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CELL TYPE SPECIFIC SPLICING of a HETEROLOGOUS EXON MEDIATED by *cis* ELEMENTS from FIBROBLAST GROWTH FACTOR RECEPTOR 2 Pre-mRNA

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Splicing of fibroblast growth factor receptor 2 (FGF-R2) pre-mRNA is an example of tightly regulated alternative splicing in which exons IIIb and IIIc are utilized in a mutually exclusive manner in different cell types. Appropriate splicing of exon IIIb in prostate cancer DT3 cells requires previously described *cis* elements which activate splicing of the upstream exon. We supposed that these elements could also repress splicing of an downstream exon (IIIc exon in the case of FGF-R2 pre-mRNA). As a model minigens containing *cis* elements in different combinations upstream of a heterologous exon from cardiac troponin I (cTNI) flanked by β–globin intron have been used. Results of transfection of these constructs in DT3 and AT3 cancer cell lines have supported our suggestion. Regulation of alternative splicing could probably be occurred through formation a stem structure between ISE-2 and ISAR sequences and its interaction with ISE-3.

Օգտագործվել են մինիգեներ, որոնք պարունակում են տարբեր համակցություններում կարդիոտրոպինի (cTNI) գենից օտարածին էքզոնի *cis* էլեմենտներ β–գլոբուլինի գենի ինտրոնների շրջանակներում: Սպլայսինգի ալտերնատիվ կարգավորումը ամենայն հավանականությամբ իրականացվում է միջուկային կառուցվածքի ձևավորման ճանապարհով ISE-2 և ISAR նուկլեոտիդ հաջորդականությունների միջև և ISE-3-ի հետ նրա փոխազդեցության միջոցով:

Сплайсинг рецептора 2 фактора роста фибробластов (РФРФ2) является наглядным примером регуляции альтернативного сплайсинга, при котором в различных видах клеток на взаимоисключающей основе используется либо экзон IIIb, либо IIIc. Обычный сплайсинг экзона IIIb в клетках рака простаты DT3 сопровождается участием ранее охарактеризованных *cis* элементов, которые активируют сплайсинг вышерасположенного экзона. Мы предположили, что эти элементы также могут репрессировать сплайсинг нижерасположенного экзона (экзона IIIc в случае пре-мРНК РФРФ2). В качестве модели мы использовали минигены, в различных комбинациях содержащие *cis* элементы чужеродного экзона из гена кардиотропина (cTNI) в окружении интронов гена β–глобулина. Результаты трансфекции этих минигенов в раковые клетки простаты DT3 и AT3 подтвердили наше предположение. Регуляция альтернативного сплайсинга, возможно, осуществляется путем формирования стволовой структуры между нуклеотидными последовательностями ISE-2 и ISAR и ее взаимодействия с ISE-3.

Key words: *gene engineering - fibroblast - splicing - exon*

Alternative splicing represents a commonly used pathway through which different gene products can be produced from a single gene. An example of alternative

splicing in which tight regulation results in a very defined cell type discrepancy in the differential expression of isoforms occurs during the splicing of the second half of the third immunoglobulin-like domain of the fibroblast growth factor receptor 2 (FGF-R2). Mutually exclusive splicing results in a mRNA containing either the 148-nucleotide IIIb exon or 145-nucleotide IIIc exon (Fig. 1), a choice which is specific for a given cell type. The isoform of FGF-R2 that results when exon IIIb is included, FGF-R2 (IIIb), is the exclusive gene product observed in normal prostatic epithelia and in a well-differentiated, androgen-dependent DT3 prostate tumor or cell line. A poorly differentiated and androgen-independent AT3 tumor exhibits a change in splicing that results in loss of FGF-R2 (IIIb) and in expression of FGF-R2 (IIIc). The markedly different growth factor specificities of the resulting receptors alter signal transduction pathways, which have been proposed to be involved in the progression of prostate cancer [1-3]. Thus, investigation of general mechanisms of splicing regulation can have more far-reaching implications towards understanding how alterations in normal cellular processes can lead to cancer development.

