% TAE-buffered agarose gel, the DNA was ligated into the pGEM®-T vector. Since this vector permits blue/white discrimination, single white colonies were picked from X-gal and IPTG-treated LB-agar plates spread with the transformed *E. coli* (strain JM-109). Colonies were then transferred into liquid media, and the plasmid DNA was isolated from overnight cultures using the PERFECTprep™ Plasmid DNA kit (5 prime 3 prime, Inc., Boulder, CO). The insertion itself and the orientation of the insert were then determined using the method as described below. First, PCR were designed which use selected pairs of oligonucleotide primers. These consisted of a combination of primers of which one primer binds to one end of the DNA insert and the other binds to the flanking region of the multiple cloning site of the vector. In this case, PCR reactions were performed using a combination of T7 promoter primers with either Uro-up or Uro-down PCR primers and SP6 promoter primers with either Uro-up or Uro-down PCR primers. As template was used a single recombinant clone containing the urocortin cDNA, termed pGEM-rURO. As it is to see in Fig. 2, the urocortin cDNA has been successfully cloned into the pGEM®-T vector and its orientation in regard to the T7 and SP6

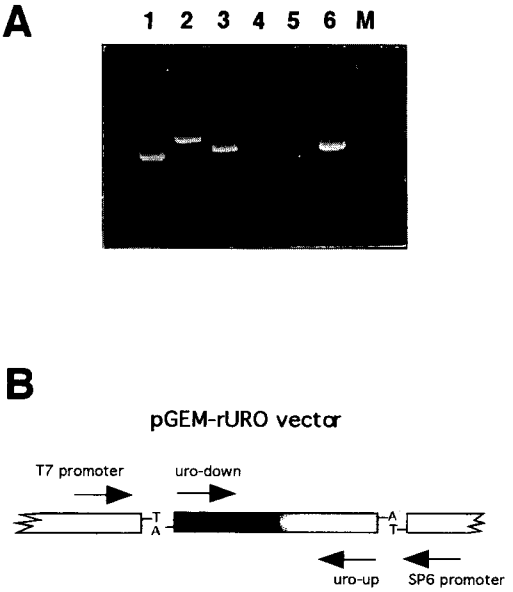

Fig. 2. PCR based analysis of the orientation of the subcloned urocortin cDNA within the pGEM-T vector.
A. The agarose-gel shows the results of the PCR using the recombinant plasmid pGEM-rURO as template and the up-mentioned oligonucleotides as primers. 1. uro-up/uro-down; 2. T7 promoter/ SP6 promoter; 3. T7 promoter/uro-up; 4. T7 promoter/uro-down; 5. SP6 promoter/uro-up; 6. SP6 promoter/uro-down. M, size marker with 100 bp differences between each band (100 bp ladder, Roche Molecular Biochemicals, Mannheim, Germany). **B.** Schematic presentation of the insert orientation as deduced from the PCR result.

promoter can be determined by the present PCR-based protocol. Judging from the agarose gel (Fig. 2A), the urocortin cDNA was inserted in the 5'-> 3' direction downstream to the SP6 promoter within the pGEM®-T vector. The same clone was then subjected to DNA sequencing to confirm the correctness of this method. The same SP6 and T7 promoter primers as used for the PCR were now used for sequencing, and it was confirmed that the identity and direction of the cloned insert were as correct as predicted from the PCR-based protocol as described above (data not shown). DNA sequencing was performed at the Genome Center, KRIBB, Taejon, using a DNA sequencing kit (Dye terminator cycle sequencing kit, Perkin Elmer). The nucleotide sequencing data further revealed that the whole nucleotide sequence of the cloned urocortin cDNA (Lewis rat) showed 100% identity to the published sequence of urocortin cDNA from SD rats, and it thereby confirms sequence conservation among different rat strains.

