肽核酸对基因调节作用的研究进展

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摘 要 肽核酸(PNA)是一类人工合成的核酸类似物，PNA 与核酸链以 Waston-Crick 碱基配对方式稳定互补结合，具有高度的亲合性、稳定性、特异性特征，PNA 能调节基因的复制、转录(或逆转录)和翻译过程，有着广泛的分子生物学效应，显示出其作为基因调节药物的应用潜力。

关键词 肽核酸，基因表达，调节
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1991 年 Nielsen 和 Egholm[1] 等人通过创造性的设计合成了核酸模拟物——肽核酸(Peptide nucleic acid，PNA)。肽核酸是以酰胺键连接而成的肽骨架替代核酸中核糖磷酸二酯键骨架构成的核酸类似物，其结构是由重复的 N-(2-氨基乙基)甘氨酸为单元通过 α-N 酰甲基与碱基相连。PNA 保留了 DNA 或 RNA 分子的碱基，碱基与骨架间隔 3 个共价键，相邻碱基间隔 6 个共价键，碱基对与螺旋轴垂直，空间大小与天然核酸相近。因此 PNA 与核酸可通过 Watson—Crick 碱基互补形式形成双螺旋的稳定复合物，或以 Hoogsteen 或反 Hoogsteen 氢键结合到有特定序列的双链大沟处形成三股螺旋结构的稳定复合物[2]，使 PNA 具有对核酸特异的识别能力与结合能力，特别对基因的表达具有较强的调节作用，被广泛应用于分子生物学和医学的研究之中。本文就近年来肽核酸对基因调节方面的研究进展作一综述。

1 PNA 与靶基因结合的特征

1.1 PNA 与靶基因结合具高度稳定性

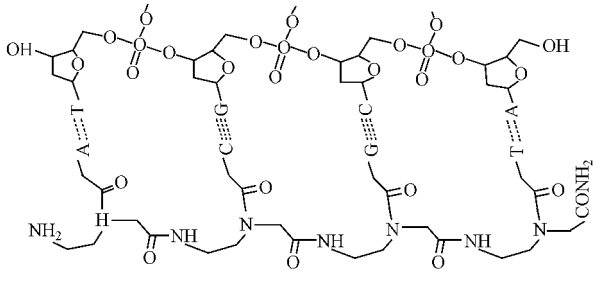

图 1 肽核酸和 DNA 的化学结构
Fig.1 The structures of PNA and DNA

从上图 PNA 分子结构分析说明 PNA 具有电中性、非手性等属性，分子中三级酰胺旋转能阈与肽键相近，在溶液中呈单一构象，碱基堆积力是维持构象的主要因素，因此 PNA 在与核酸杂交时避免了静电斥力，使形成的复合物更加稳定[3]。Egholm M[2] 把同样碱基序列的 PNA-DNA 与 DNA-DNA 双链相比，双链每增加一个碱基，Tm 将会提高 1°C(在 100mmol/LNaCl 中 15-mer PNA-DNA，Tm 69°C，15-mer DNA-DNA，Tm 54°C)，这种稳定性增加是由于 PNA-DNA 的中性骨架之间排斥力小及空间结构易于配对所致，进一步研究表明，PNA-RNA 杂交的 Tm 值较 PNA-DNA 杂交值高，而且与相同序列 PNA-DNA 相比 PNA-PNA 双链结合速率更快，解离速率相对较慢，因此 PNA 与 DNA、RNA、PNA 杂交稳定性依次为：PNA-PNA > PNA-RNA > PNA-DNA > DNA-DNA。

PNA-DNA 杂交结合强度不过分依赖于盐浓度，在低浓度盐离子强度与没有 Mg^{2+} 条件下，PNA 都能与序列 DNA 分子杂交，其解链温度较高，而同样条件下 DNA-DNA 杂交分子的稳定性较差，它受介质盐浓度的影响很大。

PNA 常与靶基因形成三股螺旋结构发挥作用。若靶基因为同聚嘌呤序列，则含纯嘧啶碱基的 PNAs 与双链 DNA 中同聚嘌呤序列互补，形成极为稳定的 $(PNAs)_2$/DNA 三链分子[4]，亦可同互补的 RNA 链形成高热稳定性的 $(PNAs)_2$/RNA 三链分子(Tm > 70°)，进一步研究发现：PNA 是以平行或反平行的双螺旋方式与核酸结合，PNA 与 DNA(或 RNA)形成的复合物倾向于反平行结合，而形成的三螺旋倾向于平行结合，且两种结合方式都得到非常稳定的复合物[5]。

