N 端融合不同长度转位肽的重组粒酶 B 抑制细胞生长作用的比较

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摘 要 采用重组 PCR 法将粒酶 B 基因的 N 端信号肽和酸性二肽编码序列去除,与两种不同长度的绿脓杆菌外毒素(PE)转位肽序列分别连接,将它们插入 pIND 诱导表达载体,通过脂质体法与 pVgRXR 辅助质粒共转染 HeLa 细胞,建立了重组 PE II-GrBa 基因的诱导表达细胞系。松甾酮 A 诱导后 Western 印迹检测到目的基因的表达,间接免疫荧光观察到表达细胞出现多核巨细胞的异常形态。两种表达的 PE II-GrBa 融合蛋白均能够切割粒酶 B 的细胞内源性和外源性底物,并且使细胞生长速度减慢。其中,PE II (aa 280-358)-GrBa 的底物切割能力和生长抑制作用较强。流式细胞仪分析这种抑制作用可能与细胞周期的 G_2 期受到阻遏有关。上述结果证实了 PE II-GrBa 融合蛋白仍然具有抑制细胞生长的作用,并且较短的转位肽对 GrBa 活性的影响较小,有助于进一步优化转位肽/细胞毒性效应蛋白重组分子的结构用于肿瘤细胞杀伤。

关键词 粒酶 B,绿脓杆菌外毒素,诱导表达,生长抑制作用

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杀伤性 T 淋巴细胞(CTL)和自然杀伤(NK)细胞通过颗粒胞吐机制(granule exocytosis mechanism)和 Fas 机制诱导异常细胞发生凋亡,维持机体的自稳状态[1]。其中粒酶 B(granzyme B, GrB)是杀伤性颗粒中最重要的丝氨酸蛋白酶,它在颗粒中以酶原形式存在,去除 N 端信号肽和酸性二肽后释放,成为活性型粒酶 B(active granzyme B, GrBa)[2]。活化后的粒酶 B 能够进入靶细胞,激活 caspase 引发级联反应,或者直接切割细胞浆和细胞核内的底物蛋白,有效启动靶细胞凋亡[3-5]。绿脓杆菌外毒素(Pseudomonas exotoxin A, PE)的第二结构域(domain II)具有转位功能[6-8]。将转位肽与活性型粒酶 B 融合,形成的嵌合蛋白可望能够高效穿膜进入细胞液,发挥细胞杀伤作用。为研究 N 端融合有不同长度的 PE 转位肽对活性型粒酶 B 功能的影响,本文构建了两种重组的 PE II - GrBa 基因,建立了诱导表达细胞系,比较了它们的表达对细胞生长的影响。

1 材料和方法

1.1 材料

1.1.1 质粒、菌种和细胞系: *E. coli* DH5α 宿主菌和人宫颈癌细胞系 HeLa 为本室保存。含有蜕皮激素反应元件的可诱导表达质粒 pIND、编码蜕皮激素受体的辅助质粒 pVgRXR 为 Invitrogen 公司产品。pCMV-sFv23e-PE40 质粒(含编码 PE 第二、第三结构域的基因序列)为陈思毅教授构建和惠赠。pUC19-GrB 克隆质粒(含 744 bp 的人全长粒酶 B 基因)由本室构建[9]。

1.1.2 试剂: 限制酶、Taq DNA 聚合酶、质粒提取试剂盒、DMEM、新生牛血清、脂质体 lipofectAMINE 2000、G418、zeocin、松甾酮 A(Pon A)及蛋白 G 琼脂糖凝胶均购自 Invitrogen 公司。PVDF 膜和 ECL Western 印迹试剂盒为 Pharmacia 产品。Ac-IETD-pNA 购自 Calbiochem 公司。山羊抗人粒酶 B 多克隆抗体和山

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羊抗人 DFF45 多克隆抗体为 Santa Cruz 产品。生物素化兔抗山羊二抗及 SABC 复合物为博士德产品。

1.2 方法

1.2.1 PE II-GrBa 融合蛋白基因诱导表达载体的构建：设计引物 INDF，5′-TTTGGATCCAATATGGGCTGGGAACAACTGGAGCA-3′；INDR，5′-TTTTCTAGATTAGTAGCGTTTCATGGTTT-3′；GINDF，5′-TTTGGATCCAATATGATCATCGGGGGACATGAGGC-3′；NLR，5′-TCCCCCGATGATGTTGGCCGCGCCGGCCTCGTC-3′；NSR，5′-TCCCCCGATGATGTCGTTGCCGGTGCCCTGCCG-3′；LGF，5′-GGCGCGGCCAACATCATCGGGGGACATGAGGCC-3′；SGF，5′-ACCGGCAACGACATCATCGGGGGACATGAGGCC-3′。以 pCMV-sFv23e-PE40 为模板，用 INDF、NLR（或 NSR）引物扩增出 PE II（aa 280-364/358）片段。以 pUC19-GrB 为模板，用 LGF（或 SGF）、INDR 引物扩增出 GrBa 片段。将这两种扩增产物等体积混合作为模板，以 INDF、INDR 为引物进行 PCR 扩增，得到 PE II（aa 280-364）-GrBa 及 PE II（aa 280-358）-GrBa 融合蛋白基因。此外，以 pUC19-GrB 为模板，以 GINDF 和 INDR 引物扩增 N 端信号肽和酸性二肽编码序列下游的 GrBa 基因作为对照。将上述重组粒酶 B 基因克隆入 pIND 诱导表达载体中，并测序证实。

1.2.2 细胞培养与转染：HeLa 细胞用 DMEM（含 10%小牛血清）在 37℃、5% CO_2 浓度条件下培养。转染前将处于对数生长期的细胞重新接种于六孔板中，待细胞达到 80%汇合时以重组表达质粒 pIND-PE II-GrBa 和辅助质粒 pVgRXR 进行共转染。共转染的目的是使 pVgRXR 在蜕皮激素类似物松甾酮 A（Pon A）的诱导下首先表达蜕皮激素核受体蛋白，然后核受体结合到 pIND-PE II-GrBa 的蜕皮激素反应元件上，启动下游 PE II-GrBa 目的基因的转录。共转染按照 LipofectAMINE 2000 试剂说明书操作。

1.2.3 诱导表达 PE II-GrBa 融合蛋白的 HeLa 细胞系的建立：转染后 24 h，加入 500 μg/mL G418、800 μg/mL zeocin 压力筛选转染的 HeLa 细胞。约 2 周后挑取单集落细胞并扩大培养。于建系细胞中加入 10 μmol /L Pon A 诱导 48 h，使之表达相应的重组粒酶 B 基因。

1.2.4 间接免疫荧光：Pon A 诱导后制备细胞爬片，4%多聚甲醛固定 30 min，再经 0.01% Triton 处理、0.3%双氧水灭活内源性过氧化物酶及血清封闭等处理后，依次加入 1∶200 稀释的山羊抗人粒酶 B 多克隆抗体，1∶100 稀释的生物素化兔抗山羊二抗，1∶100 稀释的 SABC-Cy3，荧光显微镜观察并照相，激发波长为 554 nm，发射波长为 568-574 nm。

1.2.5 免疫沉淀与 Western 印迹分析：Pon A 诱导后收集细胞，于 4℃加细胞裂解液裂解 30 min。4℃离心去除细胞碎片，上清加入 2 μg 山羊抗人粒酶 B 多抗（检测重组粒酶 B 蛋白表达）或山羊抗人 DFF45 多抗（检测切割内源性底物的酶活性），4℃振摇 1～3 h，再加入 20 μL 蛋白 G 琼脂糖凝胶，4℃振摇过夜。2500 r/min 4℃离心 5 min，洗涤沉淀 4 次，重悬于 50 μL SDS 1×上样缓冲液，进行 SDS-聚丙烯酰胺凝胶电泳。将凝胶中的蛋白质 100 V 转移 3 h 至 PVDF 膜上，5%脱脂奶粉室温封闭 2 h，依次加入 1∶200 稀释的山羊抗人粒酶 B 多抗或山羊抗人 DFF45 多抗 4℃孵育过夜，1∶100 稀释的生物素化兔抗山羊二抗室温作用 2 h，1∶100 稀释的 SABC 复合物室温孵育 1 h，PBS-T 洗后 ECL 显色。

