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不同载体表达核酶对HBV mRNA细胞内表达的阻断作用

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Effect of ribozymes on inhibiting expression of HBV mRNA in HepG2 cells

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Abstract

AIM:To study the activity of ribozymes with multiple cleavage sites and mutated ribozymes on expression of HBV mRNA in HepG2 cells.

METHODS:The triple ribozymes and two cis-ribozymes or two mutated ribozymes were inserted,respectively, into five kinds of eukaryotic plasmids,which were cotransfected into the HepG2 cells with p1.2 II plasmid carring genome of adv-subtype HBV.Cleavage effect of ribozymes on HBeAg and HBcAg were detected by ELISA and laser confocal imaging technique.

RESULTS:The transfected HepG2 cells expressed the expected ribozyme and muta-ribozyme.Intracellular level of HBeAg was surpressed variably with variety of ribozymes.The ribozyme plasmid with tRNA promoter demonstrated the highest inhibitory rate at 81 % for suppression HBeAg expression.

CONCLUSION:The ribozymes exert varied inhibitory effect on the expression of HBV in HepG2 cells depending on kinds of eukaryotic expressing plasmids.

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摘要

目的:探讨多位点自剪切核酶及突变核酶对细胞内HBV mRNA的切割作用.

方法:构建5个不同的多位点核酶及突变核酶的真核表达载体,将他们分别与乙型肝炎病毒全基因序列共转染HepG2细胞,用ELISA,共聚焦定量及图像分析的方法观察多位点核酶在细胞内对HBV mRNA切割作用.

结果:构建的真核表达载体在细胞内确可表达出多位点核酶,核酶及突变核酶在细胞内对HBV基因的表达均有抑制作用,不同表达载体的抑制率不同,以tRNA启动子的表达载体抑制效率最高,达81 %,突变核酶亦有部分反义RNA的抑制效果.

结论:抗乙型肝炎病毒核酶在细胞内可抑制HBV基因的表达,不同表达载体其核酶的表达效率不同.

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0 引言

HBV (hepatitis B virus),是一种严重危害人类健康的病原体,能够引起急、慢性乙型肝炎[1-7].而且慢性HBV携带者发生肝癌的可能性是正常人的100倍.目前的研究确信HBV是少数几种致癌病毒之一,HBV的感染、复制等生命活的依赖基因组的转录和表达.同时HBcAg在细胞内达到一定量后,可取代细胞染色体中组蛋白,而使宿主细胞的正常基因表达发生紊乱.而且HBcAg也免疫因子作用的靶位点[8].

目前常用的药物治疗方法无法阻断细胞中HBV的表达,起到细胞内的保护作用,核酶做为一种新效法已显出明显优势[9-32].前期的研究中,我们采用核酶对HBV表达进行阻断,已取得一定效果,其抑制率低于66 %.为提高核酶的剪切活性,我们采用了自剪切载体表达3个位点核酶,且对照组使用突变核酶,用不同的载体及启动子表达核酶,观察核酶对相应蛋白的抑制作用.

1 材料和方法

1.1材料 p1.2 II (含乙型肝炎病毒基因全序列),由连建奇博士惠赠,pcDNA3由冯志华博士惠赠.pCI由军事医学科学院王海涛教授惠赠.pBBS212及pCEp由本室保存.HepG2细胞由本校病理教研室惠赠.DMEM,Lipofectamine 2000购自Gibco公司.ELISA检测试剂盒购自科华公司.各种抗体购自DAKO公司.

1.2 方法 表达自载切核酶的不同真核表达载体的构建 pcDNA3-Rz123 pcDNA3-mRz12的连接,将核酶及突变核酶,用ApaI/Bst XI双酶切,回收270 bp左右的DNA片