1.2 PNA 与靶基因结合的高度序列特异性

PNA 与互补的 DNA 或 RNA 杂交时显出很强的特异性，这表现在 PNA-DNA 碱基对错配杂交分子的不稳定性比 DNA-DNA 碱基错配杂交分子的不稳定性更高。实验证实：在 15-mer PNA-DNA 双链中出现一个错误的配对时 Tm 将降低约 8 ~ 20℃(平均 15℃)，而在相应的 DNA-DNA 双链中类

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似的错误配对出现时,Tm 只降低 4 ~ 16℃(平均 11℃),PNA 与靶 DNA 之间出现一个碱基错配时,它们之间的结合效率就会明显下降,出现两个碱基错配,PNA 与靶 DNA 则完全不能杂交,因此利用 PNA-DNA 复合物的高度稳定性和序列选择性可检测 DNA 在 PCR 扩增时单碱基对的变异(在 PCR 反应体系中,加入与 DNA 模板中间重复序列互补的 PNA 片段与 DNA 模板退火杂交,从而封闭已变性的 DNA 模板中的重复序列)。

当 PNA 靶序列与引物位点重叠时,引物和 PNA 竞争引物结合位点,因为 PNA 不具备引物对 DNA 聚合酶的功能,所以 PNA 与引物位点的结合导致扩增减少,当 PNA 靶序列与引物位点相邻或相间时,PNA 的结合使得 DNA 聚合酶的延伸受阻,DNA 增殖减少,因此在 PCR 反应体系中,PNA 与引物或模板互补结合后,可导致 PCR 终止,但与一个碱基错配的模板结合后,在合适的温度范围中,由于此复合体热稳定性下降,PNA 则不能干扰 PCR 的进行,使该模板得以大量复制,或者 PCR 反应液中加入 PNA 后增殖不受影响,表明得到的 DNA 中存在突变碱基[6],实验中利用 PNA 与核酸结合的高度特异性,将 PNA 作为探针广泛采用。

1.3　PNA 与靶基因结合的高度亲和性

PNA 是由特殊的多酰胺骨架与碱基通过亚甲羰酰键连接其骨架上的,因此此分子结构不被核酸酶和蛋白酶识别、结合,能抗酶的降解[7],在人血清、细菌提取物、埃氏腹水癌细胞核细胞质抽提物中均无明显降解。它抵抗真菌蛋白酶 K,猪小肠黏膜蛋白酶分解的能力是对照肽类的 1000 和 30 倍,并且在一个相当大 pH 值范围内 PNA 都是稳定的[8],因此无论在体内或体外 PNA 都能长期存在。PNA 与 DNA 的结合导致限制性内切酶所需的特征结构发生改变,从而序列专一性地抑制内切酶对双链的切割。利用 PNA 的这种性质实验中,当富含胸腺嘧啶的纯嘧啶 PNA 通过链取代机制与 ds-DNA 杂交,使被取代的 DNA 链有单链性质,能被单链核酸酶 S1 降解,尤其当 dsDNA 上有两个相连相邻的 PNA 靶序列时,利用靶序列互补的 PNA 片段,就可使核酸酶 S1 选择性地断裂,起到限制性内切酶的作用。当同性嘧啶 PNA 寡聚体连接在一起形成所谓 PNA 夹子时,链侵染的过程能够识别双股 DNA 中短于 7 个碱基长度的同性嘌呤序列,PNA 夹子结合到双链 DNA 上,就会阻止酶的作用。Veselkov 等[9]亦使用此原理在很专一的位点上酶切了酵母菌基因组,实验中当 PNA 寡聚体连接到固定相上时,它亲和捕获碱基效果很好,Boffa[10]等曾用这种方法获得含有 CGA 重复序列的活性转录基因。

2　PNA 对基因表达的调节作用

2.1　PNA 对转录过程的调节作用

由于 PNA 与靶基因结合具高度亲合性、专一性及稳定性,PNA 反基因作用的靶是通过与每个基因组成 PNA-DNA 二聚体,而影响基因的转录、调节基因表达。有实验表明[10]:PNA 分子与含有其靶序列的人工构建质粒模板在低盐条件下预处理后再置于转录缓冲液中,加入多种 RNA 聚合酶如人 pol Ⅱ、T_3、T_4 噬菌体 RNA 聚合酶,PNA 可以抑制作用位点在 IL2-Ra 增强子上的转录调控因子 NF-κB 的转录活性,并且 PNA 结合时间先后秩序决定了 NF-κB 活性差异,*E. coli* RNA 聚合酶体系中都有效地抑制转录产物的生成。Vickers[11]研究还发现 PNA 对转录的抑制作用与它同 dsDNA 结合位置有关,若结合在转录链时,PNA 抑制作用可以达到 100% 结合在非转录链上抑制作用则会大大下降,最多不超过 50%。另外 PNA 的大小和靶序列碱基构成可以影响转录抑制率,10-mer PNA 可以抑制 PNA 转录,6-mer、8-mer PNA 的抑制效果要差些,靶序列中 G 含量增加会降低 PNA 转录抑制效果,因此,PNA 的转录抑制作用是可通过抑制聚合酶或转录因子与模板的结合,或阻止链的延伸来实现。