1.2.6 粒酶 B 酶活性测定：分别收集 Pon A 诱导前后的细胞，用冰冷的 PBS 洗涤 1 次，重悬于 300 μL 裂解液中，冰上放置 1min，13000 r/min 离心 1min，吸取上清备用。在 96 孔板每孔中加入 50 μL 待测上清，0.2 mmol/L 粒酶 B 底物 Ac-IETD-pNA，加反应缓冲液至 200 μL 总反应体系。27℃孵育 5 min，酶联免疫检测仪测定各孔 405 nm 光吸收值。相同处理的样品均设 3 孔重复实验，同时设立阴性和空白对照。

1.2.7 细胞计数：细胞消化后重悬于适量培养液中，用血球计数板进行细胞计数，计算生长抑制率。生长抑制率（%）=（对照组细胞数 - 实验组细胞数）/对照组细胞数×100%。

1.2.8 细胞周期测定：胰蛋白酶消化细胞，制成单细胞悬液，调节细胞浓度为 1×10^6 个/mL。PBS 洗涤，70% 乙醇于 4℃固定 30min。0.05% 碘化丙啶（PI）室温避光条件下染色 30 min，流式细胞仪测定各个细胞周期的细胞数。

2 结 果

2.1 PE II-GrBa 融合蛋白基因诱导表达载体的构建

将大小约 700 bp 的活性型粒酶 B（GrBa）基因 5′端连接绿脓杆菌外毒素（PE）的不同长度的转位肽编码序列，获得大小约 970 bp 和 950 bp 的 PE II（aa 280-364）-GrBa 及 PE II（aa 280-358）-GrBa 融合蛋白基因（图 1）。将上述重组粒酶 B 基因克隆入 pIND 诱导表达载体后酶切鉴定（图 2），并经 DNA 测序证实。

2.2　诱导表达 PE II-GrBa 融合蛋白的 HeLa 细胞系的建立

以 pIND-PE II-GrBa 和 pVgRXR 质粒共转染HeLa细胞，经 500 μg /mL G418 及 800 μg /mL Zeocin 筛选建系，加入 10 μmol /L Pon A 诱导后 Western 印迹检测到约 34 kD 和 33 kD 的 PE II (aa 280-364)-GrBa 及 PE II (aa 280-358)-GrBa 融合蛋白，对照 GrBa 蛋白约为 25 kD(图 3)。细胞免疫荧光观察结果表明，GrBa 基因和两种 PE II-GrBa 融合蛋白基因诱导表达后，均观察到有多核巨细胞产生(图 4)，这种异常形态是由于细胞骨架破坏、有丝分裂抑制引起的(另文发表)。

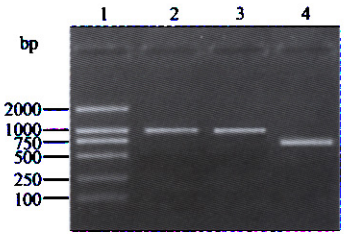

图 1　PCR 获得重组 GrB 基因

Fig.1　PCR amplification of recombinant GrB genes

1: DL-2000 marker;
2: PE II(aa 280-364)-GrBa gene;
3: PE II(aa 280-358)-GrBa gene;
4. GrBa gene

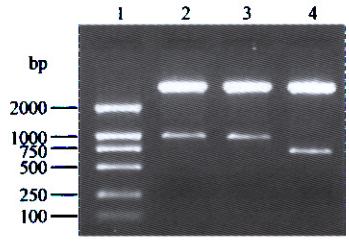

图 2　编码重组 GrB 基因的 pIND 质粒的酶切鉴定(*Bam*H Ⅰ/*Xba* Ⅰ)

Fig.2　Restriction enzyme digestion analysis of pIND expression vectors encoding recombinant GrB genes

1: DL-2000 marker;
2: pIND-PE II(aa 280-364)-GrBa/*Bam*H Ⅰ + *Xba* Ⅰ;
3: pIND-PE II(aa 280-358)-GrBa/*Bam*H Ⅰ + *Xba* Ⅰ;
4. pIND-GrBa/*Bam*H Ⅰ + *Xba* Ⅰ

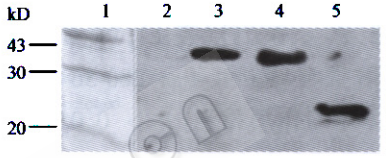

图 3　HeLa 细胞诱导表达重组 GrB 蛋白的印迹检测

Fig.3　Western blot of inducible expression of recombinant GrB proteins in HeLa cells

1: Molecular mass marker; 2: pIND;
3: pIND-PE II(aa 280-364)-GrBa;
4: pIND-PE II(aa 280-358)-GrBa;
5: pIND-GrBa

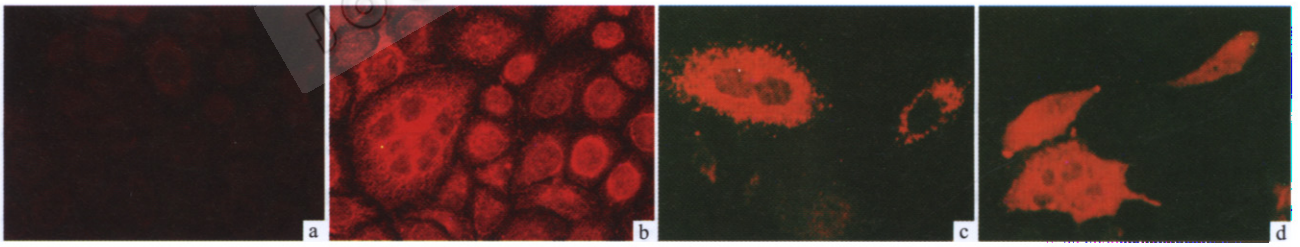

图 4　间接免疫荧光检测表达重组 GrB 蛋白的 HeLa 细胞的形态改变

Fig.4　Altered morphology of HeLa cells expressing recombinant GrB proteins detected by indirect immunofluorescence(× 400)

(a) HeLa cells transfected with pIND; (b) HeLa cells transfected with pIND-PE II(aa 280-364)-GrBa; (c) HeLa cells transfected with pIND-PE II(aa 280-358)-GrBa; (d) HeLa cells transfected with pIND-GrBa

2.3　PE II-GrBa 融合蛋白具有类似粒酶 B 的丝氨酸蛋白酶活性

DNA 片段化因子的 45 kD 亚基(DFF45)可被粒酶 B 切割，释放出 DNA 片段化因子的 40 kD 亚基(DFF40)，DFF40 能够诱导核 DNA 片段化[5]，故选择 DFF45 作为检测粒酶 B 酶活性的内源性底物。Pon A 诱导建系细胞后裂解细胞，用抗 DFF45 抗体作 Western blot，结果表明，PE II-GrBa 融合蛋白能够切割细胞中粒酶 B 的内源性底物 DFF45，将其裂解成 28 kD 的产物(图 5)。此外，将诱导后细胞的裂解液与粒酶 B 的四肽底物 Ac-IETD-pNA 孵育，测定酶活性并进行统计学分析(图 6)。结果表明，PE II (aa 280-358)-GrBa 切割外源性底物的程度与 GrBa 相当($P > 0.05$)，PE II (aa 280-364)-GrBa 的作用弱于 PE II (aa 280-358)-GrBa($P < 0.05$)。

2.4 PE II-GrBa 融合蛋白基因的诱导表达抑制细胞生长

细胞计数结果表明,与空载体转染组相比,细胞诱导表达两种 PE II-GrBa 融合蛋白后增殖速度均减慢(图 7)。统计学方差分析结果显示,空载体组与任一实验组之间的差异特别显著($P < 0.01$);PE II (aa 280-358)-GrBa 的生长抑制率高于 PE II (aa 280-364)-GrBa($P < 0.05$),与 GrBa 的生长抑制作用相当($P > 0.05$)。流式细胞仪检测诱导前后细胞周期的变化,并对同一组内采用统计学配对 t 检验,以消除诱导前细胞生长状态不同带来的干扰;同时在不同组之间进行统计学方差分析,以比较组间的差异。结果表明,空载体组诱导前后无显著差异($P > 0.05$);所有实验组诱导表达后均引起 G_2 期细胞的比例降低($P < 0.05$)(表 1),PE II (aa 280-358)-GrBa 的作用强于 PE II (aa 280-364)-GrBa ($P < 0.05$),与 GrBa 的作用相当($P > 0.05$)。