After confirming the accuracy and convenient applicability of this method, it was attempted to subclone the urocortin cDNA into an eukaryotic expression vector. Vectors for the expression of foreign genes in mammalian cells are generally much larger in their size than prokaryotic expression vectors, and therefore have only a very restricted repertoire of single-cut enzyme sites to determine the successful insertion and direction of cloned DNA. Thus it was expected that the present PCR-based method would provide a convenient alternative for the identification of recombinant clones which have inserted the urocortin cDNA in the desired direction. Furthermore, in the case of a successful cloning and expression of urocortin cDNA in mammalian cells, this would be the first report ever to have expressed this neuropeptide in eukaryotic cells. It is obvious that this recombinant material will be of great use in the further analysis of the biological function of urocortin.

Urocortin was originally discovered in the rat by screening a mid-brain cDNA library for homologue sequences to urotensin I, a fish neuropeptide[10], using an oligonucleotide probe from the urotensin I cDNA. The complete cDNA for the rat urocortin was shown to consist of a single open reading frame encoding a 122 amino acid polypeptide. Sequence analysis and comparison to other related neuropeptides, such as the corticotropin-releasing factor (CRF)[21], the frog sauvagine[12] and the urotensin I, have lead to the preliminary assumption that the mature peptide would encompass the C-terminal 40 amino acids without the last two amino acids of the precursor protein resulting in a putative C-terminal amidated 40-meric peptide[22]. However so far, the actually processed form of urocortin *in vivo* has not been identified. While synthetic urocortin peptides have been produced after the assumed structure of the cDNA and are currently used in various systems for determining the biological role of urocortin[13], since so far, the precise processing of the urocortin precursor protein into the mature peptide has not been formally established[3]. In this regard, the cloning of the whole urocortin cDNA into an eukaryotic expression vector would enable the analysis of the actually expressed form of urocortin. After direct cloning of the urocortin cDNA PCR product into the commercially available eukaryotic expression vector, pTargetT™, which is a single 3'-dT overhang vector (Promega), the orientation of the insert was instantly determined using the PCR-based method as introduced in the present study. Here in this case, the insertion of the urocortin cDNA in correct orientation, i.e. downstream to the CMV promoter and the T7 promoter region, a PCR test with the T7 promoter sequencing primer and the Uro-down primer was expected to result in the amplification of the whole cDNA sequence. However, in the case of an insertion in the reverse orientation, the same PCR would fail to amplify a product. Fig. 3 shows the result of this analysis.

Here is to see that 2 out of 6 clones have the insert in the desired orientation and 3 others in the opposite direction. Another clone (#6) didnt have an insert at all. Without further confirmation by restriction enzyme digestion and/or nucleotide sequencing of the insert, each one of the clones bearing the cDNA either in the correct or inverted orientation, termed pTargeT™-uro (+) and pTargeT™-uro (−) respectively, was transfected into eukaryotic host cells. The procedures for cell culture and maintenance were essentially performed as previously described[17,19]. Chinese hamster ovary (CHO) cells served as host cells for the eukaryotic expression of the cloned urocortin cDNA. These cells were obtained from ATCC (Rockville, MD) and were maintained in Dulbecco's modified Eagles media (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies Inc.), penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, and L-glutamine (2 mM). For transfection of the urocortin cDNA, the recombinant DNA was complexed with LipofectAMINE PLUS reagents (Life Technologies Inc.) and introduced into CHO cells following the protocol as suggested by the supplier. Cells transfected with plasmids bearing the urocortin cDNA in either the correct or reverse orientation, pTargeT™-uro (+) and pTargeT™-uro (−), were termed CHO-uro (+) and CHO-uro (−), respectively. For screening and selection of stable transfectants, from on the next day after transfection, cells were treated with 500 μg/ml G418 for at least 4 weeks. In the case of transfectants with in-