段,pcDNA用EcoR V酶切,回收做为载体.回收的片段经T4DNA聚合酶补平后,将二者进行连接,转化后挑克隆进行鉴定,鉴定采用XbaI/Hind III双酶切或XbaI单酶切.(图1) pCI-Rz123及pCI-mRz123的连接,将pGEMRz123及pGEM-mRz12用ApaI/BstXI双酶切.回收补平,pCI用EcoRI及XbaI双酶切后回收补平、连接、转化,挑取克隆,用XhoI + SalI双酶切鉴定大小及用XbaI + SalI鉴定方向.pBBS212-Rz123及pBBS212-mRz12的连接,将pBBS212及pcDNA3-Rz123及pcDNA3- mRz12均用Kpn I及Xho I进行双酶切,回收片段及载体,双粘端连接,转化.挑取克隆,鉴定用Kpn I及Xho I进行双酶切.pCEp-Rz123 pCEp-mRz12的连接,将pCEp及pcDNA3-Rz123及pcDNA3-mRz12均用Kpn I及Xho I进行双酶切,回收片段及载体,进行双粘端连接、转化,挑取克隆,鉴定用Kpn I及Xho I进行双酶切.pDCTRZA-Rz123与pDCTRZA-mRz12的连接,将pGEM-Rz123及pGEMRz-mRz12,用BstX I单切后切胶回收、补平,用Sac II酶切后回收270 bp及200 bp左右的DNA片段.将pDCTRZA质粒用BamH I单切后切胶回收、补平,用Sac II酶切酶回收载体DNA,将载体与片段进行连接、转化.挑取克隆,鉴定用Sac II、Mlu I进行双酶切.(图2) 质粒DNA转染细胞前16-20 h,用胰酶消化HepG2细胞,接种于6孔培养板(1×10^5/孔)37 ℃,待细胞生长至50-70 %融合时,取7.5 μg核酶及突变核酶质粒DNA及7.5 μg p1.2 II质粒DNA溶于100 μL无血清DMEM中,混匀;取Lipofectamine 20 008 μL加无血清DMEM中,混匀.缓慢混合,室温25 min,加无血清DMEM800 μL至总体积1 mL,混匀.用无血清DMEM洗细胞1次,加入上述转染液于培养板(1 mL/well),37 ℃培养16 h,换完全培养液,传代,48 h后更换选择性培养液,筛选出阳性克隆后,混合克隆,次日收集细胞.细胞用冰浴的PBS缓冲液冲洗3次,加1 mL含10 mmol/LEDTA的PBS缓冲液消化细胞,加入1-10 mL PBS悬浮细胞,计数离心,去除上清,悬浮细胞于0.25 mmol/L Tris-HCl (pH7.5) (100 μl/10^6细胞),上清即为胞质裂解液,用于测定HBeAg、ELISA检测按说明书进行.A值在450 nm下读取.结果均以阳性孔A值/阴性孔A值(P/N)表示,抑制率按下式计算:抑制率=(实验孔P/N值－对照孔P/N值)÷(对照孔P/N值－2.1)× 100 %免疫荧光及激光共聚焦的检测转染的细胞爬片后固定,滴加HBcAg抗体IgG,37 ℃,30min,洗涤5次,滴加荧光抗体37 ℃,30 min,吹干,封片,镜检,用激光共聚焦显微镜观测,定量.免疫细胞化学用SABC法检测(参见说明书),共转染HepG2细胞染色结果判定,呈棕色者为阳性,不着色者为阴性,依据其着色的深浅,代表HBcAg表达的量的多少,不同载体转染各选择5份标本,40倍镜下随机各选择50个细胞,测定其胞度的灰度,计算其均数及标准差,所有结果均以SPLM软件进行t检测.细胞的总RNA用异硫氰酸胍法提取,用^{32}P标记单链DNA的5′末端,打点杂交方法参见说明书.