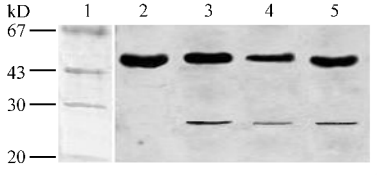

图 5 蛋白印迹检测重组 GrB 的表达引起 DFF45 的切割

Fig.5 Western blot of DFF45 cleavage in HeLa cells caused by the expression of recombinant GrB proteins

1:Molecular mass marker; 2:pIND; 3:pIND-PE II(aa 280-364)-GrBa; 4:pIND-PE II(aa 280-358)-GrBa; 5:pIND-GrBa

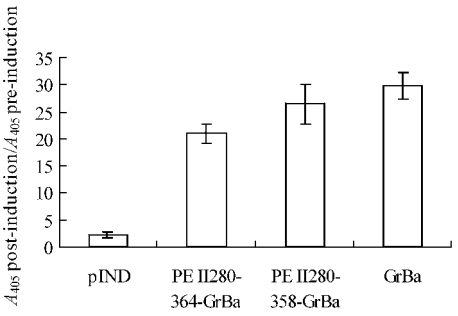

图 6 诱导表达的重组 GrB 蛋白的丝氨酸蛋白酶活性检测

Fig.6 Inducible expression of recombinant GrB proteins in HeLa cells led to higher serine proteinase activity

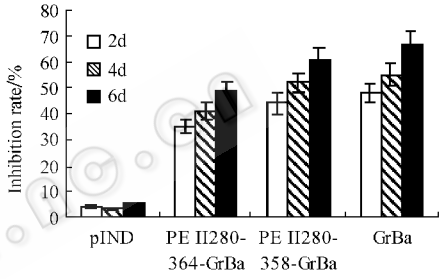

图 7 诱导表达重组 GrB 蛋白的 HeLa 细胞出现生长抑制

Fig.7 Inducible expression of recombinant GrB proteins in HeLa cells caused cell growth inhibition

表 1 表达重组 GrB 蛋白的 HeLa 细胞出现 G_2 期抑制

Table 1 Inducible expression of recombinant GrB proteins in HeLa cells caused decrease of G_2-phased cells

Cell group	vector	PE II$^{280\text{-}364}$-GrBa	PE II$^{280\text{-}358}$-GrBa	GrBa
Decrease of G_2 cells after induction/%	−0.8±0.2	5.7±0.52	8.6±0.2	8.9±0.3

3 讨论

研究表明,凋亡效应蛋白基因的表达能够导致不可逆的细胞死亡,不同的效应分子启动不同的凋亡途径[10,11]。粒酶 B(GrB)是淋巴细胞特异性表达的一种丝氨酸蛋白酶,能够直接激活 caspase 级联反应,或者通过激活独立于 caspase 之外的其它凋亡途径,有效清除异常细胞[2-5]。因此,以粒酶 B 诱导细胞死亡将为杀伤肿瘤细胞提供一种新的方法。由于活性形式的粒酶 B(GrBa)需要在靶细胞的细胞液中发挥作用[12,13],故构建转位肽与 GrBa 融合的重组分子有望跨越内吞体膜屏障、进入细胞液并杀伤细胞。

绿脓杆菌外毒素(PE)具有转位结构域,其作用机制是,在内吞体酸性条件下,PE 分子 Arg279 和 Gly280 之间的肽键断裂,释放催化结构域至胞浆[6]。关于 PE 转位结构域的长度界定尚有争议。最早认为 aa 253-412 为内吞体膜转位所必需。有研究表明,结构域 II(aa 253-364)足以完成膜转位作用[7]。Taupiac 等[8]实验证明,截短的结构域 II(aa 253-358)转位效率更高。本文将 PE aa 280-364 和 PE aa 280-358 的编码序列分别与 GrBa 基因相融合,比较两种 PE 肽段对 GrBa 活性的影响。

建立诱导表达重组 GrBa 蛋白的 HeLa 细胞系意义在于,一方面 GrBa 对转染细胞有杀伤作用,非诱导表达的细胞无法建系,另一方面瞬时表达不能排

除过量表达对细胞的毒性干扰，故采用可诱导表达系统能够比较准确的研究重组 GrBa 蛋白对表达细胞的作用。

首先，通过蛋白印迹检测证实了 GrBa 基因和两种 PE II-GrBa 融合蛋白基因的诱导表达。间接免疫荧光染色观察到表达几种目的蛋白的细胞均出现多核巨细胞的异常形态。我们以往的研究表明，瞬时表达 GrBa 的多核巨细胞的细胞骨架出现异常、有丝分裂产生多极纺锤体(另文发表)。上述观察结果初步表明 PE 肽段的存在基本不影响 GrBa 对转染细胞的作用。进一步的研究表明，N 端融合 PE 肽段的 GrBa 重组蛋白能够切割粒酶 B 的细胞内源性和外源性底物，提示这种融合方式基本不影响丝氨酸酶活性的发挥。在两种 PE II-GrBa 融合蛋白中，PE II (aa 280-358)-GrBa 的底物切割能力较强，与 GrBa 的酶活性相当，推测原因是较短的 N 端肽段对 GrBa 活性中心折叠形成的空间位阻更小。对诱导后的存活细胞进行计数，结果两种 PE II-GrBa 融合蛋白均使细胞生长速度减慢，带有较短 PE 肽段的融合蛋白的生长抑制率较高。流式细胞仪分析这种抑制作用可能与细胞周期的 G_2 期受到阻遏有关。

本研究证实了 N 端融合部分 PE 转位肽的 GrBa 重组蛋白仍然能够抑制细胞生长，并且具有较短转位肽的融合蛋白的作用较强，为进一步优化转位肽/促凋亡蛋白重组分子的结构用于肿瘤治疗作出了有益的探索，使其既具有高效的膜转位功能，同时还尽量不影响效应分子杀伤肿瘤细胞的活性。

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Growth Inhibitory Effects of Recombinant Granzyme B Containing Different N-terminal Translocating Peptides

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Abstract　Translocating protein and translocating peptides have therapeutic potential against tumors by exposing the cytotoxic

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domains of toxic proteins to the cell cytosol. The aim of this study is to investigate the effect of N-terminally fused PE translocating peptides on granzyme B (GrBa) activity. PE II-GrBa fusion protein genes were constructed by replacing N-terminal signal and acidic dipeptide sequence of human granzyme B gene with two truncated translocating sequences of *Pseudomonas* exotoxin A (PE II aa 280-364/358) by recombinant PCR, and then cloned into pIND inducible expression vector. The resulting pIND-PE II-GrBa expression vectors were co-transfected with assistant plasmid pVgRXR into HeLa cells through lipofectamine, followed by selection on G418 and zeocin. The resistant cells were collected and induced with ponasterone A. Western blot analysis demonstrated that ponasterone A induction caused the expression of PE II-GrBa fusion proteins, and indirect immunofluorescence detected giant sized multinucleated cells, suggesting cytoskeletal and mitotic abnormalities as reported in our previous studies. Western blot, enzymatic activity assay and cell counting analysis indicated that two types of PE II-GrBa fusion proteins were capable of cleaving both endogenous and exogenous substrates of granzyme B, and inhibiting the growth of cells. The PE II (aa 280-358)-GrBa was shown to have higher serine protease activity and stronger growth inhibitory effect. Such inhibition was presumably associated with G_2 arrest as determined by cell cycle analysis. These data prove that PE II-GrBa fusion proteins have cell inhibitory effect similar to GrBa, and that the shorter PE-derived peptide exerts less influence on GrBa activity. This study helps to optimize the construction of recombinant protein comprising translocating peptides and cytotoxic molecules for tumor cell killing.

Key words granzyme B, *Pseudomonas* exotoxin A (PE), inducible expression, growth inhibitory effect

Identification of a Lens-specific *cis*-acting Element within the Basal Promoter of the Human Lens Intrinsic Membrane Protein MP19 Gene(LIM2)

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Abstract Using overlapping and mutant oligonucleotides as probes, gel mobility assays and competition experiments identified a sequence from −47 to −32 bp upstream of the LIM2 CAP site, which a lens protein complex bound with high affinity which appeared to bind only to the "sense" strand of the double-stranded DNA molecule. This sequence consisted of a string of four guanine residues followed by seven other nucleotides (AACCTAA) and followed by another four guanines, *i.e.* GGGGAACCTAAGGGG, called the Hsu element. Promoter-CAT constructs containing this sequence or mutations of the sequence indicated that the Hsu element is located within the basal promoter, and is essential for expression of the LIM2 gene. The trans factors binding to the Hsu element are present throughout development, and appear to be lens-specific. Since the LIM2 gene promoter does not contain a classic TATA box, the Hsu element may serve as the site for binding the RNA polymerase complex.