研究发现[12] T_3、T_4 噬菌体聚合酶转录体系中转录启动时,瞬时的'转录泡'(Transcription bubble)使 RNA 与模板的结合更易于发生,进而抑制了转录的进一步进行,这样在一定条件下 PNA 可以在生理盐浓度下发挥转录调节作用。从理论上讲,基因转录亦是一个生物信号放大的过程,少量 PNA 若与 DNA 结合可抑制转录进行,而 PNA 若与 RNA 结合时,大量 PNA 必须与细胞中大量的 mRNA 结合,才可抑制翻译,因此以 DNA 为靶点的反基因研究更具有应用潜力,可能通过抑制基因转录而阻止肿瘤细胞中原癌基因的表达。

PNA 除对转录具有负的调节作用外,也可对转录进行正调节[13],在转录体系中,RNA 聚合酶能利用 PNA-DNA 结合作用产生的单链 DNA,在 PNA 链取代反应发生的位点为起点转录生成分子,而且 PNA 介导的转录强度与 LacUV *E. coli* 启动子起始的转录强度相当。

总之,PNA 对转录的调节具有双向性,分别实现对转录的序列专一的终止或启动需要不同的特定条件。一般只需单个 PNA 分子和处于模板链上的较短靶序列的相互结合即可实现对转录的抑制,而转录的启动需较大的或多个 PNA 靶序列位于两条 DNA 链上,因此可以推断利用 PNA 控制不同条件,进行序列专一性转录的人工正负调节。

2.2　PNA 对翻译过程的调节作用

PNA 对翻译过程主要起反义调节作用,即 PNA 与 mRNA 结合形成三螺旋结构,从而阻碍 80S 核糖体与 mRNA 结合或形成空间位阻防碍核糖体在链上的移动,而不被 RNase H 酶降解。有许多实验对 PNA 的反义性质作研究,Hanvey[13]等将 PNA($HT_3ACT_2NH_2$)在兔网状细胞溶解物中与含互补序列 mRNA 孵育,对翻译产物的分析结果表明,随反应液中 PNA 浓度的增加,完整翻译产物 36kD 蛋白的生成减少,终止于结合位点的部分翻译产物 22kD 蛋白的生成增加,因此 PNA 与 mRNA 的靶位点结合阻抑了翻译的进行。采用微注射法将 PNA 直接注射到培养的细胞核中,定靶于 SV40 抗原 mRNA 的 5′末端非翻译区,15-mer 同聚嘧啶 PNA 可以抑制 99% 的 T 抗原在 CV-1 细胞中表达,而且三链形式的$(PNA)_2$-RNA 同双链形式的 PNA-RNA 一样具有反义调节作用,都不依赖于 RNase H 而发挥作用。比较 PNA 和寡核苷酸的反义作用发

现,结合于 mRNA 起始密码子(AUG)附近的寡核苷酸,即使在翻译反应中加入 *E. coli* 的 RNase H,反义寡核苷酸也不能抑制翻译的进行,表明 PNA 比普通寡核苷酸的翻译调节作用强。同时还发现,当进入胞内的 PNA 浓度低于 5μmol/L 时,反义作用的效果除了呈剂量依赖性和靶向特异性外,与 PNA 的长度也有关系,在 PNA 的浓度固定为 1μmol/L 时,10-mer、15-mer 和 20-merPNA 进入细胞核后,蛋白质的翻译相应受到了 0%、40%和 50%的抑制,表明 PNA 的长度越长,反义作用越强。

反义 PNA 不仅可以抑制病毒的翻译,也可以阻碍细菌中蛋白的生成。针对核糖体 RNA 功能和结合位点的反义 PNA 可以抑制大肠杆菌的翻译。以上研究说明 PNA 无论在体内或体外,其抑制作用不仅有剂量依赖性而且有基因特异性,PNA 对翻译的抑制在起始密码子区可能通过形成三螺旋阻碍 80S 核糖体与 mRNA 结合,而在编码区 PNA 与 mRNA 结合的复合物可通过阻碍核糖体在 RNA 链上移动。

