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Construction of an inducible nitric-oxide synthase gene transferring vector mediated by retrovirus[1]

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KEY WORDS nitric-oxide synthase; complementary DNA; gene expression regulation; eukaryotic cells; recombinant DNA; retroviridae; genetic vectors; restriction mapping; polymerase chain reaction

AIM: To construct an inducible nitric-oxide synthase (iNOS) gene transferring vector mediated by retrovirus. **METHODS**: Recombinant DNA and polymerase chain reaction (PCR) amplification techniques were used. **RESULTS**: With 2 steps of molecular cloning, the full-length cDNA encoding macrophage iNOS was isolated from plasmid pKSiNOS and subcloned into intermediate vector pSP72, adjusting the restriction enzyme sites in both 5′- and 3′-flanking ends of insert fragment. The retroviral vector pLNCXiNOS which contains iNOS coding region, cytomegalovirus promoter and neomycin resistance (neo^r) gene was further constructed. The authenticity of insertion size and orientation of iNOS sequence was verified by restriction mapping and PCR analysis with iNOS gene-specific primers. **CONCLUSION**: Retroviral expression vector carrying iNOS fragment is obtained, which provides a material to establish a model of iNOS gene-modified neurons.

Nitric oxide as an intracellular messenger participates in neurotransmission, vascular dilation, and antimicrobial defense[1]. Nitric oxide synthase (NOS) catalyzes the formation of NO and *L*-citrulline (*L*-Cit) from *L*-arginine (*L*-Arg) and O_2[1]. Three principal isoforms of NOS have been cloned, including neuronal (nNOS), endothelial (eNOS), and macrophage (macNOS) or inducible NOS (iNOS). Expression of iNOS can be induced by cytokines or lipopolysaccharide (LPS) in macrophages, hepatocytes, and astrocytes[2]. Excessive NO produced by iNOS is responsible for the nonspecific cytotoxicity[3].

Early work in mechanisms of opioid tolerance and dependence focused upon opioid receptors and second messengers[4]. NOS inhibitor attenuates μ- or δ-opiate tolerance and dependence[5], and prevents naloxone-precipitated withdrawal signs in morphine-dependent mice[6]. Chronic administration of *L*-Arg accelerates development of opioid tolerance[7]. NO is involved in modulation of opioid analgesia. Thus development of specific iNOS inhibitors may be beneficial to treat this condition. Based on previous work that nNOS was expressed in NG108-15 cell[8], this study was to construct retroviral vector with iNOS gene to establish a model of iNOS gene-modifying neural cells, investigating the role of NO/NOS in opioid tolerance and dependence.

MATERIALS AND METHODS

Enzymes and chemicals Restriction endonucleases were purchased from Promega Co, New England Biolabs Co, and Boehringer Mannheim Co. T_4 DNA ligase, T_4 DNA polymerase, Taq polymerase, Klenow fragment of DNA polymerase, and dNTPs were obtained from Boehringer Mannheim Co. DNA purification kit, GlassMax DNA isolation system, was from Gibco BRL. DNA molecular weight markers, 1 kilobase (kb) DNA ladder and SPP1 DNA/EcoR Ⅰ, were purchased from Boehringer Mannheim Co. All other reagents were products from Sigma or Fluka Chemical Co. Other chemicals were of AR.

Plasmids and strains The plasmid pKS iNOS, a gift from Dr S H Snyder and Dr D S Bredt at Johns Hopkins Medical Institute, contained the full-length cDNA encoding iNOS from interferon-γ (IFN) and LPS-stimulated RAW264.7 macrophages which was cloned into a unique Not Ⅰ site in pBluescript KS Ⅱ (+) plasmid[9]. The retroviral vector pLNCX was generously provided by Dr SHEN Yong-Quan at Institute of Basic

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Medical Science, CAMS. Plasmid pSP72 and strain *Escherichia coli* DH5α [supE44 ΔlacU169 (φ80 lacZ ΔM15) hsd R17 recA1 endA1 gyrA96 thi-1 relA1] were provided by Institute of Microbiology, Chinese Academy of Sciences.