Key words lens fiber membrane, MP19, LIM2 gene; promoter, TATA box, *cis*-acting element

The lens fiber cell membrane contains a group of four proteins, called major intrinsic membrane proteins, which are characterized by their quantity in the lens membrane (at least 95% of the total membrane proteins are made up by these four), by their structural properties (all contain multiple transmembrane sequences), by function (three appear to function as transport-type proteins, while the last has an unknown function), and by their involvement with cataract[1-3].

The second most abundant fiber cell intrinsic membrane protein, MP19, has, as yet, no known function. However, this protein has recently been linked to the dominant mouse lens cataract, To3 (total lens opacity)[3]. The gene coding for human MP19 (LIM2) has been isolated and complete sequence analysis has been carried out[4]. The normal control of transcription of MP19 mRNA appears to be regulated by separate developmental and differentiation events[5,16]. For the above reasons, therefore, it was of interest to investigate the MP19 gene (LIM2) upstream promoter region in order to determine the nature of transcriptional control of this gene. We report here the identification of a lens-specific cis-acting element within the LIM2 basal promoter that may serve as an RNA polymerase complex binding site.

1 MATERIAL AND METHODS

1.1 LIM2 Upstream Sequence Analysis

Restriction enzyme analysis of a 46 kb cosmid clone (f24596) containing the entire LIM2 gene and at least 7.5 kb of upstream sequence[7] demonstrated that *Bam*HⅠ digestion released a roughly 2.3 kb fragment which contained the 5′-end of the LIM2 gene (about 325 bp) and about 2 kb of upstream sequence. This *Bam*HⅠ digested fragment was purified using agarose gel electrophoresis and cloned into the *Bam*HⅠ site of pBluescript SK, and sequence analysis carried

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Abbreviations: LIM, lens intrinsic membrane; bp, base pair(s); CAT, chloramphenicol acetyl tranferase; MP, membrane protein; CAT, transcription start site.

out using automated DNA sequencing (Alf Express, Pharmacia), following company procedures for cycle sequencing analysis. A total of 1 kb of LIM2 upstream sequence was determined, using synthetic oligonucleotide primers to "walk" through the sequence.

1.2 Oligonucleotide Design and Production

Oligonucleotides were synthesized using a Beckman synthesizer and purified using micro-bore HPLC.

1.3 Probe Labeling for DNA/Protein Binding Assays

The PCR product or each synthesized single-stranded oligonucleotide was end-labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]-ATP (specific activity 3 000 Ci/mmol) for 45 minutes at 37℃ [17,18] in a 30 μL reaction containing 6 μL 5 × forward reaction buffer (350 mmol/L Tris-HCl, pH 7.6, 50mmol/L $MgCl_2$, 500mmol/L KCl, and 5 mmol/L 2-mercaptoethanol). The labeling reaction was stopped by adding EDTA to 5 mmol/L or by heat inactivation (10 min at 65℃). The labeled PCR DNA was purified by NICK column (Sephadex G – 50, Pharmacia). The labeled single-stranded oligonucleotides were purified by XTreme Oligonucleotide Purification Kit purchased from Pierce. The purifications with NICK columns and XTreme matrix also separated out unincorporated isotopes. Both procedures used followed the manufacturer's instructions.

The radioactive double-stranded oligonucleotides were made from the labeled single-stranded ones. By adding KCl to a final concentration of 50 mmol/L, two relevant labeled single-strand oligonucleotides (equal volume or equal moles) were mixed together and incubated at 85℃ for 2 minutes, then cooled to room temperature.

Finally, ^{32}P incorporation was tested for all probes made from PCR product, the synthesized single- and double-stranded oligonucleotides. Usually the specific activity of labeled probes was about 10^8 cpm/μg of DNA.

1.4 Nuclear Extract Preparation

Timed fertilized chicken eggs were supplied by Hyline International (Mansfield, GA). The eggs were obtained 2 days post fertilization and were placed in a 37℃ incubator to allow the embryos to develop to specific stages before use. Chick embryos were removed from the egg at different days of development (8, 10, 12, 14, 16, and 18 days post fertilization, which called as E8, E10, E12, E14, E16 and E18 in text below, respectively), then lenses and other organs (brain, heart, liver, and limb) were removed and used for preparation of nuclear or cell protein extracts.

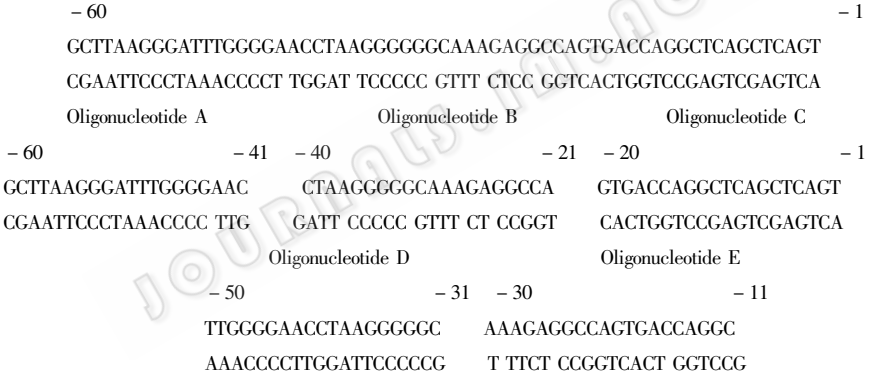

Fig.1 Sequence of the immediate upstream region of LIM2, from – 60 to – 1, showing the sequence of the overlapping double-stranded oligonucleotides used in gel retardation assays

(A) Overlapping double-stranded oligonucleotides were synthesized from the region of – 60 to – 1 upstream of the LIM2 CAP site. Each oligonucleotide was 20 bases in length. Figure 1 illustrates the oligonucleotides synthesized over the region of – 60 to – 1;

(B) A double-stranded oligonucleotide, called as the Hsu element, was synthesized from the region of – 47 to – 32 upstream of the LIM2 CAP site. As indicated in the text and figures, the single-strand coding and noncoding sequences and their relevant mutations of the Hsu element were synthesized to use in gel retardation assays;

(C) Primers used for PCR were synthesized from the nucleotide sequence of the upstream region of the human LIM gene

Nuclear extracts from chicken organs (including lens) were prepared using a modification of the technique described by Andrews and Faller[8]. After the various organs were removed from the animal, they were quickly put in cold 1 × phosphate-buffered saline (PBS) buffer. The organs were washed in 1 × PBS once. The organs were then dissected and put into 20 ~ 100 μL of ice-cold buffer A (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 2 μg/mL chymostatin). DTT and protease inhibitors were added just before use. The samples were vortexed vigorously for 1 to 3 minute(s) and allowed to swell on ice for 15 to 30 minutes. Then an equal volume of buffer D (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 840 mmol/L NaCl, 50% glycerol, 0.4 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 2 μg/mL chymostatin) was added and homogenized thoroughly for 2 ~ 3 minutes using a pellet pestle (Kontes hand-held minihomgenizer with disposable plastic pestle), and incubated on ice for another 20 ~ 30 minutes. The protein extracts were centrifuged in the cold for 2 minutes at ~ 14 000g (13 000 r/min in table-top centrifuge). The supernatants were transferred to fresh microfuge tubes and

used immediately, or stored as aliquots at −80℃ or colder. Protein extracts were not reused after thawing. The protein concentration for each extract was determined[9] and constant amounts of protein were used in each assay.

1.5 Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCR) were carried out following the method of Saiki, Mullis and Faloona[10-12], with minor changes. Reactions were carried out in a 100 μL volume containing 10~20 ng of DNA template (pBluescript S_K-plasmid containing the 5′ end of the LIM2 gene, about 350 bp, and about 1.7 kb of upstream sequence), 100 ng of each oligodeoxynucleotide primer, dATP, dGTP, dCTP and dTTP, (2.5 mmol/L), 1× reaction buffer (20 mmol/L Tris-HCl, pH 8.75, 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/mL bovine serum albumin), and about 2.5 units of *Pfu* DNA polymerase (Stratagene).