2.3 PNA 抑制逆转录调节基因的表达

研究发现 PNA 能够抑制 mRNA 逆转录成 cDNA,选择适当的 PNA 浓度就可专一性抑制逆转录活性,而不影响翻译[14]。Koppelhus[15] 在研究体外 PNA 对 HIV-1gag 基因逆转录的作用时,设计了与 RNA 的纯嘧啶序列 AAAGAAAA 平行互补的 PNA、反平行互补 PNA 和将两种 PNA 连接起来的 bis-PNA,并研究分别加入不同浓度的 PNA 对逆转录的调节(见表 1)。而相同条件下与相同序列硫代寡核苷酸却不能抑制逆转录反应,由此得出在一定浓度范围内 PNA 能选择性地抑制逆转录而不影响翻译。如果选择合适的 PNA 浓度,就能做到专一性抑制逆转录活性,而无其他毒性,并推断 PNA 对逆转录的调节阻断是 PNA 空间阻碍效应的结果。

表 1 PNA/RNA 不同浓度对逆转录的调节

Table 1 Regulation of reverse transcription in different concentration of PNA/RNA

PNA/RNA 浓度		逆转录情况	逆转录产物
60	互补平行 PNA	完全阻断	无产物
	反平行 PNA	完全阻断	无产物
	bis-PNA	完全阻断	无产物
6	互补平行 PNA	部分阻断	部分及完整产物
	反平行 PNA	部分阻断	部分及完整产物
	bis-PNA	完全阻断	部分产物
0.6	互补平行 PNA	近乎完全进行	完整产物
	反平行 PNA	近乎完全进行	完整产物
	bis-PNA	部分阻断	部分产物

此外,研究发现 PNA 能通过特异性识别人端粒酶的 mRNA而抑制端粒酶的活性,Norton[16] 针对人端粒酶设计了一组与其互补的 PNA,PNA 对端粒酶活性的抑制作用有高度的序列选择性,而硫代寡核苷酸则以非序列选择性方式抑制端粒酶活性,因此 PNA 是一种很有前途的抗肿瘤端粒酶活性的药物。

3 结束语

综上所述,由于 PNA 具有良好的生物稳定性及对核酸靶序列的高度亲和性和专一性,因此 PNA 显示了对基因表达有效的调节作用,目前已成为分子生物学研究的有力工具[17]。但由于 PNA 是人工合成的核酸类似物的大分子,它不易穿过细胞膜和血脑屏障,制约了它的实际应用。最近国内外研究表明,将 PNA 与不同的载体偶联能改善细胞对 PNA 的摄入状况,特别是 PNA 与某些多肽序列结合后直接运送 PNA 穿过细胞膜,进入细胞后很快与多肽脱开,不影响 PNA 与细胞质中 mRNA 结合,或者进入细胞核内与 DNA 相互作用,这对 PNA 在生理条件下调节基因的表达、PNA 的应用有重要意义。目前把 PNA 作为基因调节药物用于基因水平治疗肿瘤、爱滋病、肺结核等[18~20] 已成为生物医药界研究的热点,具有广阔的应用前景。因此预计随着人们对 PNA 在基因表达调节方面研究的进一步深入,它在生命科学中的作用将会更加重要。

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The Development of Peptide Nucleic Acid in Gene Regulation

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Abstract　Peptide nucleic acid(PNA) is a kind of artificial DNA mimic. PNA hybridizes with DNA or RNA by means of Watson-Crick's base- pairs complementary with high stability, affinity and selectivity. PNA not only regulates DNA replication, but also adjusts DNA transcription (or reverse transcription) and translation. Many applications have been explored as a new kind of molecular biological tool and a gene-targeting strategy.

Key words　peptide nucleic acid (PNA), gene expression, regulation

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安捷伦化学分析部更名为生命科学与化学分析部
——Chris van Ingen 访谈

2002 年 2 月 5 日,安捷伦化学分析部(CAG)宣布更名为生命科学与化学分析部(LSCA)。为此,我们采访了安捷伦生命科学与化学分析部(LSCA)的高级副总裁兼总经理 Chris van Ingen 先生,就该部门的名称变动及其为生命科学领域带来的影响展开了讨论。

问:您宣布化学分析部更名为生命科学与化学分析部(LSCA)。更名的原因都有哪些呢?

答:我们认为,以前的名称'化学分析部'已经成为安捷伦在生命科学市场中扩大品牌无形资产价值的障碍,生命科学客户把我们看成一家传统的化学分析仪器公司。我们需要有新的名称,以反映安捷伦提供越来越多的生命科学产品和服务,同时巩固我们在化学分析市场中的领先地位。

问:安捷伦参与开发生命科学解决方案已经有多长时间了?