Recombinant DNA manipulation Standard molecular biological techniques were used to manipulate and analyze nucleic acids[10]. Plasmid DNAs were prepared by the alkaline lysis method and purified by CsCl ultracentrifugation. DNA was digested with restriction endonucleases as recommended by the manufacturer. Restriction fragments were analyzed by electrophoresis on 0.8 % agarose gels and purified by GlassMax DNA isolation system according to the manufacturer's instruction. T_4 DNA ligase was added to the mixture for ligation at 16 ℃ for overnight. The reaction products were transformed into competent *E coli* DH 5α cell which was previously prepared with $CaCl_2$. The transforming mixture was selected on LB agar plates with ampicillin 100 $mg \cdot L^{-1}$. The transformants were screened by plasmid minipreparation. All recombinant clones were further identified by extensive restriction mapping and DNA amplification.

PCR amplification The oligonucleotides corresponding to the bases 1824-2275 of published DNA sequence of iNOS[9] were designed and synthesized at Institute of Microbiology, Chinese Academy of Sciences. A pair of iNOS-specific DNA primers were 5′ DNA primer (upstream primer, P1), 5′ TACAGCTGAAGCACTAGCCAGGGAC, and 3′ DNA primer (return primer, P2), 5′ ATAACGTTGGAAGTGAAGCGT-TTCGG3′, which added a Sal Ⅰ site to the 5′-end and a Hind Ⅲ to the 3′-end. Reaction mixture included 50 $pmol \cdot L^{-1}$ of each primer, 20 ng of the plasmid DNA template, dNTPs 200 $\mu mol \cdot L^{-1}$, $MgCl_2$ 1.5 $mmol \cdot L^{-1}$, Taq polymerase buffer (Tris·Cl 10 $mmol \cdot L^{-1}$, pH 9.0, KCl 50 $mmol \cdot L^{-1}$, Triton X-100 0.1 %), and 0.5 unit Taq polymerase in 50 μL total volume, which was finally overlaid with 200 μL mineral oil. In addition, a positive control with template DNA of plasmid pKSiNOS and a negative control without template DNA were set up in each experiment. The mixtures were denatured at 94 ℃ for 5 min, followed by amplification for 30 cycles: denaturation at 94 ℃ for 1 min, annealing at 62 ℃ for 1 min, and extension at 72 ℃ for 1 min, then completed the extension at 72 ℃ for 10 min. Amplified product (10 μL) was analyzed by 1.2 % agarose gel electrophoresis, followed by ethidium bromide staining.

RESULTS

Construction and screening of intermediate vector pSPiNOS pLNCXiNOS, the retroviral vector for stable expression of iNOS gene in mammalian cells was constructed as follows. pKSiNOS was digested with Sac Ⅱ and Hind Ⅲ, and 3′ overhand of Sac Ⅱ site was removed with T_4 DNA polymerase. A 4165 bp blunt-ended Hind Ⅲ fragment that contained the full-length cDNA of macNOS was isolated, and inserted into Sma Ⅰ - Hind Ⅲ polylinker sites in intermediate plasmid pSP72. Forty-one colonies were screened by alkaline lysis, one positive clone obtained was designated pSPiNOS (Fig 1).

pSPiNOS was subjected to the digestion by restriction endonucleases, Hind Ⅲ, Hind Ⅲ + Cla Ⅰ, Not Ⅰ, BamH Ⅰ + Cla Ⅰ, EcoR Ⅰ, EcoR Ⅴ, Hinc Ⅱ, Hinc Ⅱ + Cla Ⅰ, respectively. For instance, two fragments of 2404 bp and 4186 bp were obtained after digestion with Hinc Ⅱ + Cla Ⅰ, the former was plasmid pSP72 fragment and the latter, the insert fragment. These results showed that the insert size and orientation of iNOS gene in pSPiNOS were the same as that of deduct from restriction map (Fig 2).

Using the iNOS-specific primers, a 451 bp product amplified by PCR in pSPiNOS was completely similar to that in positive control pKSiNOS, indicating the existence of iNOS gene in recombinant (Fig 3).

Construction and identification of recombinant iNOS retroviral vector The retroviral plasmid pLNCX was cut with Hind Ⅲ, and the cohesive end was filled in with klenow fragment to generate a blunt end. After a second digestion with Cla Ⅰ, a 6607 bp blunt-ended Cla Ⅰ vector fragment, which contained neomycin phosphotransferase (neo^r) gene, human cytomegalovirus (CMV) immediate early promoter, pBR322 origin and Ampr, was ligated with 4000 bp Hinc Ⅱ + Cla Ⅰ cDNA fragment from pSPiNOS in orientation-specific insertion to make pLNCXiNOS (Fig 4).

After the plasmids of 5 positive clones 3#, 9#, 14#, 20#, 22# were respectively extracted and verified by cleavage with EcoR Ⅰ or Kpn Ⅰ, they were found to be identical (Fig 5).