Samples were amplified using a DNA thermal cycler (Perkin-Elmer). The first cycle was 5 min at 95℃, 1 min at 60℃, and 2 min at 72℃. The samples were then processed through 42 temperature cycles consisting of 1 min at 94℃ (denaturation), 1 min at 60℃ (annealing), and 2 min at 72℃ (elongation). The last elongation step was lengthened to 10 min. Finally, the samples were soaked at 4℃. The PCR products were cleaned up using the QIAquick Nucleotide Removal Kit (QIAGEN), following the manufacturer's instructions.

1.6 Gel Mobility-Shift Assay[13-16]

Binding reactions were carried out in shift binding buffer containing 4% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10mmol/L Tris-HCl (pH 7.5). The probe (85 000 cpm) was added to each reaction mix. In initial experiments, increasing quantities of poly (dI-dC) from 25 ng to 3200 ng was added to each corresponding reaction mix, except the control. In experiments to characterize protein binding during development or for tissue-specificity, a constant 1 000 ng poly (dI-dC) was added to each reaction mix, except the control. Competition assays also used a constant 1 000 ng poly (dI-dC). For competition experiments, unlabeled oligonucleotide was used. Where appropriate, unlabeled competitor was used at between a 10 fold to 5000 fold molar excess.

After incubation of the protein binding reactions at room temperature for 10 min, 60 μg of nuclear protein extract was added to the reactions in a final 30 μL reaction volume, except the control. The whole reaction was incubated for another 40~50 min at room temperature. The reactions were stopped by addition of 3 μL of 10× loading buffer (250mmol/L Tris-HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol). The resultant DNA-protein complexes were resolved by electrophoresis through a non-denaturing 4% or 5% polyacrylamide gel in 89 mmol/L Tris-HCl (pH 8.0), 89 mmol/L boric acid and 2 mmol/L EDTA. Gels were prerun at 50 mmol/L for 30 min, then run with samples at the same current for 1.5 to 2.0 hours. After the gel was soaked in 5% glycerol for 15 min, the gel was then transferred to a cellulose sheet and dried (Gel Drying Kit, Promega), and autoradiography was carried out using Kodak film at −70℃.

1.7 CAT constructs and assays

The Hsu element sequence was mutated by introducing a random sequence of the same size in place of the Hsu element sequence in the basal promoter region of the LIM2 gene (−200 to −1). Both normal and mutant basal sequences were cloned into the promoterless CAT vector pSVOATCAT[17], respectively. This construct containing mutant Hsu element was called as HBM-200 and its relative normal construct as −200bp. Three different length fragment of basal promoter containing normal Hsu element (−140bp, −100bp and −60bp) were constructed into the vector, pSVOATCAT, and termed as constructs −140bp, −100bp and −60bp, respectively. Each fragment was synthesized using PCR, with the 3′ end of the fragments being identical and ending at residue +53 (to include all of the LIM2 first exon). The 5′ end of each fragment was different and the resulting PCR products ranged in size from 113 bp to 253 bp. CAT constructs and assays were carried out using the general procedures of Chepelinsky *et al*[18], and Klement *et al*[19], with the modification of Cassinotti and Weitz[20], to improve sensitivity. Transient transfections were carried out as described by Klement *et al*[19], using Lipofectamine instead of calcium phosphate. The chick embryo lens epithelial cell cultures were used as described by Borras, *et al*[21]. Cells were co-transfected with plasmid P409 containing the β-Gal gene to monitor transfection efficiency. Forty-eight hours after transfection, the cells were harvested and assays for CAT and β-Gal activity were carried out.

2 RESULTS

Fig. 2 shows the sequence of 1093 bp upstream of the LIM2 CAP site (marked as +1). Also included is the 53 bp first exon (noncoding) of the LIM2 gene. A search of known transcription factors was made of this upstream sequence using the Internet TFSEARCH program (Kyoto University). No known vertebrate transcription factor binding sequences were found throughout this sequence when the program was set to search for 100% matches. In order to determine if lens nuclear proteins bound to DNA sequences immediately upstream of the LIM2 CAP site, we prepared several overlapping 20-mer double-stranded oligonucleotides between the region of −60 through −1, as shown in Fig. 1. These oligonucleotides were labeled and used in gel retardation assays with lens nuclear proteins. As seen in Figure 3, two separate proteins (or protein complexes) appeared to bind to double-stranded oligonucleotide "B" (Fig. 3A). Similar protein complexes also appeared to bind to double-stranded oligonucleotide "D" (Fig. 3B). But, the other three double-stranded oligonucleotides, "A", "C", and "E", appeared to not bind any protein, as observed (Figure 3C, oligonucleotide "A" is an example). And, the bind-

ing of protein complex onto oligonucleotides“B”and“D”did not appear to be affected by titration with poly (dI-dC). Increasing concentrations of poly (dI-dC) from 25 ng to 3200 ng did not appear to have any effect on protein binding (Fig. 3A and B).

```
      -1080                -1060                -1040                -1020
TTATGGGAAGAAAGAAGCTCGAGGTCCAGAGCCACCCCTGGAAACACCACTGGCTGCTGAGGACTGGGAGACAGAGTACT
AATACCCTTCTTTCTTCGAGCTCCAGGTCTCGGTGGGGACCTTTGTGGTGACCGACGACTCCTGACCCTCTGTCTCATGA

         -1000                -980                 -960                 -940
AGGACCTTGGAGGGTCAGGCACCTGAGATTGCAGTCACCCAGGAGGGTTTTCCTGGGGCCCAGAGTGATCTCAGCCCTGT
TCCTGGAACCTCCCAGTCCGTGGACTCTAACGTCAGTGGGTCCTCCCAAAAGGACCCCGGGTCTCACTAGAGTCGGGACA

         -920                 -900                 -880                 -860
CAGAAGAACCCCAAGAAACATCTTCCATCCTCCCCTCTCCCCAAAACCACAATCCCAGGAGTAGCTCCTGTGAGGCACTT
GTCTTCTTGGGGTTCTTTGTAGAAGGTAGGAGGGGAGAGGGGTTTTGGTGTTAGGGTCCTCATCGAGGACACTCCGTGAA

         -840                 -820                 -800                 -780
GGTGAACCTGACCTAAGTCACCTGAATCCCAAGTGAGAAGAAAATCCTAAACCTCGTACCCAAACCTGTCTCCTTGATGG
CCACTTGGACTGGATTCAGTGGACTTAGGGTTCACTCTTCTTTTAGGATTTGGAGCATGGGTTTGGACAGAGGAACTACC

         -760                 -740                 -720                 -700
CCTCAACCCCATTCCCAATGCCATCCCCAACCACAACCACAATCCTTACCTCATTCTCAACCCCCAGGGCTCCTCCAACC
GGAGTTGGGGTAAGGGTTACGGTAGGGGTTGGTGTTGGTGTTAGGAATGGAGTAAGAGTTGGGGGTCCCGAGGAGGTTGG

         -680                 -660                 -640                 -620
CCATTCCCTATCCTCTCCCTAATCCCAAAGCCACTCCTACCTGCCTCTCTCTTCCCCACTCTCAATACCCTGTTCCCAAA
GGTAAGGGATAGGAGAGGGATTAGGGTTTCGGTGAGGATGGACGGAGAGAGAAGGGGTGAGAGTTATGGGACAAGGGTTT

         -600                 -580                 -560                 -540
CCCATTCATTCCCATCCCTGTCACCAGTCCCAGCTCCACTTCTGCGCCTCAACCCTAACCCACCCTCTTTACCAACCCCT
GGGTAAGTAAGGGTAGGGACAGTGGTCAGGGTCGAGGTGAAGACGCGGAGTTGGGATTGGGTGGGAGAAATGGTTGGGGA

         -520                 -500                 -480                 -460
TTCTCCTCATTGCCTGTGTACCTCAGCCCATCTTTAATGTCCTCTGAATCTACAGCCCCAAAGAGCTCCATCCTCAACCT
AAGAGGAGTAACGGACACATGGAGTCGGGTAGAAATTACAGGAGACTTAGATGTCGGGGTTTCTCGAGGTAGGAGTTGGA

         -440                 -420                 -400                 -380
CAGCCACAGCCCTGTGTCCATCCCCACAAAGAACCGCATTGGCCCCACATACCCAGACACCTGTGACTCCCCACCCCACT
GTCGGTGTCGGGACACAGGTAGGGGTGTTTCTTGGCGTAACCGGGGTGTATGGGTCTGTGGCACTGAGGGGTGGGGTGA

         -360                 -340                 -320                 -300
GCCTGCTCTTCAGGTAGAAAGCCCTGGTTGAACAATAAACGCGTGACTCTCCCTGAATCTTGCGTGTGTGCGCGCGCCCG
CGGACGAGAAGTCCATCTTTCGGGACCAACTTGTTATTTGCCGCACTGAGAGGGACTTAGAACGCACACACGCGCGCGGGC
                              FnuD II                              Kha I
         -280                 -260                 -240                 -220
GTGTGTGTGTGTGTGCGTGTGTGCGTGCTTACACAGCTCGGACAAAGCCAGGGTTGCTTTGAGCAAGGCTGGCGACA
CACACACACACACACACGCACACACGCACGCAATGTGTCGAGCCTGTTTCGGTCCCAACGAAACTCGTTCCGACCGCTGT
CA-repeat
         -200                 -180                 -160                 -140
AGGTATTGGGTTCCGGGGAGGGTCACAGAGGTCAGTGAGTGGGTGGGGGGAGCCCCCTGGATGCCTGCTTCAGCGAATTC
TCCATAACCCAAGGCCCCTCCCAGTGTCTCCAGTCACTCACCCACCCCCCTCGGGGGACCTACGGACGAAGTCGCTTAAG
          Hpa II/Msp I
         -120                 -100                  -80                  -60
CATTTGAGGCCTCTGCCACCGCTGCATTTCTGCCTGCTGGAAGCTGCTGGGTAGTGGGACCATTGTGTAGGGAGGCTTAA
GTAAACTCCGGAGACGGTGGCGACGTAAACGACGGACGACCTTCGACGACCCATCACCCTGGTAACACATCCCTCCGAATT

         -40                  -20                  +1                   +20
GGGATTTGGGGAACCTAAGGGGGCAAAGAGGCCAGTGACCAGGCTCAGCTCAGTCTGCACAGAGGGGCCGTGGCAGAAGG
CCCTAAACCCCTTGGATTCCCCCGTTTCTCCGGTCACTGGTCCGAGTCGAGTCAGACGTGTCTCCCCGGCACCGTCTTCC

         +40
CAGAAGGAGGGCTCGGGCAGGCTCTGCCACTCAG
GTCTTCCTCCCGAGCCCGTCCGAGACGGTGAGTC
```