答:多年来,安捷伦一直以各种形式为'生命科学'提供仪器解决方案。由于我们的重点是基因表型性状和蛋白质研究,所以我们需要定位成一家'生命科学'公司。生命科学也是安捷伦的战略增长计划之一。

问:您个人对公司在未来发展方面有什么看法?

答:从个人来看,我对这一业务很乐观,同时我认为,我们运营的市场在营业收入和市场份额方面都会有长期的可盈利增长。基因表型性状、蛋白质结构和药物发现和开发是安捷伦公司服务的三大市场。这一技术的潜力是惊人的,将对人们的生活产生巨大影响。这是一种原动力,一种令人激动的动力,当然也是'梦想成真'的根源。

问:目前 LSAC 为生命科学市场提供哪些产品和服务?这些产品和服务具有哪些特殊功能?

答:我们为制药、生物技术和科研人员提供的主要解决方案集中在四大平台上,即 DNA 微阵列、微型应用流体技术、液体色谱技术、质谱测量技术以及相关的用品、服务和生物信息学(软件系统)。每个平台拥有大量的专门解决方案,以满足广大客户的特殊需求。

与生命科学公司建立合作关系是一种行之有效的方法,我们可以随时跟踪生命科学的最新进展,保证安捷伦提供的产品和服务能够满足现在和未来的各种需求。

问:未来几年内,我们可望看到哪些生命科学技术?

答:安捷伦最近宣布其在研究新技术方面的两大重要科研合作。一个是安捷伦实验室正在与哈佛大学合作,开发突破性的纳米微孔技术,以更快地分析作为所有生命组织蓝图的 DNA 和其它核酸;另一个是安捷伦实验室与科罗拉多大学和美国国防部高级研究计划署进行合作,为核酸的化学合成开发突破性的两步工艺。在不久的将来,这一技术可以降低我们制造 DNA 微阵列的复杂性和成本。从长期来看,这两项新技术将会变革疾病特征描述和药物发明。

(熊一飞　供稿)

A Transgenic Mouse That Targets the Expression of Cre Recombinase in Pancreatic Tissue

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Abstract The transgenic mice that express Cre recombinase in a tissue specific manner is a powerful tool in generating the conditional gene knockout mice. The rat insulin promoter was cloned target the expression of Cre in pancreatic tissue. The Cre gene was modified by adding the nuclear localization signal and the sequence for initiation by eukaryotic ribosomes at 5′ terminal of the Cre gene. Cre gene was linked to the intron of human growth factor gene. This construct was introduced into the mouse eggs using microinjection. Seven mice were identified as founders carrying the Cre gene by PCR. The results of RT-PCR showed that the transgenic mouse from one founder could transcribe the foreign gene in pancreas. The Southern blot analysis indicated that the Cre recombinase expressed in pancreas of the transgenic mouse was functional.

Key words: transgenic mouse, cre recombinase, pancreas

The bacteriophage P1 Cre recombinase, which is a site-specific DNA recombinase, recognizes specific 34bp sequences called LoxP sites and catalyses recombination between two LoxP sites[1]. Recombination can excise the intervening DNA segment between two same direction LoxP sites. Cre recombinase is a 38 kD protein and belongs to λ integrase superfamily. Since the first introduction of the Cre/LoxP system into the eukaryotic genome by Sauer in 1988 this excisive feature of the Cre recombinase has become a powerful tool for the conditional and tissue or cell-specific deletion of genes[2]. In 1994, Gu *et al*. established the first cell-specific gene knockout mice using the Cre/LoxP system[3].

The complete disruption of embryonic development related genes frequently resulted in embryonic lethality, which makes it impossible to obtain the adult animals. Tissue specific gene targeting can overcome the shortage of the conventional gene knockout technology. The conditional gene targeting mice (Floxed mice) could be obtained by inserting the LoxP sites into the introns flanking the most important functional domain of the target gene through homologous recombination, since the LoxP sites are not going to affect the transcription and translation of the target gene. Tissue specific gene knockout mice could be generated by crossing the conditional gene targeting mice with the tissue or cell specific Cre transgenic mice. The target gene could be disrupted through the Cre/LoxP dependent recombination in the specific tissues or cells. The target gene maintains functional in other tissues or cells in which Cre does not express[4].

Our previous researches show that Smads gene family can directly transduce the transforming growth factor-β (TGF-β) signals from the membrane to the nucleus and involve in pancreas cancer. Targeted disruption of murine Smad2, Smad4 and Smad5 gene resulted in embryonic lethal[5]. To further study the function of Smads in tumorigenesis of pancreas cancer, we have to generated pancreatic tissue specific gene knockout mice. The establishment of the transgenic mouse that targets the expression of Cre recombinase in pancreatic tissue will lay a solid foundation for producing the pancreatic tissue specific gene knockout mice[6].