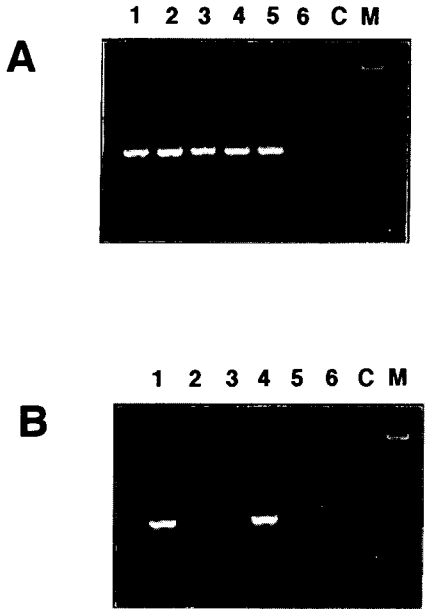

Fig. 3. PCR analysis of the subcloned rat urocortin cDNA into the pTargeT vector.
A. PCR amplification of the subcloned rat urocortin cDNA using the PCR primer uro-up and uro-down. **B.** PCR-based determination of the orientation of the subcloned rat urocortin cDNA using the T7 sequencing primer and uro-down. The numbers of individual clones are as indicated on the agarose gel. M, size marker with 100 bp differences between each band (100 bp ladder, Roche Molecular Biochemicals, Mannheim, Germany); C, negative control (H_2O instead of plasmid).

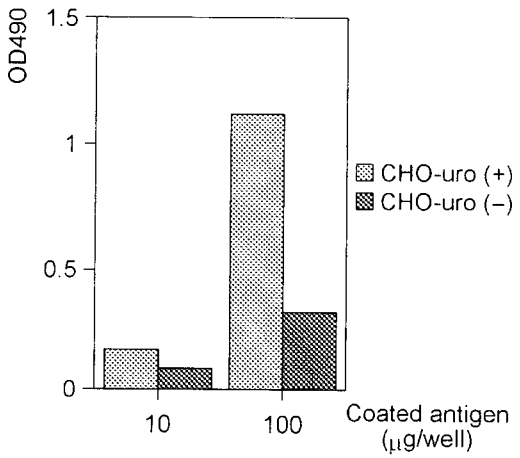

Fig. 4. Whole cell lysate ELISA of urocortin cDNA transfected CHO cells.
CHO cells, which had been transfected with either pTargeT -uro (+) or pTargeT -uro (−), were selected upon their resistance to G418. The transfectants were then lysed with cell lysis buffer containing 0.5% NP-40, and the protein content of the cell lysate was determined by the Bio-Rad protein assay kit. After dilution of the cell lysate in ELISA coating buffer, each 10 μg and 100 μg of whole cell lysate were used to coat 96-wells. Recombinantly expressed urocortin was then detected with saturating amounts of anti-urocortin antibodies and peroxidase-conjugated secondary antibodies.

serts in their correct orientation, the cells would produce recombinant urocortin, but not those cells, which have the urocortin cDNA in the opposite direction to the promoter.

To determine the successful expression of urocortin, first, Western blot analysis was performed. For this, cell lysate of both CHO-uro transfectants were separated in an SDS-PAGE, blotted onto nitrocellulose membrane, and then detected using anti-urocortin antibodies[14]. However, no specific signal was detected in the blots (data not shown), which even didnt change after increasing the amount of cell lysate or enhancing the concentration of the anti-urocortin antibodies (data not shown). An interesting aspect of this experiment was that synthetic urocortin which has been included in the blot as control was also not detectable with the anti-urocortin antibodies. Since the titer and specificity of the anti-urocortin antibody has been previously confirmed[14], it was concluded that urocortin, either in its synthetic or recombinant form, might not be detectable by conventional Western-blots. Judging from this result, and since there had been also other observations that small peptides are hardly to detect in conventional Western blot analysis[4,6], an alternative method for proving the successful expression of urocortin in transfected cells was tried out. For this, whole cell lysate of transformed CHO-cells were used as coating antigens in an ELISA, and the plastic-bound urocortin was then detected using anti-urocortin antibodies[14]. First, cells were harvested from culture plates and then incubated for 30 min on ice in lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, with freshly added 1 mM PMSF). The insoluble fractions were removed by centrifugation, and the protein content of the supernatant was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The cell lysate was then diluted in ELISA coating buffer (0.1 M Na-carbonate, pH 9.5) and used for the coating of 96-well flat bottom MaxiSorb™ ELISA plates (NUNC, Roskilde, Denmark). After overnight incubation at 4℃, excess reagents were removed by extensive washing with TBS/0.05% Tween-20, and the presence of urocortin was then detected by incubation with anti-urocortin antiserum. The antibody concentration was hold constant while the amounts of coated antigens varied in a descending manner. Specifically bound antibodies were then detected with peroxidase conjugated rabbit anti-mouse Ig antibodies (Sigma), and the subsequent addition of the corresponding substrates for colormetric analysis (*o*-phenylenediamine and H_2O_2). As expected, cell lysate of CHO-cells transfected with the pTargeT™-uro (+) vector showed a strong positive signal (Fig. 4), but no signal was seen in the cell lysate of their counterparts, the CHO-uro (−) cells. This result is in agreement to the determination of the orientation of the insert, since CHO-uro (−) cells should express the urocortin cDNA in the inverse orientation, and thereby must not express recombinant urocortin. It is expected that the urocortin transfectants might now serve as an excellent source for the isolation of recombinant urocortin. Furthermore, due to the eukaryotic nature of the expression system, the *in vivo* processed and expressed form of urocortin will now be able to be exactly defined.