Fig.2 Nucleotide sequence of the upstream region of the human LIM2 gene, including the first 53 bp of the first noncoding exon. Methylation islands are indicated by underlining of both strands. The CA-repeat is marked by lines above and below the sequence. Numbers, both + and -, indicate nucleotide position relative to the first nucleotide in the CAP site.

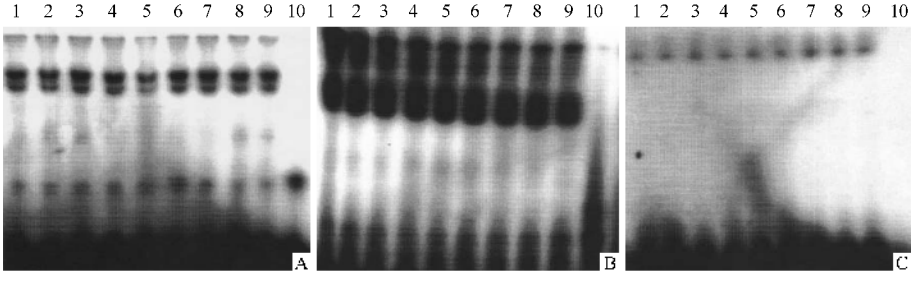

Fig.3 Gel retardation assay using overlapping 20-mer double-stranded oligonucelotides, as described in Fig. 1. Panel A is oligonucleotide“B”from Fig. 1. Panel B is oligonucleotide“D”from Fig. 1, and panel C is oligonucleotide“A”from Fig. 1. Oligonucleotides were labeled and incubated with lens nuclear proteins as outlined in the Methods and Materials. Radiolabeled probes (~ 10^8 cpm/μg) were incubated with 60 μg of chicken lens nuclear protein extracts (E12). The poly (dI-dC) added in lanes 2 to 9 were 25, 50, 100, 200, 400, 800, 1600, 3200 ng, respectively. Lane 10 was probe only, and lane 1 was probe + lens protein without poly (dI-dC).

As can be seen in Fig. 1, we synthesized the oligonucleotide termed as" Hsu element "covered the region of -47 to -32 from the LIM2 CAP site, which contained in the majority of oligonucleotide " D "and the first half of oligonucleotide" B "appeared to be the common denominator between the two. Hsu element coding strand called as oligonucleotide # 2," 5′-GGGGAACCTAAGGGGG-3′ ", and the noncoding strand called as oligonucleotide # 7," 5′-CCCCCTTAGGT-TCCCC-3 ". It was found that only the coding strand of the Hsu element bound two protein complexes (Fig. 4, left panel) while the noncoding strand not bind any protein (Fig. 4, right panel).

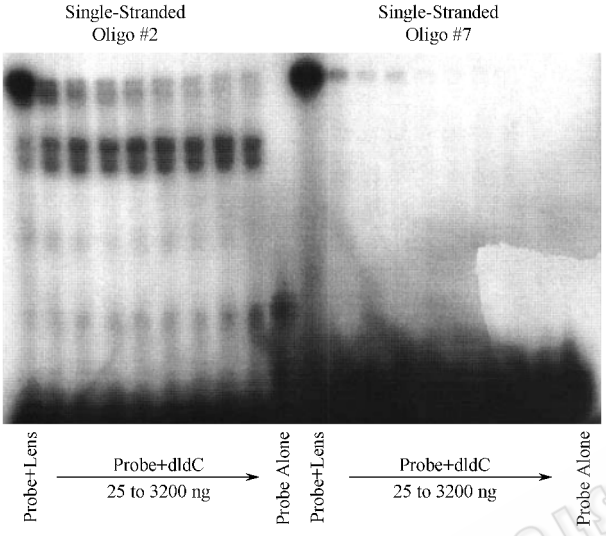

Fig.4 Gel retardation assays of nuclear proteins which bound to single-stranded oligonucleotides # 2 and # 7 (20 bp in length, # 2 was the sense strand and # 7 was the complement)

These oligonucleotides covered the region of -47 to -32 from the LIM2 CAP site. Assay conditions were as described in Fig. 3.

To demonstrate that lens nuclear proteins bound to a double-stranded configuration within Hsu element, a radioactive gel shift reaction was carried out using lens nuclear proteins, in which radioactive single-stranded oligonucleotide # 7 was run alone (Fig. 5, panel A) or made a double-stranded oligonucleotide with radioactive oligonucleotide # 7 (noncoding strand) and non-radioactive oligonucleotide # 2 (coding strand) (Fig. 5, panel B). The protein binding in Fig. 5B occurred identical to that seen with double-stranded oligonucleotide " B "or" D "in Fig. 3A and 3B. The single-stranded antisense (noncoding) strand failed to bind lens nuclear protein (Fig. 5A). Thus, lens nuclear proteins appeared to bind only the sense stand in a double-stranded configuration.

Nuclear proteins were extracted from chick brain, heart, limbs, and liver, and compared to lens proteins in a gel retardation assay. Fig. 6 shows that all of the nonlens tissues appear to contain the faster migrating complex of the two (Fig. 6, band B) seen in lens extracts. The slower migrating complex (Fig. 6, band A) did not see in brain, limb and liver extracts. Though the slower migrating complex could be seen in heart extracts, the band in both shape and migration appeared to be different from that of lens extracts comparing lane 5 with lane 2 or lane 3 in Figure 6. By using the lens nuclear extract from chicken E8, E10, E12, E14, E16 and E18 respectively, the presence of protein binding to the Hsu element during chick lens development was investigated. As shown in Figure 7, both lens protein complexes bound to the Hsu element that were similar to that seen in Figure 3A, appeared to be present in all of the stages of chick lens development.