1 MATERIALS AND METHODS

1.1 Materials

The pIC-Cre covering the Cre gene was kept in our lab[7]. The pBS-hGH covering the human growth hormone (hGH) gene was kindly provided by Dr. Mark A. Magnuson[8]. The LoxP reporter mouse was kept in our lab. Kunming White mice were prepared by the Center of experiment animals of the Academy of Military Medical Sciences.

Restriction endonucleases, T4 DNA liganse and Taq DNA

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polymerase were purchased from Promega and Sino-American Companies. The chemical reagents, PMSG (pregnant mare serum gondadotropin) amd hCH (human chorionic gondotropin) were purchased from Sigma Company.

M2 and M16 mediums were prepared according to reference[9].

1.2 PCR primers

The PCR primers were designed by us and synthesized by Institute of Biotechnology.

RIP primer P1 5′ AGGATCCCCCAACCACTCCAAGT 3′

RIP primer P2 5′ TTAGGGCTGGGGGTTACTGAATC 3′

Cre primer P1 5′ GCCTGCATTACCGGTCGATGC 3′

Cre primer P2 5′ CAGGGTGTTATAAGCAATCCC 3′

Cre primer PA 5′ TTGCCTGCATTACCGGTCGATGC 3′

Cre primer PB 5′ TTGCACGTTCACCGGCATCAACG 3′

1.3 The construction of vector

The manipulation of the expression vector was carried out as described in reference [10].

1.4 Embryo collection

4 weeks old Kunming white female mice were used for superovulation. Females were injected 10IU PMSG intraperitoneally at noon on day one and were injected 10IU hCG at noon on day three, then were crossed with fertile stud males. Vaginal plugs were checked before noon on day four. The pregnant mice were sacrificed by cervical dislocation and fertilized eggs are removed from the swollen ampullae of the Fallopian tubes by dissection. The embryos are freed from attached cumulus cells by brief incubation in the presence of hyaluronidase. Wash the isolated embryos four times with M2 medium. Transfer the embryos from the M2 medium into the M16 culture and keep the plate in the incubator (37℃, 5% CO_2).

1.5 Microinjection

Embryo injection and pipettes preparations were made as described. Typically 20 ~ 30 embryos were processed at a time. One by one, they were picked up by gentle suction onto a holding pipette and injected with the injection needle. DNA was injected into the pronucleus and successful injection was indicated by swelling of the pronucleus. Embryos that had been injected were returned to the incubator for 30 ~ 60min in M16 medium. Transfer the embryos from M16 to M2 medium before implantion. Load the embryos into the transfer pipette. Embryos were reimplanted into the oviduct of anaesthetized pseudopregnant females. Offspring were born after successful implantion.

1.6 PCR analysis for transgenic mice

The genomic DNA was prepared from mouse tail using standard method. PCR was carried out using corresponding Cre primers.

1.7 RNA preparation and RT-PCR

Total RNA was isolated from the pancreatic tissue of adult Cre mice using TRIZOL (GibcoBRL). One microgram of RNA was reverse transcribed using SUPERSCRIPT-RT from Gibco BRL and oligo(dT)-primers. An aliquot of 2 μL of the RT-reaction was used in a 50 μL PCR reaction.

1.8 Southern-blot analysis

The genomic DNA was prepared from all kinds of tissues and digested with *Eco*R V. Southern-blot analysis was carried out using appropriate probe.

2 RESULTS

2.1 The amplification of the rat insulin promoter by PCR

The rat insulin promoter (RIP) was amplified from rat genomic DNA using PCR. RIP primer P1 and RIP primer P2 were designed based on rat insulin gene 5′ transcription regulation sequence according to reference[11]. The 705bp PCR product was cloned to puc19 vector. The nucleotide sequence of the rat insulin promoter fragment was confirmed by sequence determination.

2.2 The modification of the 5′ terminal of Cre gene

Nucleotide sequences surrounding the translation initiation codon of the Cre gene were modified using a PCR method. An oligonucleotide (ACCATGGGCCCAAAGAAGAAGAGAA AGGTTTCGAATTTACTGACCGTACAC) was used as a 5′ primer for PCR amplification, by which the optimal sequence for initiation by eukaryotic ribosomes (ACCATGG) and the nuclear localization signal peptide (Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val, corresponding to the nucleotide GGC-CCA-AAG-AAG-AAG-AGA-AAG-GTT) was introduced into the N-terminus of the Cre enzyme[12,13]. The 3′ primer for PCR is GAAGATAATCGCGAACAT . PCR fragment was blunt-ended by Klenow fill-in and digested with *Nru*I, then the extracted fragment replaced the *Sal*I to *Nru*I fragment in the 5′ terminal of Cre gene. The modified Cre gene was confirmed by sequence analysis.