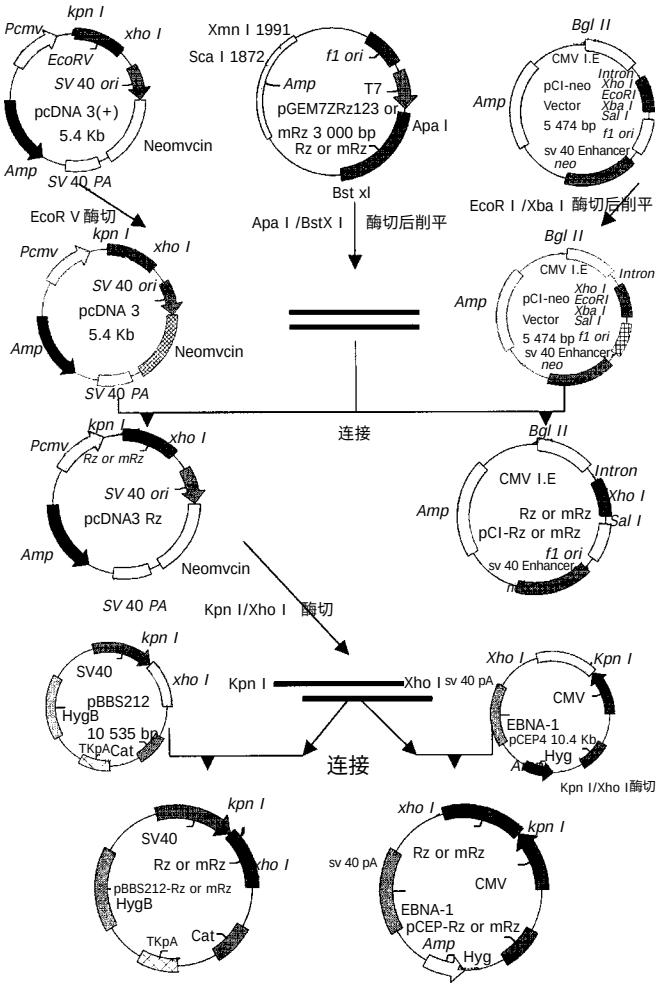


图1 四种不同载体的核酶及突变体的构建流程图

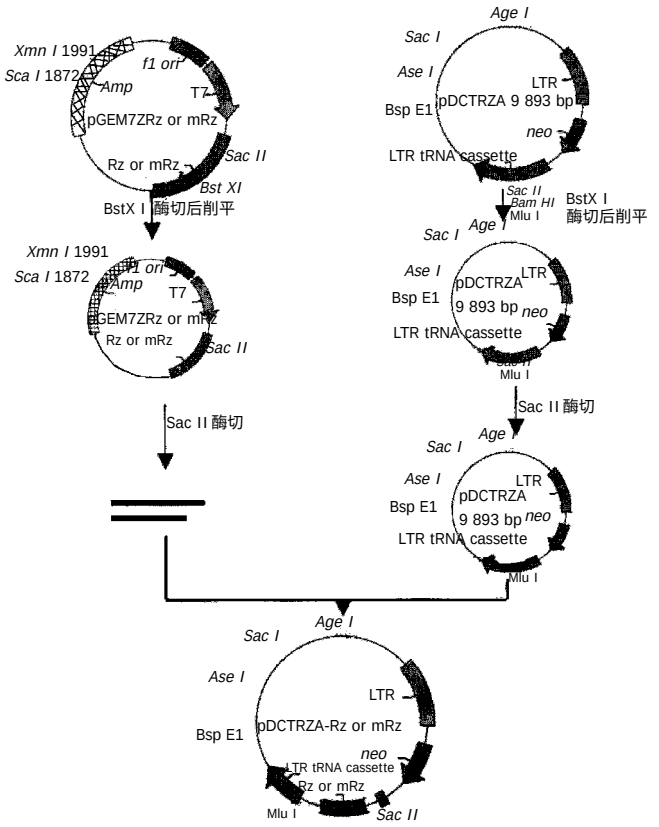
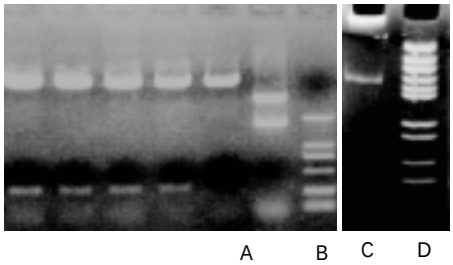