In conclusion, the generation of recombinant urocortin in its naturally processed form will provide a novel tool for the functional analysis of urocortin, and it is also to be expected that the PCR-based protocol for the determination of correct insertion of PCR fragments as described in this study will serve as a rapid and convenient alternative for screening recombinant clones.

Acknowledgments

This work was supported in part by a grant (NN1010) from the Ministry of Science and Technology (MOST), Korea.

REFERENCES

1) Chomczynski P and Sacchi N (1987): Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* **162:** 156-159.
2) Clark JM (1988): Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res,* **16:** 9677-9686.
3) Donaldson CJ, Sutton SW, Perrin MH, Zorrigan AZ, Lewis KA, Rivier JE, Vaughan JM and Vale WW (1996): Cloning and characterization of human urocortin. *Endocrinol,* **137:** 2167-2170.
4) Gillot DJ, Nouri AME, Compton SJ and Oliver RTD (1993): Accurate and rapid assessment of MHC antigen upregulation following cytokine stimulation. *J Immunol Meth,* **165:** 231-239.
5) Goldberg HA and Warner KJ (1997): The staining of acidic proteins on polyacrylamide gels: Enhanced sensitivity and stability of Stains-All staining in combination with silver-nitrate. *Anal Biochem,* **251:** 227-233.
6) Hawkes R (1986): The dot immunobinding assay. *Methods Enzymol,* **121:** 484-491.
7) Hengen PN (1995): Cloning PCR products using T-vectors. *Trends Biochem Sci,* **20:** 85-86.
8) Kuhnlein P, Park JH, Herrmann T, Elbe A and Hunig T (1994): Identification and characterization of rat gamma/delta T lymphocytes in peripheral lymphoid organs, small intestine, and skin with a monoclonal antibody to a constant determinant of the gamma/delta T cell receptor. *J Immunol,* **153:** 979-986.
9) Kwak JH and Kim MY (1995): Construction of T-vector for direct cloning and expression of cloned genes in *Escherichia coli. Anal Biochem,* **228:** 178-180.
10) Lederis K, Letter A, McMaster D, Moore G and Schlesinger D (1982) Complete amino acid sequence of urotensin I, a hypotensive and corticotropin-releasing neuropeptide from Catostomus. *Science,* **218:** 162-165.
11) Marchuk D, Drumm M, Saulino A and Collins FS (1990): Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res,* **19:** 1154.
12) Montecucchi PC and Henschen A (1981): Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei. Int J Pept Protein Res,* **18:** 113-120.
13) Moreau JL, Kilpatrick G and Jenck F (1997): Urocortin, a novel neuropeptide with anxiogenic-like properties. *NeuroReport,* **8:** 1697-1701.
14) Na SY, Park JH, You KH and Kim KL (1999): Generation of self-antigen reactive, anti-urocortin specific antibodies by immunization of recombinantly expressed urocortin fusion proteins. *Mol Cells,* **9:** 587-595.
15) Ozawa M, Oki Y, Watanabe F, Iino K, Masuzawa M, Iwabushi M and Yoshimi T (1998): Effect of urocortin and its interaction with adrenocorticotropin (ACTH) secretagogoues on ACTH releases. *Peptides,* **19:** 513-518.
16) Park JH, Mitnacht R, Torres-Nagel N and Hunig T (1993): T cell receptor ligation induces interleukin (IL) 2R beta chain expression in rat CD4, 8 double positive thymocytes, initiating an IL-2-dependent differentiation pathway of CD8 a+/b- T cells. *J Exp Med,* **177:** 541-546.
17) Park JH, Cho EW, Lee YJ, Hahm KS and Kim KL (1997): Generation and characterization of a novel fusion partner cell line for the production of human macrophage hybridoma. *Hybridoma,* **16:** 551-556.
18) Park JH, Choi EA, Cho EW, Hahm KS and Kim KL (1998): Maltose binding protein (MBP) fusion proteins with low or no affinity to amylose resins can be single-step purified using a novel anti-MBP monoclonal antibody. *Mol Cells,* **8:** 709-716.
19) Park JH, Cho EW, Shin SY, Lee YJ and Kim KL (1998): Detection of the asialoglycoprotein receptor on cell lines of extrahepatic origin. *Biochem Biophys Res Commun,* **244:** 304-311.
20) Spina M, Merlo-Pich E, Chan RKW, Basso AM, Rivier J, Vale W and Koob GF (1996): Appetite-