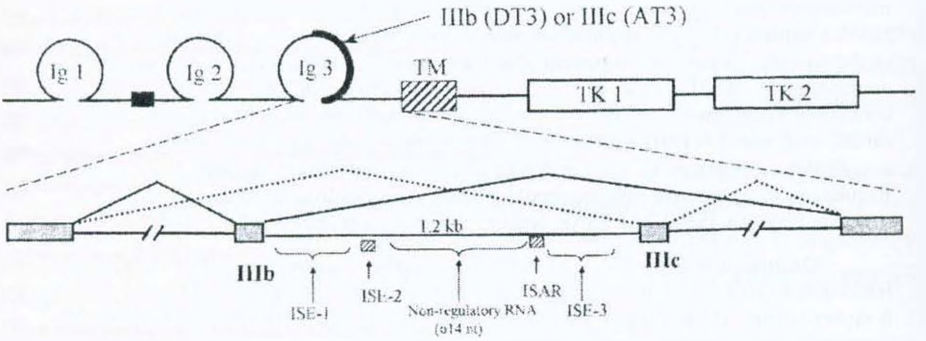

Fig. 1. Schematic representation of alternative splicing variants of FGF-R2. At top is a protein domain map with the region encoded by exons IIIb or IIIc indicated. At bottom is a map of the pre-mRNA. Hatched boxes represent deifined intronic splicing elements and regions containing other elements are indicated by brackets. The horizontal arrows and dashed line underneath the pre-mRNA represent the sequence that was positioned in a heterologous intron for transfection studies.

Studies of rat and human FGF-R2 splicing have revealed the presence of several *cis* elements, which can alter the splicing of mutually exclusive alternative exons IIIb and IIIc [4-8]. We have previously characterized ISAR (for Intronic Splicing Activator and Repressor), a *cis* element located in the intron between exons IIIb and IIIc of the rat gene. This element dramatically increases the splicing efficiency of the upstream IIIb exon in DT3 cells and represses the splicing of the downstream IIIc exon [6,9]. ISAR was not shown to have an effect on the relative inclusion of either exon in AT3 cells. We have identified a second element, ISE-2 (for Intronic Splicing Enhancer), which is located 736 nt upstream of ISAR. A role in the regulation of FGF-R2 splicing can also play ISE-1 and ISE-3, other possible intronic *cis* elements.

A convenient method to assess the function of the above mentioned elements is to determine the ability of each individual element to modulate the splicing pattern of a heterologous exon [10].

Materials and methods. **Plasmid construction.** The plasmid DNAs used in this study were all made on the base of pPIP11 adenoviral splicing construct described previously [6]. pI-11(-H3)PL minigene was derived from pI-11 by digestion with Hind III restriction endonuclease. The digested ends were further blunted with *Pfu* polymerase (Stratagene) and religated with T4 DNA ligase. The resulting plasmid was created by cloning annealed oligonucleotides Poly F (5'-CTAGAGGCCGTCGACGCTAGCGCGGCCGCGATAT CATCGATACTAGTGGCC-3') and Poly R2 (5'-TCGAGGGCCACTAGTATCGATGA TATCGCGGCCGCGCTAGCGTCGA CGGCC-3') in *Xba I* and *Xho I* sites of pI-11(-H3) plasmid. As a source of heterologous exon a splicing construct from Tom Cooper (Baylor Univ.) that consists of truncated exon (28 nt) of cardiac troponin I (cTNI) flanked by β–globin intron was used. This sequence was cloned in Cla I and Xho I sites of pI-11(-H3)PL minigene. Plasmids studied containing sequences from intron 8 upstream of heterologous exon were obtained following amplification with forward primers 3BF-Two and 3BF-Four, and reverse primer Int 2R2 using templates pI-11-FS:Δ3 and pI-11-FS:Δ4 [9].

PCR amplification. PCR from DNA templates was performed with 5 ng of plasmid DNA and *Pfu* DNA polymerase (Boehringer Mannheim) according to the supplier's recommendations. Amplifications were performed with a Perkin-Elmer GeneAmp PCR System 9700. A typical cycle consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. After completion of the final cycle, a final extension was done at 72°C for an additional 7 min.