2.3 Generation of the insulin-Cre recombinase construction

The modified Cre gene was placed under the control of the rat insulin promotor and followed by the human growth hormone (hGH) intron and poly(A) signal (Fig.1). The resulting RIP-Cre-hGH transgene was released from the vector as a 4kb *Kpn*I-*Not*I fragment using a Promega DNA Clean-up gel extraction kit. The isolated fragment was diluted to 2 μg/mL in a modified TE buffer (10mol/L Tris, pH7.5, and 25mmol/L EDTA) for microinjection.

2.4 Generation of transgenic mice

Transgenic mice were produced using microinjection. The isolated RIP-Cre-hGH DNA fragment was microinjected into the pronuclei of mice embryos. The injected embryos were reimplanted into the pseudopregnant females. Offspring were born after successful implantion.

2.5 Identification of the Cre transgenic mice

Tail DNA from potential founder mice was screened by PCR using the Cre primer P1 and Cre primer P2, which amplify a 480bp fragment. Thermal cycling consisted of a 5 - min denaturation at 95 ℃

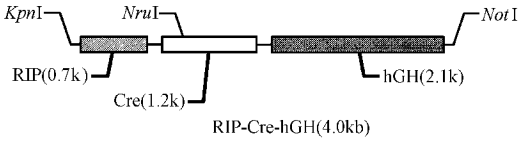

Fig.1 Structure of the RIP-Cre-hGH transgene
The rat insulin promoter is linked to the coding sequence of the Cre gene followed by an intron and polyadenylation site from the human growth hormone gene

Table 1 The results of microinjection

Selected Embryos	Injected Embryos	Transplanted Embryos	Receptor Mice	Birth Mice	Positive Mice
2000	700	600	24	27	7

followed by 30 cycles of 30 denaturation at 94℃, 15 annealing at 58℃, and 30 s extension at 72℃. Cre primer PA and Cre primer PB were used to check the positive results, which amplify a 368bp fragment at 68℃ for annealing (Fig. 2). It was demonstrated that 7 founder mice were obtained.

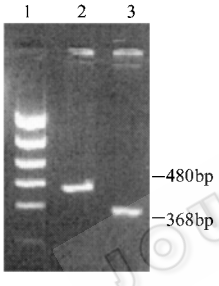

Fig.2 Identification the positive Cre founder mice using PCR
1. PCR Marker 2. PCR product using Cre primer P1 and P2 3. PCR product using Cre primer PA and PB

2.6 Reverse trancriptase-PCR assay of Cre-hGH mRNA

Total RNA was isolated from mice pancreatic tissue. cDNA synthesis was carried out using Promega reverse transcription system. After cDNA synthesis, PCR was carried out in 50μL reactions containing 5μL of 10X PCR mix, 1.5mmol/L $MgCl_2$, 40μmol/L dNTP, 2.5 units of Taq DNA polymerase, and 10 pmol of the primers 5′- GAAGCCTATATCCCAAAGGAA-3′ and 5′- ACTGGAGTGGCAACTTCCAG-3′, which amplify a fragment of 530 bp in one RIP-Cre-hGH transgenic mice. The other 6 positive mice didn't amplify the PCR product. Because the PCR primers are located in the third and fifth exon of hGH respectively, the unspliced mRNA should amplify a fragment of 880 bp (Fig.3). The advantage of having the primers in the hGH portion is that PCR product span a splice junction. In this way, the RT-PCR reactions only detect RNA, and not contaminating genomic DNA. The 530 bp PCR fragment indicated that this transgenic mouse transcribed the foreign DNA in pancreas and the pre-mRNA was spliced correctly.

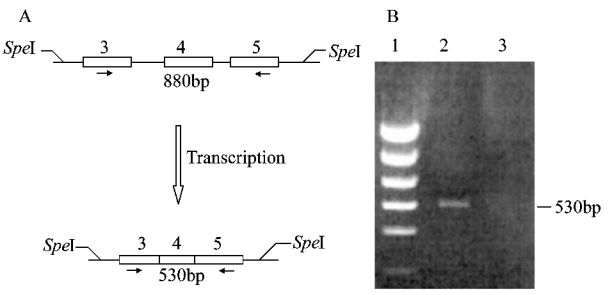

Fig.3 A: Structure of the hGH gene spliced after transcription. Boxes represent the exons and arrows represent the primers used for RT-PCR. B: Expression analysis of Cre-hGH transgenic mice using RT-PCR. A 530 bp band represents the spliced hGH mRNA
1. PCR Marker; 2. Positive transgenic mouse; 3. Negative control mouse