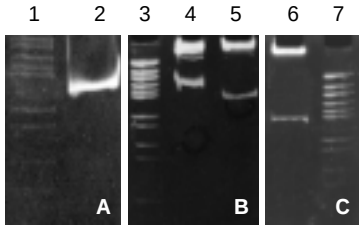
图2 pDCTRZA-Rz或mRz构建流程图

2 结果

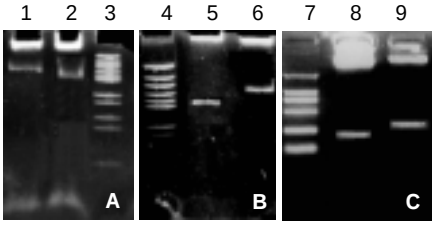
2.1 构建的不同载体鉴定 pcDNA3- Rz123 与 pcDNA3-mRz12 质粒,用不同的单酶或双酶酶切鉴定结果与预想结果相同，说明质粒构建成功(图 3).pCI-Rz123 及 pCI-mRz12 质粒，pBBS212-Rz123 及 pBBS212-mRz12，pCEp-Rz123 及 pCEp- mRz12，pDCTRZA-Rz123 及 pDCTRZA-mRz12，用不同的单酶或双酶切鉴定结果与预计结果相吻合，证明构建质粒成功(图 4,5).



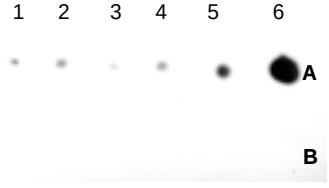
A:Xho I/Hind III 酶切; B:DL2 000 Marker;
C:Xba I 酶切; D:pGEM7Z Hea IIIMarker
图 3 pcDNA3-Rz123 酶切鉴定图



1:pGEM7Z Hea III Marker ; 2:Xho I/Hind III 酶切; 3、7: pGEM7Z Hea III Marker; 4:Xho I/Sal I 酶切;5:XbaI /Sal I 酶切; 6:Xho I/Sal I 酶切
图 4 pcDNA3-mRz12(A)、pCI-Rz123(B0)及 pCI-mRz129 (C)质粒酶切鉴定图.



1:pBBS212-Rz123; 2:pBBS 212-mRz12; 3，4:7Marker; 5:pCEP-mRz12;6:pCEP-Rz123;7:pDCTRZA -mRz12;8:pDCTRZA-Rz123
图 5 pBBS212-Rz123 pBBS 212-mRz12(A) pCEP-Rz123 及 pCEP-mRz12(B). pDCTRZA-Rz123 及 pDCTRZA -mRz12(C)的酶切鉴定图.



1:pcDNA;2:pCI; 3：pBBS212;4:pCEP;5:pDCTRZA;6:对照组;
A:核酶组;B:突变核酶组
图 6 核酶在细胞中的表达（RNA 打点杂交图）

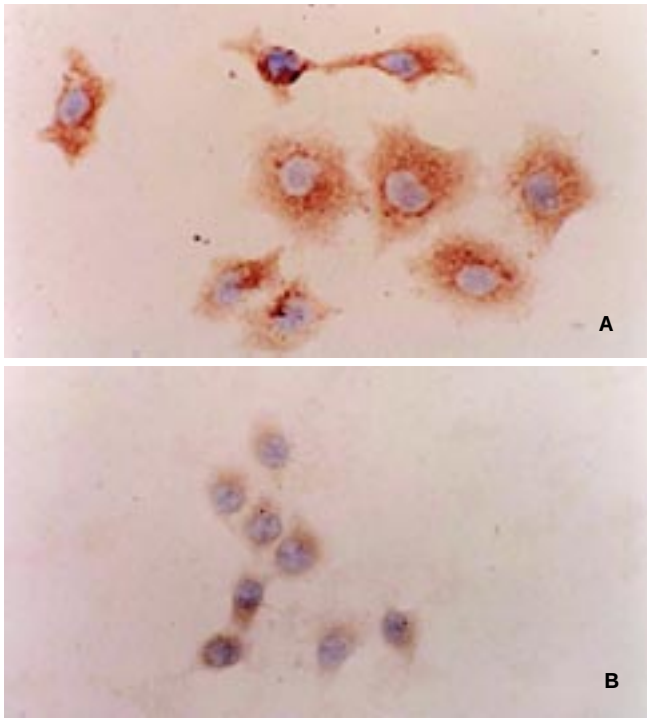
2.3 多位点核酶对细胞质中乙型肝炎病毒核心蛋白表达的抑制作用 在将p1.2 II 与多个核酶的不同载体以及突变核酶，空载体分别转染 HepG2 细胞，转染 1wk 后胞质中可见到 HBeAg 的表达.待筛选出阳性克隆后，取胞质裂解液进行ELISA测定.由表中可见,不同的载体表达核酶,对 HBeAg 的表达有不同的抑制率,以 pDCTRZA 为最高,为 81 %,其次为 pBBS212 76 %,最低为 pCEp,且突变核酶亦有一定的抑制率，说明其具有部分反义 RNA 的功能(表 1).