Only a 10607 bp fragment was yielded when pLNCXiNOS was cleaved by Cla Ⅰ. Three bands of 2206 bp, 3382 bp, and 5019 bp were derived when digested with Hinc Ⅱ + Xba Ⅰ, two of which (2206 bp and 3382 bp) were identical to those from plasmid pLNCX cut by Hinc Ⅱ + Xba Ⅰ, indicating that a 5019 bp band contains iNOS insert. To check for orientation and sequence integrity of the insert, the recombinant plasmid was digested by BamH Ⅰ + Cla Ⅰ to generate 3 fragments (1456 bp, 3356 bp, and

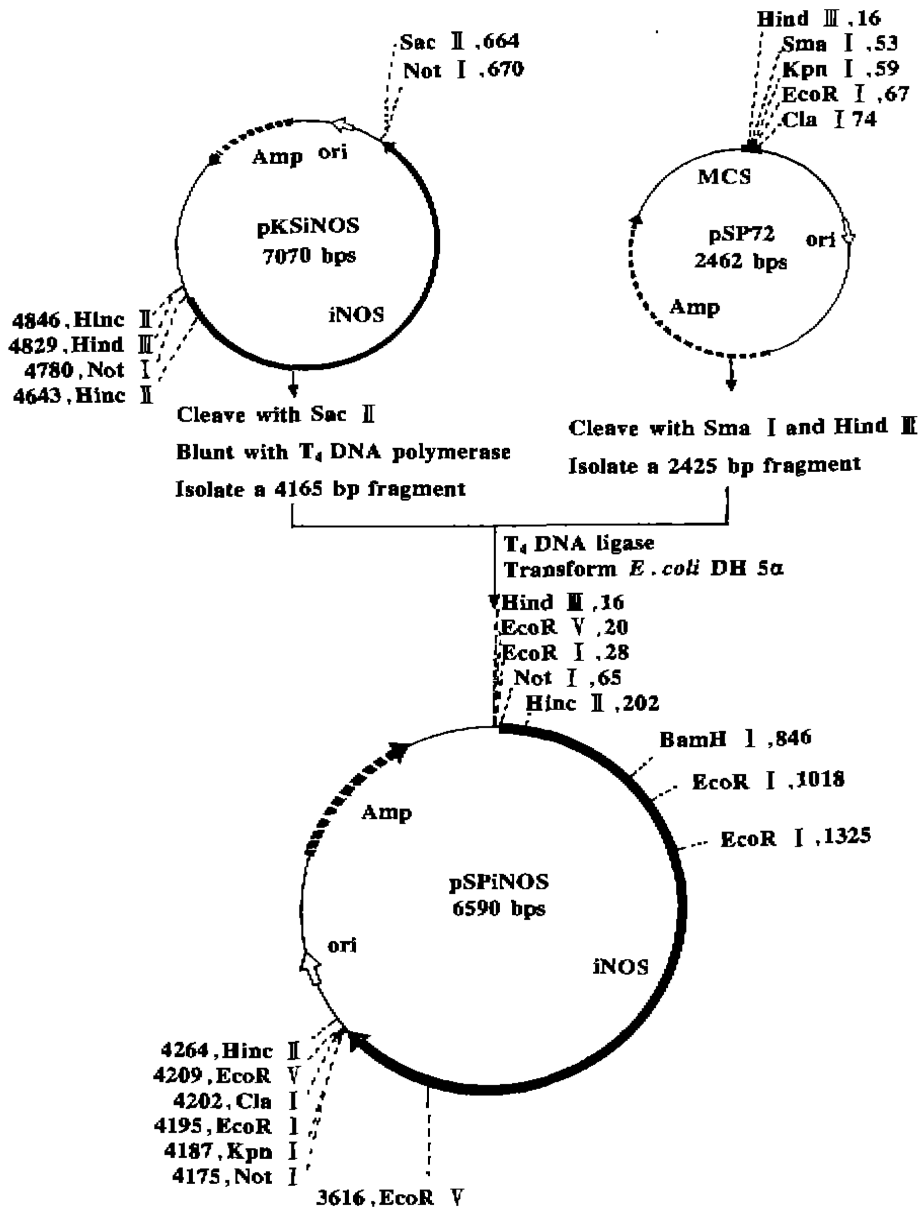

Fig 1. Strategy and partial restriction map for subcloning of iNOS gene into intermediate plasmid pSP72, Dark boxes, iNOS coding region; Stippled (shaded) boxes, Amp^r; Open boxes, the replication origin. MCS is multiple cloning site. Arrows show the direction of transcription. Numbers indicate nucleotide positions.