Cell culture and transfection. AT3 and DT3 cells were maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). All transfections were performed in 35-mm-diameter wells with 5 μl of Lipofectamine (Gibco) according to the supplier's recommendations. Each well was seeded with 4 X 10^5 cells 20 to 24 hr prior to transfection. Stable transfections were performed with 2 μg of linearized minigene DNA, and after 24 to 48 h the cells were trypsinized and reseeded in 75-cm^2 flasks containing Geneticin (Gibco) at an active concentration of 400 μg/ml, Penicillin (100 U/ml) and Streptomycin (100μg/ml). Selection was performed until isolated colonies were obtained (usually 14 to 20 days). Pooled colonies were then harvested for RNA preparation.

RNA purification and RT-PCR analysis. Total cellular RNA was isolated from transfected cells by the method of Chomczynski and Sacchi [11]. Two micrograms of total RNA was heated to 100°C, chilled on ice, and reverse transcribed in a reaction volume of 20 μg containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1o mM dithiothreitol, 1 mM deoxynucleoside triphosphates, 100 ng of random hexamers, 2 U of RNasin (Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco) at 37°C for 1 h. Samples were then heated to 90°C for 5 min and then chilled on ice. Two microliters of each reverse transcription (RT) reaction mixture was amplified in 100-μl PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 200 μM (each) dATP, dGTP, and dTTP, 50 μM dCTP, 100 nM each primer, 10 200 μCi of [α-^{32}P]dCTP, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). The primers used were PIP10-F (5'-CCCGGGGGTACCGGGCGAATTCG AATTCGAGCTCACTC-3') and PIP11-R (5'-CCCGGGACTAGTAAGCTTAGGCTCT TGGCGTT-3'). Amplification conditions consisted of an initial denaturation at 94°C for 5 min, followed by 25 to 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 7 min. Aliquots representing equal amounts of each PCR reaction mixture were loaded directly on nondenaturing 5% polyacrylamide gel at 100 V to 4-5 h, followed by drying and exposure to Amersham Hyperfilm-MP. Analysis was performed with a Molecular Dynamics PhosphorImager.

Results and discussion. Study of alternative splicing is complicated by the fact that regulation requires the presence of several *cis* elements. It appears that the factors that interact with these elements in combination determine the splicing pattern in a given cells. It is often not clear whether a defined RNA element or group of elements independently affects splicing efficiency or whether some of these elements must act in concert. In the case of FGFR2, a number of elements besides ISE-2 and ISAR are clearly involved in modulating splicing efficiency. In our previous studies of rat FGFR2 transcripts we have also identified a couple of other

intronic *cis* elements such as ISE-1 and ISE-3, downstream of exon IIIb, that affect splicing of this pre-mRNA. Studies of a group analyzing human FGFR2 splicing have identified three intronic elements (termed IAS-1, IAS-2, and IAS-3, for Intron Activator of Splicing) [4,7]. While IAS-2 and IAS-3 are highly similar to the rat elements we call ISE-2 and ISAR, respectively, IAS-1 is an uridine rich element that binds the ubiquitous TIA-1 protein and promotes splicing to the exon IIIb 5'-splice site in a non-tissue-specific manner. It thus appears that IAS-1 activates exon IIIb splicing, but that tissue specific activation mediated by other *cis* elements (such as dsRNA-SE and ISE-3) is required to result in efficient cell specific exon IIIb splicing. Rat FGFR2 also contains an uridine stretch downstream of the exon IIIb 5'-splice site, ISE-1, and although the sequence is not identical to IAS-1, it is likely that a similar phenomenon activates exon IIIb splicing in rat transcripts as well.

We chose to limit study of RNA *cis* elements to sequences in the intron between exon IIIb and exon IIIc (intron 8) that have been shown to activate upstream exon IIIb splicing. Some of these elements were supposed to decrease the splicing efficiency of the downstream exon IIIc. To confirm this hypothesis minigenes were generated in which FGFR2 intron 8 sequences were positioned upstream of T.Cooper's R35(28) heterologous exon [10] and tested for ability to repress splicing (Fig. 2). These minigenes were transfected into DT3 and AT3 cells, followed by RT-PCR to determine levels of exon inclusion versus skipping. Because a deletion of 614 nt from the middle of this intron had previously been shown to be inessential for splicing regulation, we did not include this region in any minigenes.