suppressing effects of urocortin, a CRF-related neuropeptide. *Science,* **273:** 1561-1564.

21) Turnbull AV and Rivier C (1997): Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. *Proc Soc Exp Biol Med,* **215:** 1-10.

22) Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE and Vale W (1995): Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature,* **378:** 287-292.

=국문초록=

중합효소 연쇄반응에 근거한 벡터 클로닝된 DNA조각의 방향성 결정 및 이를 이용한 랫트 Urocortin의 진핵 세포주상에서의 발현과 클로닝의 수행

생명공학연구소, 단백질공학실,

박정현 · 이윤정 · 나신영 · 김길룡†

양끝이 같은 형태로 이루어진 DNA조각들은 벡터에 두 가지 방향으로 삽입될 수 있다. 기존의 방법으로는 이들의 방향성을 알아내기 위하여 제한효소의 처리 혹은 DNA염기서열 분석법이 수행되어졌는데, 이들은 적절한 제한효소 인식부위의 부재 혹은 높은 가격과 많은 샘플 수 등으로 그 이용범위가 어느 정도 제한되어 있었다. 본 연구에서는, 벡터에 클로닝 된 DNA조각의 방향성을 결정하기 위한 새로운 실험기법과 이에 따르는 구체적인 방법을 기술하고 이의 직접적인 이용을 보고하고 있다. 통상적인 염기서열 분석용 oligonucleotide primer와 중합효소 연쇄반응용 (PCR) primer를 이용한 PCR에 기초한 이 방법은, 여러 후보 클론의 플라스미드 DNA를 주형으로 하여 한 차례의 반응으로, 원하는 방향으로의 DNA조각이 삽입된 클론을 찾아낼 수 있게 한다. 이 실험기법의 용이함과 정확성은 최근에 보고된 바 있는 랫트의 신경 펩타이드인 urocortin의 cDNA를 재조합 발현 벡터상에 클로닝하고 분석하는 것으로 증명할 수 있었다. 이 같은 방법으로 찾아진 유전자 재조합 클론들은 추가적인 실험을 통하여 CHO 세포주에 transfection 되었는데, 이들이 실제로 urocortin을 발현함은 면역효소 측정법으로 검증될 수 있었고, 이를 통하여 최초로 이 40개의 아미노산으로 이루어진 짧은 펩타이드를 진핵 세포상에서 재조합 단백질의 형태로 발현시키는 데 성공하였다.

[대한의생명과학회지 **6(1): 73-82, 2000**년 **3**월]

†별책 요청 저자