5795 bp), and cut by EcoR Ⅰ into DNA fragments of 307 bp, 1613 bp, 2811 bp, 2870 bp, and 3006 bp (Fig 6).

It was suggested that the plasmid contained iNOS gene in sense orientation and its restriction mapping was consistent with the sequence of prediction. Amplification with P1P2 primers resulted in an approximate 451 bp product (Fig 7), indicating that there was iNOS sequence in retroviral vector pLNCXiNOS.

DISCUSSION

The previous study showed that a cDNA sequence encoding murine macNOS from pKSiNOS required to be isolated by digestion with Hinc Ⅱ and Sac Ⅱ[9], while the retroviral vector pLNCX only has unique Hind Ⅲ, Hpa Ⅰ, and Cla Ⅰ restriction sites in multiple cloning site (MCS) and has no compatible termini with the iNOS DNA fragment[11]. Thus, it may be difficult to construct recombinant plasmid

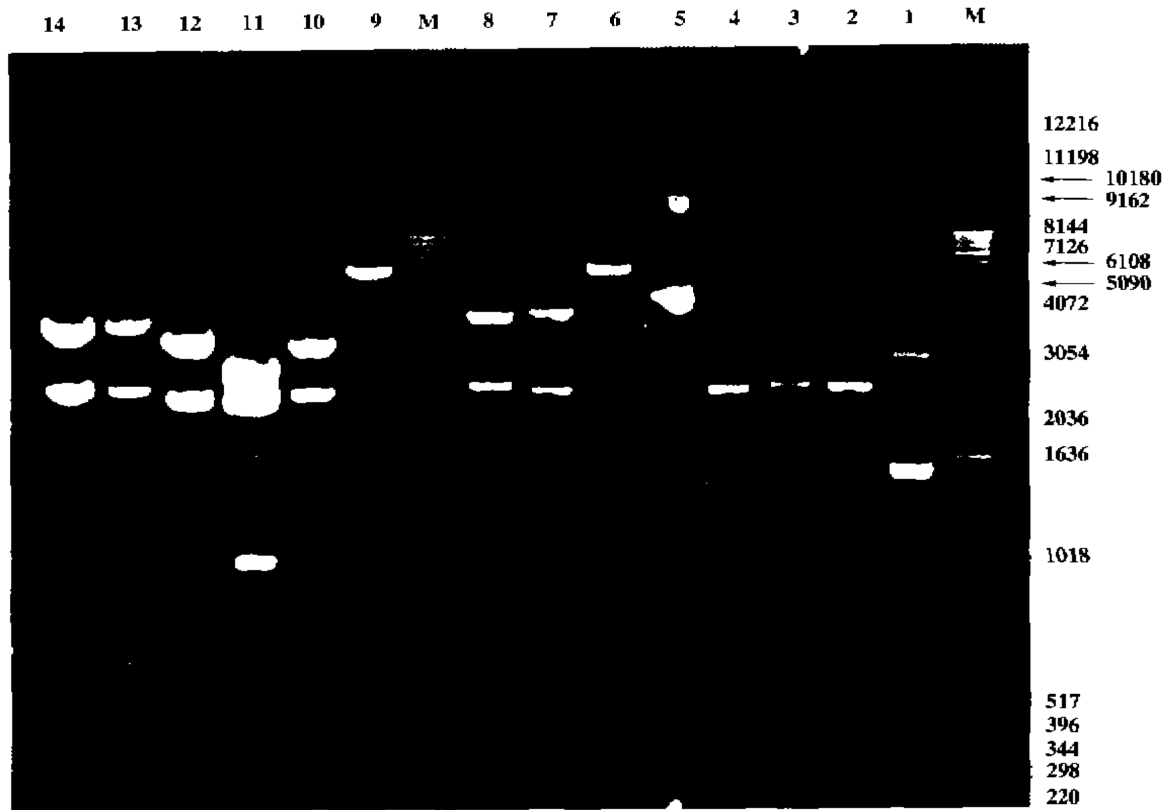

Fig 2. Restriction enzyme analysis of plasmid DNA. M: 1 kb DNA ladder; 1: pSP72 uncut; 2: pSP72/Cla Ⅰ; 3: pSP72/Hind Ⅲ; 4: pSP72/Hind Ⅲ + Cla Ⅰ; 5: pSPiNOS uncut; 6,9: pSPiNOS/Hind Ⅲ; 7: pSPiNOS/Hind Ⅲ + Cla Ⅰ; 8: pSPiNOS/Not Ⅰ; 10: pSPiNOS/BamH Ⅰ + Cla Ⅰ; 11: pSPiNOS/EcoR Ⅰ; 12: pSPiNOS/EcoR Ⅴ; 13: pSPiNOS/Hinc Ⅱ; 14: pSPiNOS/Hinc Ⅱ + Cla Ⅰ.