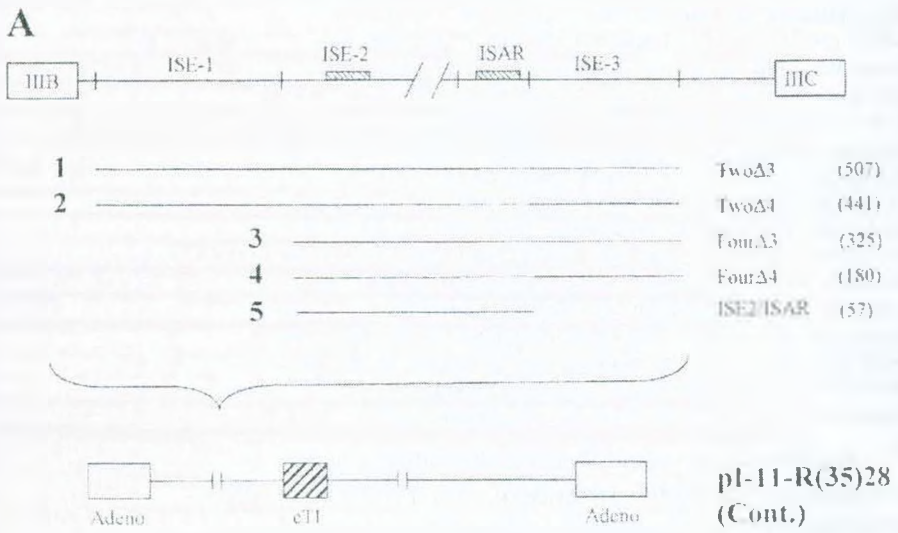

Fig. 2 Intron 8 elements from FGF-R2. Solid lines represent portions of this intron that were relocated upstream of the c TNI exon. Dashed lines indicate deleted sequence followed by the size of the repositioned intron sequence.

As seen in Fig 3, regions of FGFR2 intron 8 that include both ISE-2 and ISAR sequences followed by ISE-3 decreased dramaticly splicing of the downstream exon from 61.4 (control) to 24.2 for pI-CX-R35(28)-TwoΔ3 and 10.4 percent for

pI-CX-R35(28)-FourΔ3 in DT3 cells. We suppose that ISE-3 can perform a role of not only intronic splicing enhancer but also intronic splicing silencer (ISS) for a downstream exon. On the other hand, a sequence containing ISE-1, ISE-2 and ISE-3 but not ISAR did not repress splicing of the heterologous exon. In AT3 cells, insertion of these sequences did also not decrease splicing of the downstream exon, and in fact an increase in exon inclusion was even seen. Thus, *cis* elements in this intron, which include ISE-2, ISAR, and ISE-3, efficiently repress splicing of a downstream heterologous exon in a cell-specific manner. Deletion of the ISAR sequence resulted in increase in exon inclusion from 24.2 to 71.1 percent for TwoΔ3 and TwoΔ4 series, and from 10.4 to 30.1 percent for FourΔ3 and FourΔ4 series plasmids. However, a 57 nt fragment containing ISE-2 and ISAR only did not repress downstream exon splicing in DT3 cells, consistent with a possible requirement for ISE-2 and ISAR to cooperate in order to repress or/and activate splicing. Although variable levels of inclusion were observed in AT3 cells with these constructs, it should be noted that inclusion of these sequences never significantly decreased splicing of that of the control.