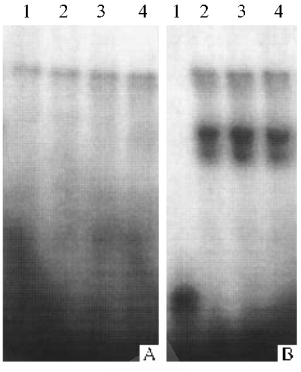

Fig.5 Gel retardation assays uere used to demonstrate that lens nuclear proteins bound to a double-stranded configuration within the Hsu element

Radioactive single-stranded oligonucleotide # 7 (nonsense strand of Hsu element, panel A) was run alone or made double-stranded with non- radioactive oligonucleotide # 2 (sense strand of Hsu element, panel B), and gel shift analysis carried out using lens nuclear proteins.

In each group, the radiolabeled probe (-10^8 cpm/μg) was incubated with 60 μg of chicken lens nuclear protein extracts (E12). Lanes 1 in both panels was probe alone. Lanes 2 were a probe + lens nuclear protein. Lanes 3 were probe + lens nuclear protein + 500 ng of poly (dI-dC), and lanes 4 were probe + lens nuclear protein + 1000 ng of poly (dI-dC).

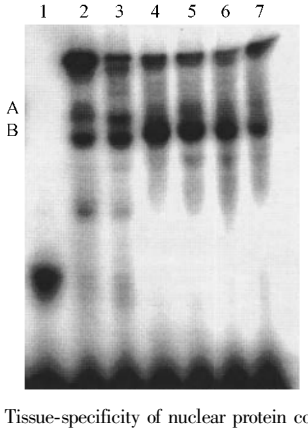

Fig.6 Tissue-specificity of nuclear protein complexes binding to the Hsu element

Radiolabeled Hsu element (-10^8 cpm/μg) was incubated with 60 μg of nuclear protein extracts from different chicken tissues: Lens (lanes 2 and 3), brain (lane 4), heart (lane 5), limb (lane 6), and liver (lane 7). The content of poly (dI-dC) added in all lanes was 1 000 ng, except lanes 1, and 2. Lane 1 was probe only and lane 2 was probe plus chicken lens nuclear protein (E10).

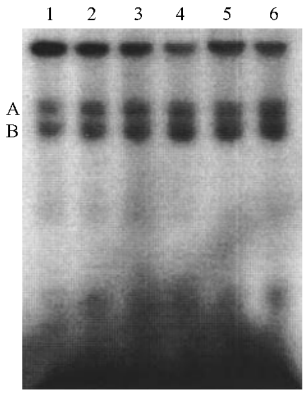

Fig.7 Lens nuclear protein binding to the Hsu element during development

Radiolabeled Hsu element, (nucleotides −47 to −32, -10^8 cpm/μg) was incubated with 60 μg of lens nuclear protein extracts from chicken E8 (lane 1), E10 (lane 2), E12 (lane 3), E14 (lane 4), E16 (lane 5), and E18 (lane 6), respectively. The content of poly (dI-dC) added in each lane was 1 000 ng. A and B indicated the two Hsu element lens protein-DNA complexes.

Fig. 8 shows the protein binding patterns of single-stranded Hus element from the normal sequence −47 to −32 (5′-GGGGAACCTAAGGGGG-3′) and three mutants. Two of which have an altered inner sequence with the shell of poly“Gs”left alone (i.e., 5′-GGGGCTAAAACGGGGG-3′, and 5′-GGGGCATAACAGGGGG-3′); one which replaced the poly“Gs”with poly“Cs”(i.e., 5′-CCCCAACCTAACCCCC-3′), which would be present in the complementary strand, but keeping the inner sequence the same as the normal coding sequence. As can be seen in the middle two panels of Fig. 8, in all cases where the poly“Gs”were left intact, protein binding remained very similar to the normal shown in left panel of Fig. 8, although the bands appeared to be more diffuse than that in the normal Hsu element. However, when the normal inner sequence was left intact and the poly“Gs”were changed to poly“Cs”, no binding whatsoever was observed (Fig. 8, far right panel). When the complementary sequences were synthesized for each of the oligonucleotides tested in Fig. 8 and protein binding assays carried out, it was observed that whenever poly“Cs”formed the shell, regardless of the inner sequence, no binding was observed (Fig. 9, left panel and middle two panels). However, when the outer shell was composed of poly“Gs”and the inner sequence was the complement of the normal sequence, a new and faster migrating protein binding band was observed (Figure 9, far right panel), seemed to same as the C band seen in the middle two panels of Fig. 8.

Fig. 10 shows the competition experiments between the normal Hsu element oligonucleotide and several different Hsu element mutants by using gel shift analysis. Radioactive normal Hsu element (5′-GGGGAACCTAAGGGGG-3′) was competed with nonradioactive mutant (5′-GGGGTTGGATTGGGGG-3′) in order to determine if both the normal Hsu element and the mutants were binding the same protein complexes. Interestingly, the faster migrating complex of the two (band B in Fig. 10) was competed out much faster at a lower mutant quantity than the slower complex (band A in Fig. 10), and all binding protein was removed from the radioactive normal Hsu element at between 1 000 and 5 000-fold excess of nonradioactive mutant.

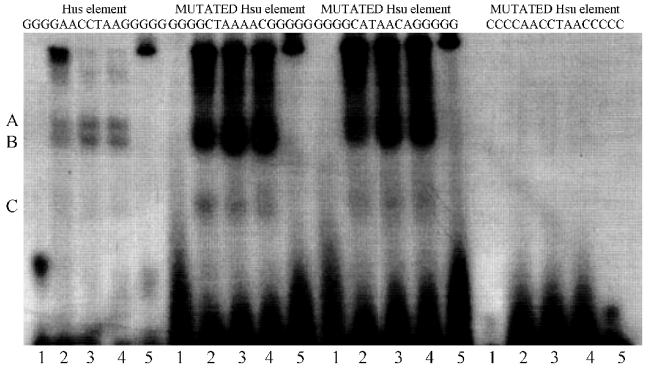

Fig.8 Gel retardation assays of nuclear proteins binding to Hsu element coding strand and various mutations of this sequence

In each group, radiolabeled Hsu element or different mutations ($\sim 10^8$ cpm/μg) were incubated with 60 μg of chicken lens nuclear protein extracts (E8) (lanes 2 – 4) or with 60 μg of crystallin (lane 5). Lane 1 was probe only, and lanes 2 – 5 were probe and lens nuclear protein extract. Lanes 3, 4 and 5 contained 500 ng, 1000 ng, and 500 ng poly (dI-dC), respectively. A, B, and C indicate lens protein-DNA complexes.

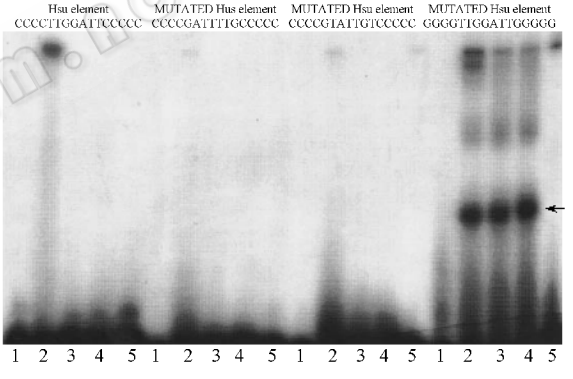

Fig.9 Gel retardation assays of nuclear proteins binding to Hsu element complementary strand and various mutations of this sequence

In each group, radiolabeled Hsu element complement or mutations (−108 cpm/μg) were incubated with 60 μg of chicken lens nuclear protein extracts (E8) (lanes 2 – 4) or with 60 μg of crystallin (lane 5). Lane 1 was probe only, and lane 2 – 5 were probe and lens nuclear protein extract. Lanes 3, 4 and 5 contained 500 ng, 1000 ng, and 500 ng poly (dI-dC), respectively. Arrow indicated a new and faster migrating lens protein-DNA complexes.

CAT activity of both normal and mutant basal constructs (−200bp and HBM − 200) containing Hsu element were compared to three normal upstream constructs (−140bp, −100bp and −60bp) in transfected chick lens cell cultures. The promoter-less CAT vector pSVOATCAT did not contain LIM2 promoter and acted as a control. Comparing with the low promoter activities exhibited by the constructs (−140bp, −100bp and −60bp) containing normal Hsu element, the construct (−200bp) normal basal promoter showed a more high promoter activity. As illustrated in Figure 11, however, the construct

(HBM – 200) containing the mutant Hsu element sharply displayed an over 75% decrease in CAT activity compared to the normal basal promoter (– 200bp) sequence.