containing iNOS gene. In general, cloning strategies should modify the terminus of fragment by DNA-modifying enzyme. For example, 5′ protruding end is able to be filled in by klenow fragment and 3′ overhead is removed by T_4 DNA ligase[10]. However, the recombinant frequency for blunt-ended ligation appears to be very low. The background of non-recombinant clone such as tandem array of DNA insertion may be much high. An exogenous DNA is able to insert into plasmid in either sense or antisense orientation so that ligation directions of DNA fragment in positive cloning have to be identified. In the present study, to select suitable enzyme site, obtain sticky end and increase recombinant efficiency, we take advantage of plasmid pSP72 with various restriction sites in MCS, yielding an intermediate plasmid pSPiNOS. Further, cDNA sequence from macrophage iNOS was successfully subcloned to the plasmid pLNCX via ligation of cohesive-ended DNA to get a recombinant pLNCXiNOS. In addition, a 5′-untranslation region (UTR) structure that has G + C enrich domain to form hairpin loop could affect mRNA processing, localization, stability or translation efficiency. To express active iNOS at a high level, the 137 bp 5′-UTR of iNOS gene was deleted by the digestion with Hinc Ⅱ in pKSiNOS. Hence, the characteristic of pLNCXiNOS may contain 54 bp 5′-UTR, 487 bp 3′-UTR sequence, and the longest open-reading frame (ORF) that consists of 3432 bp, bases 192 – 3623, and encodes 1144 amino acids. The flanking sequences G or A(– 3) and C(+ 4) of an in-frame ATG transcription initiation codon in this plasmid are consistent with a Kozak's consensus sequence for an eukaryotic transcription initiation site[12].

The replication defective retroviral vector pLNCX belongs to a kind of vector with internal promoters (VIP), which is named for its order of genetic elements: L, long terminal repeat (LTR); N, neo

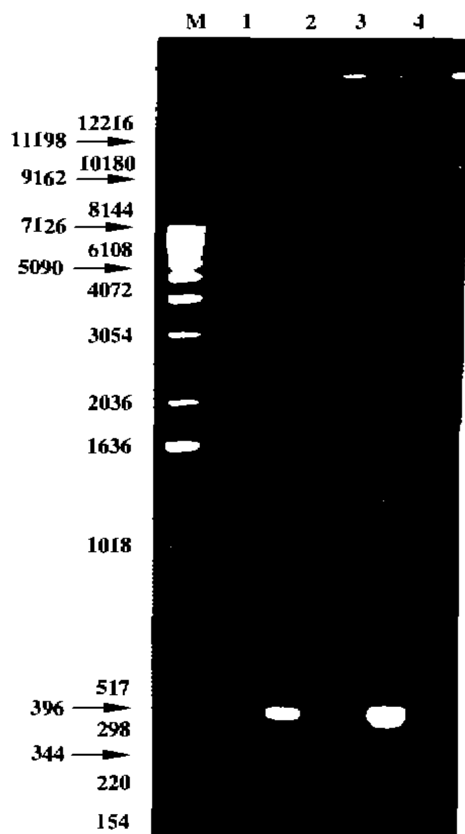

Fig 3. Analysis of PCR amplified fragment (1.2 % agarose). M: 1 kb DNA ladder; 1: negative control; 2: pKSiNOS; 3: pSP72; 4: pSPiNOS.