2.7 Assessment of Cre recombinase using Southern blot analysis

The RIP-Cre transgenic mouse was mated with the LoxP/LoxP reporter mouse to get the Cre and LoxP double transgenic mouse. To determine the efficiency and specificity of Cre recombinase the bigenic mice having RIP-Cre and LoxP/+ transgenes were sacrificed and different tissues were removed to prepare genomic DNA according to Hogan *et al*. The efficiency of Cre recombinase was usually assessed by Southern blot analysis. DNA was digested with *Eco*R V, and the blots were probed with a 2.6kb DNA fragment located at the 3′ end of the LoxP anchored targeting gene. 9.5kb band was found in wild type. The band was shift down from 9.5kb to 4.4kb in the LoxP anchored allele because a new *Eco*R V site was introduced with the LoxP site. As Cre/LoxP recombination occurred between two LoxP sites, the introduced *Eco*R V site disappeared and the bolt band became 7.1kb (Fig. 4). We detected the 7.1kb band in pancreatic tissue. We can still found 4.4kb band because the Cre recombinase only expresses in part of pancreatic cells. The presentation of 7.1kb band demonstrated the transgenic mouse expressed functional Cre recombinase in pancreatic tissue.

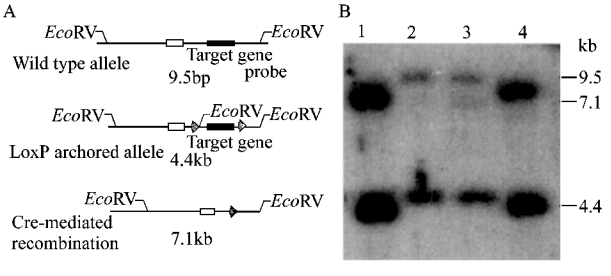

Fig.4 (A) Schematic representation of the strategies used to produce the flox target locus and detect Cre-mediated recombination. The short black line indicates the probe used to detect recombination. (B) Southern blot analysis the target locus before and after Cre-mediated recombination. Genomic DNA from various tissues was digested with EcoRV. (Lane 1 ~ 4 are brain, intestine, pancreas and muscle respectively.) A 7.1kb brand represents the recombination result mediated by Cre

3 DISCUSSION

In order to construct the transgenic mice that the foreign Cre gene only expresses in pancreatic tissue. There are several barriers have to clear up. Firstly, the expression of Cre must be controlled in pancreatic tissue. Secondly, the substrate of Cre recombinase is DNA, the Cre protein has to span the nuclear membrane and accumulate to the nucleus. Thirdly, Cre gene comes from procaryotic P1 phage and can't express effectively in eukaryotic transgenic mice. We found the appropriate methods to solve these corresponding barriers. The rat insulin promoter (RIP) was amplified using PCR, which can dictate the expression of the Cre in pancreatic tissue. The nuclear localization signal (NLS, Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val) suffices to promote efficient nuclear accumulation of cytoplasm protein. The NLS introduced Cre protein is synthesized in the cytoplasm, from which the mature polypeptides migrate rapidly to the nucleus. In 1986, Kozak[12] identified ACCATGG as the optimal sequence for translation initiation in eukaryotic ribosomes. We add the sequence ACCATGG to the upstream of procaryotic Cre gene. The modified Cre gene will express efficiently in transgenic mice. As to hGH, the added hGH aid the expression of Cre. The mechanism is not clear. We like the idea that there are sequences in hGH that determine nucleosome spacing just like an enhancer, and there is some experimental evidence for this[14~16]. We finally established a transgenic mouse model for expression Cre recombinase in pancreatic tissue. All this also demonstrated the construction of Cre expression vector is successful.

We only got a transgenic mouse from 7 founder mice. Not every transgenic founder mouse expresses Cre recombinase effectively due to the unpredictable nature of chromosomal position effects. If promoter or protein encoding sequences of the transgene have been compromised during the integration process, the transgene will be essentially inactive. Intact transgenes can also remain silent if the genomic integration site is located in a heterochromatin region, which is transcriptionally inactive. Alternatively, integration close to highly active genes may have a boosting effect on the transgene expression; this situation may lead to background expression[17,18]. From our experiment, we also found Cre expressed slightly in liver, spleen and some other tissues besides pancreatic tissue due to background expression.[19]

In recent years, the Cre/LoxP system has been widely used for tissue specific ablation of a particular gene. The expression of Cre recombinase is controlled either by using a tissue specific promoter or in a ligand dependent manner. Under this situation the ablation of the target gene can be restricted in a particular cell type by tissue specific expression of the Cre recombinase. A series of Cre transgenic mice have been established in America and Europe. It is just the beginning in domestic research.

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