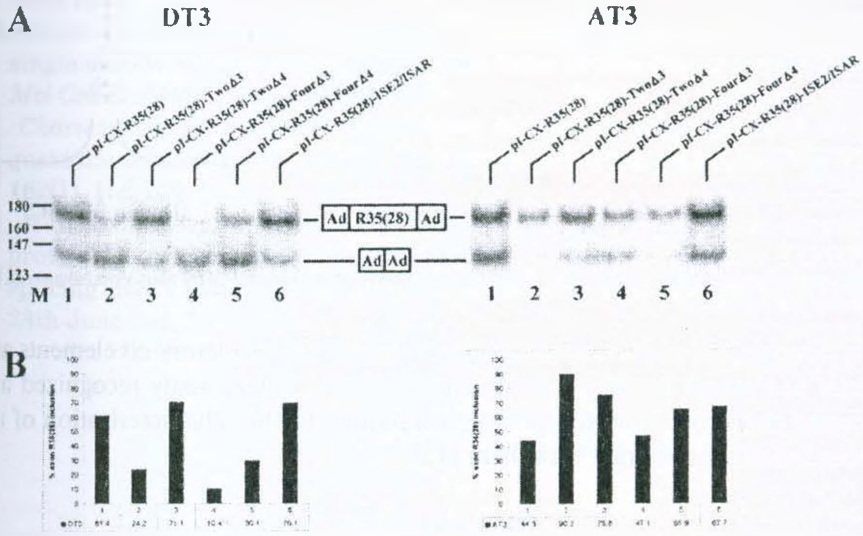

Fig. 3. Intron 8 sequences from FGF-R2 can repress *in vivo* splicing of heterologous cTroponin I exon located downstream in DT3, but not AT3 cells. **A.** RT-PCR of RNA from DT3 and AT3 cells stably transfected with minigenes containing intron 8 elements from FGF-R2 that were relocated upstream of the cTNI (R35(28)) exon. Boxes labeled *Ad* indicate the adenoviral exons. **B.** Graphical representation of percent exon inclusion.

Studies performed by another group investigating splicing of human FGFR2 demonstrated a sequence that was highly similar to the rat ISAR that was also essential for exon IIIb splicing; a sequence they termed IAS-3 [7]. These studies also implicated a second element, IAS-2, located approximately 700 nt upstream of IAS-3 and furthermore suggested that these elements form a secondary structure that is involved in activation of exon IIIb splicing. We identified a rat sequence, ISE-2, highly similar to IAS-2 which is located 736 nt upstream ISAR. Alignment of

ISE-2 with ISAR was consistent with formation of a putative RNA secondary structure although one not identical to that proposed from the human sequence elements (Fig. 4). This was also consistent with the observation that a minimal 20 nt ISAR was involved in exon IIIb activation since additional nucleotides did not contribute to formation of this stem structure. We suppose that in our model, the stem structure formed between ISE-2 and ISAR is required not only for activation of an upstream exon splicing but also for repression of a downstream exon splicing.

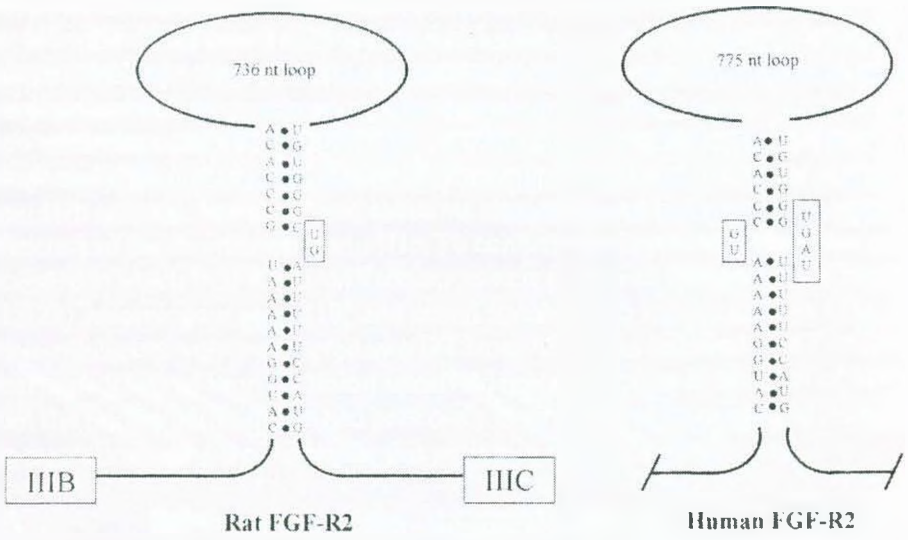

Fig. 4. Putative secondary structure between ISE-2 and ISAR in rat and human FGFR2 transcripts (sequences that do not base pair are boxed).

Elucidation of the mechanism by which several regulatory *cis* elements are, directly or through forming a secondary structure, differentially recognized and processed during pre-mRNA splicing will require further characterization of the protein factors that interact with them [12].

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