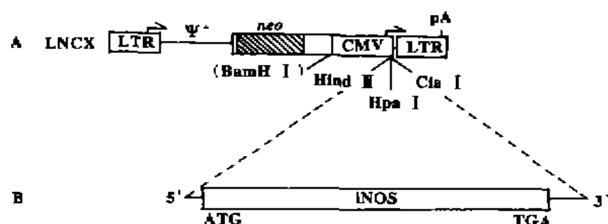

Fig 4. Construction of retroviral plasmid pLNCXiNOS. A. Retrovial vector pLNCX shows to contain LTR, CMV promoter, neo^r gene, retroviral packaging signal (Ψ$^+$) and polyadenylation signal (pA), and restriction sites for cDNA insertion. B. Structure of cDNA sequence in iNOS. Fine lines represent the 5′-UTR (137 bp-191 bp) and 3′-UTR (3624 bp-4110 bp). Open box represents iNOS coding region; Position of translation start codon (ATG) and in-fame stop codon (TGA) are indicated.

resistance gene (neo^r); C, CMV promoter; and X, unique cloning site for inserted cDNAs[11]. In this report, internal promoters in recombinant vector pLNCXiNOS consist of the LTR promoter driving expression of selectable marker neo^r gene and the strong CMV promoter driving transcription of iNOS cDNA. Besides, the vector also contains polyadenylation signals (poly A) and the extended retroviral packaging signal (Ψ$^+$). It may be used to transfer the iNOS gene into mammalian cells and take advantage of its strong promoter to express iNOS at a high level, which will provide a tool to study iNOS structure, function and regulation.

Biochemical studies of iNOS have initially relied on protein purification from IFN-γ and LPS-stimulated macrophages, which may produce low amounts of protein. To screen a selective iNOS inhibitor, sufficient enzyme isolated from activated macrophages has remained prohibitively expensive. Lowenstein *et al* subsequently cloned and transiently expressed macNOS in kidney 293 cells[9]. Recently overexpression of functional macNOS has been achieved in both *E coli* and baculovirus-insect cell system, and the resultant recombinant iNOS appears to be pharmacologically indistinguishable from the native enzyme[13-15]. The retrovial recombinant with iNOS coding region has been made. We have presently been transfecting into neuroblastoma × glioma NG108-15 cells with pLNCXiNOS, in which a significant level of NOS activity was measured by conversion of L-[^{3}H] Arg to L-[^{3}H] Cit (data to be published). These iNOS-expressing cells, which permit the relatively inexpensive generation of active iNOS for studying the pharmacological and biochemical properties of enzyme and screening novel iNOS inhibitors, may be used to determine whether NO effects on opioid tolerance and dependence.

REFERENCES

1 Xu W, Charles IG, Liu L, Moncada S, Emson P. Molecular cloning and structural organization of the human inducible nitric oxide synthase gene (NOS2). Biochem Biophys Res Commun 1996; 219: 784-8.

2 Adachi H, Iida S, Oguchi S, Ohshima H, Suzuki H, Nagasaki K, *et al*. Molecular cloning of a cDNA encoding an inducible calmodulin-dependent nitric-oxide synthase from rat liver and its expression in COS1 cells. Eur J Biochem 1993; 217: 37-43.