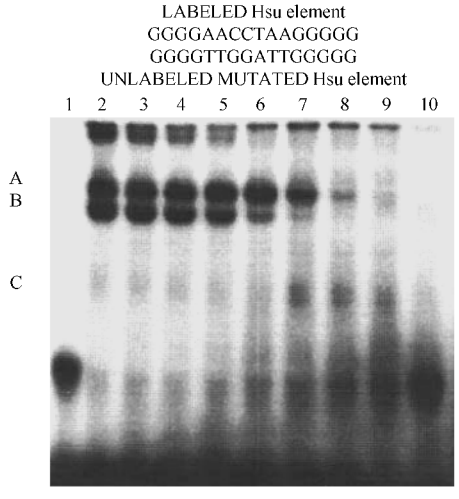

Fig.10 Gel-shift analysis of competition experiments using radioactive Hsu element and nonradioactive mutated Hsu element sequence

Lane 1 was probe only. In lanes 2 to 10, radiolabeled Hsu element (-10^8 cpm/μg) was incubated with 60 μg of chicken lens nuclear protein extracts (E12) and 1 000 ng of poly (dI-dC), respectively. Each nonlabeled mutant sequence was added in different concentration as indicated in the figure. A, B, and C indicated the lens protein-DNA complexes.

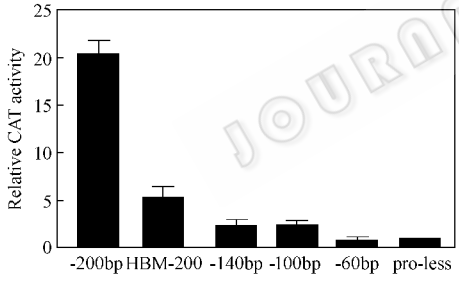

Fig.11 Relative promoter activity of various LIM2 upstream sequences cloned into the promoterless CAT vector pSVOATCAT, as outlined in the Materials and methods

3 DISCUSSION

The search with TFSEARCH program did not find any 100% matched binding sequence for a known transcription factor within 60 nucleotides upstream of the LIM2 CAP site. Interestingly, no TATA or CAAT box sites were observed within this region, which is usually the binding site for the transcription complex, including RNA polymerase. Therefore, it is necessary to determine DNA sequences immediately upstream of the LIM2 CAP site where lens nuclear proteins could bind. To reach the question raised, the assays for DNA-protein interaction in this study were used to identify the lens-specific cis-acting element within basal promoter of LIM2 gene.

When several overlapping 20-mer double-stranded oligonucleotides between the region of – 60 through – 1 of LIM2 promoter were used as the substrate that lens nuclear proteins bound, as shown in Figure 3A and 3B, the gel retardation assays obviously showed that two lens nuclear complexes bound onto the region of – 50 to – 31. Increasing concentration of non-specific poly (dI-dC) up to 3200 ng, the DNA-protein interactions were still stable. Therefore, it seemed that the binding onto the site (region) was lens protein-specific.

The sense-strand "Hsu element" covered the region of – 47 to – 32, shorter than that of – 50 to – 31, but two protein complexes could bind onto this double-stranded oligonucleotide in an identical fashion as seen in Figure 3A. Since by far the majority of all proteins within the lens cell are composed of crystallins, this protein may very well be a contaminant in the lens nuclear protein preparations and cause artifactual binding of crystallins to DNA. To test this possibility, a mixture of purified lens crystallins was run in parallel will several of the gel retardation assays, with completely negative results (not shown). It appeared that crystallins were not involved with the observed protein binding to the oligonucleotides. This then appeared to be the sequence that was binding lens nuclear proteins only.

In Fig. 4, the two protein complexes binding onto the coding strand of Hsu element did not be effected by the increasing concentrations of poly (dI-dC) from 25 ng to 3200 ng as seen in Fig. 3A. Therefore, both experiments shown in Figures 3 and 4 indicated that this sequence, from – 47 to – 32 upstream of the LIM2 CAP site, appeared to be the only sequence in the immediate upstream region to bind lens nuclear proteins. Other preliminary studies stated that within 160 bp upstream of the LIM2 CAP site, no other nuclear protein binding was observed (unpublished). This Hsu element may, indeed, serve as an RNA polymerase complex binding site.

Since only the coding strand appeared to be responsible for binding protein, it was suspected that this sequence of Hsu element might be only a single-stranded protein binding site, and not overly important for transcription. To check the raised question, we therefore carried out an experiment as seen in Fig. 5 in which the double-stranded oligonucleotide consisted of a radioactive noncoding strand and nonradioactive coding strand. The radioactive gel shift reaction observed in Fig. 5 clearly revealed that the lens nuclear proteins must bind to the sense strand in a double-stranded configuration. This is not an unusual occurrence in mammalian genes. Such single-stranded DNA binding proteins usually appear to act as transcriptional repressers[22 – 26].

Both pattern and migration of band A seen in Fig. 6 appeared to show a difference in the DNA-protein interaction onto Hsu element between lens extracts and other tissue extracts. However, further work is required to prove the tissue-specificity of lens nuclear protein complexes binding to the Hsu element and the significance of this observation. Fig. 7 indicated that the presence of protein binding to the Hsu element during chick lens development presented in approximately the same proportions and quantity throughout development. Apparently,

the trans factors for the Hsu element within the LIM2 promoter are always present, as would be expected of an RNA polymerase complex binding site.

Examination of the sequence from −47 to −32 (Hsu element) indicated that the poly"Gs"on each end of the sequence might form a "shell", perhaps binding to one protein complex with the inner sequence (AACCTAA) binding to the other complex. To demonstrate the raised hypothesis, we therefore synthesized a series of mutated oligonucleotides to see the binding pattern of lens protein onto the Hsu element. When the guanines at either ends of the Hsu element were altered, no DNA-protein interactions were seen (Fig. 8, far right panel). As seen in the middle two panels of Fig. 9, the protein binding assays using the relevant complementary oligonucleotides for each of mutations tested in Fig. 8 supported the conclusion above.

Clearly, the poly"G"shell was essential for protein binding as illustrated in Fig. 8 and 9. The mutations in the inner sequence of the Hsu element that left the guanines intact could produce a new and faster migrating protein (complex) as seen in the middle two panels of Fig. 8 and far right panel of Fig. 9, meaning that the inner sequence could play a role to assist and strength the binding of nuclear extract onto the Hsu element. As shown in Fig. 10, it was interesting that as the nonradioactive mutant sequence increased in quantity for the competition assays, a new binding complex began to appear, which migrated much faster (band C in Fig. 10). This band was very similar in mobility to the band observed when the complementary Hsu element inner sequence was combined with poly"Gs"in the outer shell (Fig. 9, far right panel). It appeared that the complementary inner sequence of the Hsu element may bind a certain portion of the protein complex, possibly stabilizing the overall complex.

The mutant oligonucleotides contained poly"Cs"in the outer shell failed to compete with the normal Hsu element for protein binding. When radioactive normal Hsu element was competed with a mutated sequence contained poly"Gs"in the outer shell and the complement of the normal inner sequence, a clear competition for binding protein was observed (no shown). Both competition experiments also further supported the conclusion above.

The necessity of the Hsu element for lens nuclear protein binding could be reflected in their promoter activity. An over 75% of CAT activity could be dropped down when mutated the Hsu element in the normal basal promoter (n−200) of LIM2 gene. The relative promoter assays of various LIM2 basal promoter regions shown in Fig. 11 indicated that this sequence of Hsu element is essential for promoter activity.

We have demonstrated that an immediate upstream sequence within the LIM2 basal promoter between −47 to −32 (Hsu element) could bind two lens nuclear protein complexes. This sequence appeared to be very important for LIM2 promoter activity, since mutation of this sequence decreased LIM2 promoter activity by over 75%. The proteins binding to the Hsu element appeared to only bind to the sense strand, however this binding occurred while the DNA was double-stranded. Mutational analysis indicated that the poly"G"shell was of primary importance for protein binding. This sequence may serve as the primary binding site for the RNA polymerase complex, since the LIM2 gene promoter does not contain a consensus TATA box sequence. Further work is required to prove the lens-specific nuclear protein complexes binding to the Hsu element and their significance in the regulation of LIM2 expression.

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