表 1 不同载体多位核酶对 HBeAg 表达的作用(P/N 值,抑制率, $\bar{x} \pm s$)

分组	pCDNA3	pCI	pBBS212	pCEP	pDCTRZA
核酶组	2.6±1.3(0.73)	2.9±0.7(0.61)	2.5±0.4(0.76)	3.1±0.4(0.55)	2.6±1.1(0.81)
突变核酶组	3.6±1.0(0.18)	3.7±0.7(0.21)	3.4±0.1(0.26)	3.9±1.0(0.21)	3.8±1.0(0.39)
空载体组	3.9±0.1	4.2±0.1	3.9±1.10	4.4±0.1	4.9±0.3

2.4 免疫荧光及激光共聚焦的结果 取突变组及核酶组细胞分别进行免疫荧光测定，荧光强度差异明显.将其进行共聚焦定量,以下列公式计算:抑制率= 1-核酶组像素密度/突变核酶组像素密度，经计算，其最高抑制率可达 73.2 %.

2.5 免疫组化及图像分析结果 取突变组、空载体组及核酶组两种细胞抗原表达有明显差异(图7).将此细胞进行图像分析,亦表明二者有差异.



A:空载体组 B:核酶表达组
图 7 核酶对细胞内 HBV 表达的抑制作用.

3 讨论

近年来,在细胞内应用核酶抑制基因表达已取得了令人瞩目的成果,尤其抗 HIV 核酶已在 $CD4^+$淋巴细胞中表达,并将进行回输人体进行研究.因此尽管核酶的酶效率较低,比蛋白质低几个数量级,但在细胞和活体研究中,却发现他可以降低靶 RNA 90 %以上.可见核酶用于抑制基因表达和基因治疗有着广阔的前景.乙型肝炎病毒(HBV)的复制必须经过由细胞的 RNA 多聚酶转录成多

拷贝的3.5 kb RNA,以这一RNA前基因组为模板,通过逆转录,自DR1区开始合成负链DNA,因此前基因组mRNA是关键的一步,所设计的核酶如能正确切割mRNA,则可抑制乙型肝炎病毒的复制和表达，从而起到胞内免疫的作用.到目前为止,关于细胞内核酶抗HBV的研究尚不多[32],Beck所用核酶没有在细胞内做到有活性的表达,而Welch则用串联核酶表达，做到最高抑制率83 %,南非学者也用荧光蛋白来检测细胞内核酶对HBV的抑制作用,证实确可抑制HBV的表达.

本室前期的工作亦证实对HBV C区基因的表达有明显的抑制作用.同时在前期工作的基础上,我们增加了自剪切序列及核酶的数量，用自剪切来包装核酶，避免其两侧的附加序列影响到核酶的切割活性，使切割核酶与靶RNA作用后更易脱落下来，进行新一轮的切割.增加核酶数量则使其能在不同的位点破坏靶RNA，避免病毒变异造成的单一核酶切割作用的丧失,也可避免结合蛋白掩盖切割位点.细胞内核酶的表达,关键在于启动子的选择，Cotton将核酶基因克隆在蛙蟾tRNA met及密码环中，提高了核酶的转录水平.Baier利用tRNA启动子可使核酶基因高效表达，同时tRNA的结构稳定，可抵抗核酸酶的降解，因此被认为是细胞内表达核酶的理想启动子.本研究即将核酶克隆于含有tRNA启动子的真核载体中.本研究的结果也印证了上述理论,杂交证实转染细胞中有核酶的mRNA存在，ELISA等方法检测发现核酶及突变核酶对HBV的表达均有抑制作用.pcDNA3，pCI，pBBS212， pCEp， pDCTRZA所达核酶对HBV C基因抑制率为73 %,61 %,76 %,55 %,81 %.最高者为pDCTRZA即tRNA启动子所启动表达的核酶.这也说明核酶作为一种抗HBV的手段是可行的.

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