3 Zang MW, Fang F, Liu JS. NO-cGMP signal transduction system. Basic Med Sci Clin 1996; 16: 408-19.

4 Wang Z, Bilsky EJ, Porreca F, Sadee W Constitutive μ opioid

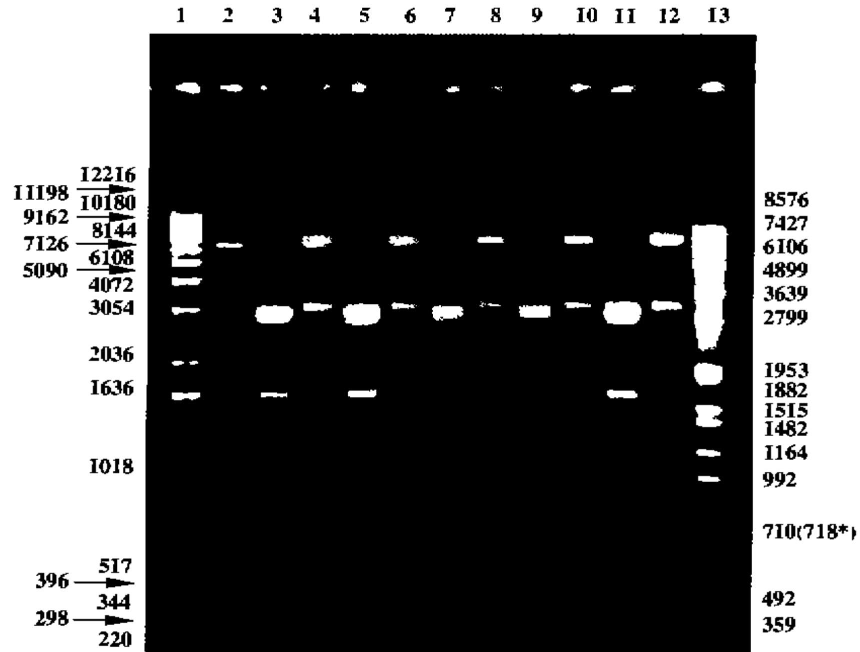

Fig 5. Identification of recombinant plasmid with restriction endonucleases. 1: 1kb DNA ladder; 2: pLNCX digested with Hind Ⅲ; 3,5,7,9,11: pLNCXiNOS 3#, pLNCXiNOS 9#, pLNCXiNOS 14#, pLNCXiNOS 20#, and pLNCXiNOS 22# digested with EcoR Ⅰ, respectively. 4,6,8,10,12: pLNCXiNOS 3#, pLNCXiNOS 9#, pLNCXiNOS 14#, pLNCXiNOS 20#, and pLNCXiNOS 22# digested with Kpn Ⅰ, respectively. 13: SPP1 DNA/EcoR Ⅰ.

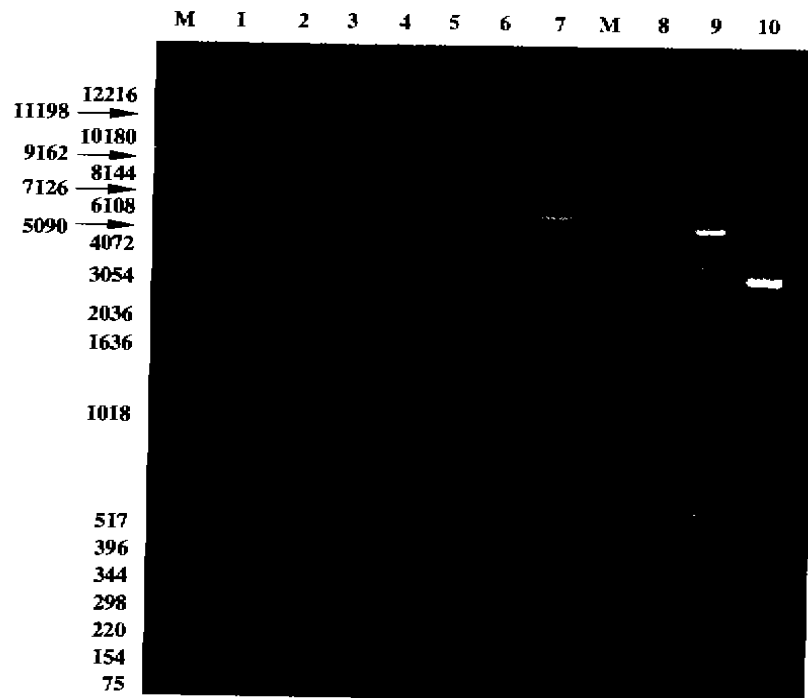

Fig 6. Restriction mapping analysis of plasmid pLNCX and pLNCXiNOS. M: 1 kb DNA ladder; 1: pLNCX uncut; 2: pLNCX/Cla Ⅰ; 3: pLNCX/Hinc Ⅱ + Xba Ⅰ; 4: pLNCXiNOS uncut; 5,8: pLNCXiNOS/Cla Ⅰ; 6: pLNCXiNOS/Hinc Ⅱ + Xba Ⅰ; 7: pLNCXiNOS/Kpn Ⅰ; 9: pLNCXiNOS/BamH + Cla Ⅰ; 10: pLNCXiNOS/EcoR Ⅰ.

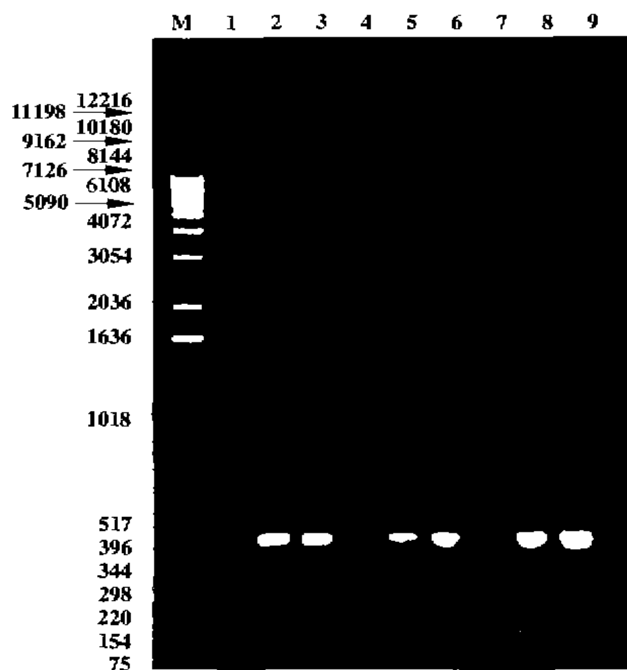

Fig 7. Agarose gel electrophoresis of PCR products amplified from plasmid DNA with iNOS-specfic primer (P1P2). M: 1 kb DNA ladder; 1: Negative control without DNA template; 2: Positive control with pKSiNOS; 3: pSPiNOS; 4: pLNCX; 5: pLNCXiNOS 3#; 6: pLNCXiNOS 9#; 7: pLNCXiNOS 14#; 8: pLNCXiNOS 20#; 9: pLNCXiNOS 22#.

receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci 1994; 54: PL339 – 50.

5 Elliott K, Minami N, Kolesnikov YA, Pasternak GW, Inturrisi CE. The NMDA receptor antagonists, LY274614 and MK801, and the nitric oxide synthase inhibitor, N^G-nitro-*L*-arginine, attenuate analgesic tolerance to the mu-opioid morphine but not to kappa-opioids. Pain 1994; 56: 69 – 75.

6 Cappendijk SLT, de Vries R, Dzoljic MR. Inhibitory effect of nitric oxide (NO) synthase inhibitors on naloxone-precipitated withdrawal syndrome in morphine-dependent mice. Neurosci Lett 1993; 162: 97 – 100.

7 Babey AM, Kolesnikov Y, Cheng J, Inturrisi CE, Trifilletti RR, Pasternak GW. Nitric oxide and opioid tolerance. Neuropharmacology 1994; 33: 1463 – 70.

8 Liu ZQ, Qiang WN, Zhang F, Zang MW, Liu JS. The expression of rat brain constitutive nitric oxide synthase in NG108-15 cell. Acta Acad Med Sin 1997; 19: 11 – 7.

9 Lowenstein CJ, Glatt CS, Bredt DS, Snyder SH. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. Proc Natl Acad Sci USA 1992; 89: 6711 – 5.

10 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

11 Miller AD, Miller DG, Garcia JV, Lynch CM. Use of retroviral vectors for gene transfer and expression. Methods Enzymol 1993; 217: 581 – 99.

12 Kozak M. An analysis of 5-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987; 15: 8125 – 48.

13 Wu C, Zhang J, Abu-Soud H, Ghosh DK, Stuehr DJ. High-level expression of mouse inducible nitric oxide synthase in *Escherichia coli* requires coexpression with calmodulin. Biochem Biophys Res Commun 1996; 222: 439 – 44.

14 Fossetta JD, Niu XD, Lunn CA, Zavodny PJ, Narula SK, Lundell D. Expression of human inducible nitric oxide synthase in *Escherichia coli*. FEBS Lett 1996; 379: 135 – 8

15 Moss DW, Wei X, Liew FY, Moncada S, Charles IG. Enzymatic characterization of recombinant murine inducible nitric oxide synthase. Eur J Pharmacol 1995; 289: 41 – 8.

逆转录病毒介导诱导型一氧化氮合酶基因转移载体的构建[1]

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关键词 一氧化氮合酶；互补 DNA；基因表达调控；真核细胞；重组 DNA；逆转录病毒；遗传载体；限制性酶切图谱；聚合酶链式反应

目的：构建逆转录病毒介导的诱导型一氧化氮合酶(iNOS)基因转移载体. **方法**：应用 DNA 重组和 PCR 扩增技术. **结果**：通过二步克隆，从质粒 pKSiNOS 分离编码巨噬细胞 iNOS 的全长 cDNA，亚克隆于中间载体 pSP72，调整插入片段二端酶切位点；进而构建含 iNOS 基因、巨细胞病毒启动子和 neo 抗性基因的逆转录病毒载体 pLNCXiNOS. 限制性酶切分析和 PCR 鉴定证实，重组体 iNOS 插入片段的大小和方向正确. **结论**：获得含 iNOS 基因逆转录病毒表达载体，为建立 iNOS 基因修饰的神经细胞模型，研究 NO/NOS 在阿片耐受依赖中的作用奠定基础.