Exon 3 of the α folate receptor gene contains a 5′ splice site which confers enhanced ovarian carcinoma specific expression

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Abstract The human folate receptor (FR) is overexpressed in ovarian carcinoma. FR transcripts are heterogeneous due to the use of two promoters, P1 and P4, and alternative splicing of exon 3. RNase protection assay and RT-PCR revealed higher levels of the transcripts that include exon 3 in lines and specimens from ovarian carcinoma. A P1–chloramphenicol acetyltransferase (CAT) construct containing exon 3 demonstrated efficient reporter expression only in ovarian carcinoma. 5′ and 3′ deleted variants of the P1–CAT construct were analyzed by RT-PCR of the exogenous transcripts and reporter activity. A 5′ splice site and 35 bp downstream intronic region of exon 3 appeared to regulate enhanced FR expression in ovarian carcinoma. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Transcription regulation; Alternative splicing; α Folate receptor; Ovarian carcinoma; Gene therapy

1. Introduction

The human α folate receptor (αFR), a 38 kDa membrane glycoprotein, is selectively overexpressed in 90% of non-mucinous ovarian carcinomas and in some other malignant tissues. The receptor has also been detected in the epithelium of the proximal tubules of kidney and a few other normal cell types [1], but not in normal ovarian surface epithelium. In ovarian carcinoma cell lines [2] and normal human tissues [3], the abundance of αFR transcripts is proportionate to receptor expression, suggesting that transcriptional regulation modulates at least in part expression of the receptor. The gene is composed of seven exons spanning approximately 6.7 kb. The open reading frame (ORF) is encoded by exons 4 through 7, while the reported 5′ non-coding sequence (NCS) of the cDNA isoforms are encoded by exons 1 through 4 [4]. The αFR transcripts cloned from cDNA expression libraries from different cell lines share common ORF and 3′ NCS but show unique 5′ termini [5,6]. This heterogeneity relies on the use of at least two promoter regions, P1, upstream and internal of exon 1 and P4, upstream of exon 4 [4]. An additional mechanism of transcript diversity, i.e. the inclusion of 66 bp of alternatively spliced exon 3, was evidenced in the cDNA sequences of two different P1-derived αFR transcripts (clones #31 and #4/6) isolated from an IGROV1 ovarian carcinoma cDNA expression library [7].

Here we point out that the inclusion of alternatively spliced exon 3 of the αFR gene is enhanced in ovarian carcinoma. We suggest that in the 5′ splice site and downstream 35 bp intron region of exon 3 lies a control element that promotes its efficient inclusion and is one of the mechanisms that contributes to the FR expression specificity in ovarian carcinoma.

2. Materials and methods

2.1. Materials

The following human tumor cell lines were used: the serous ovarian carcinomas IGROV1 (J. Benard, Institute G. Roussy, Villejuif, France), OVCAR3, SKOV3 (both from ATCC), OAW42 (DKFZ Germany), OVCA432 (R. Knapp, Dana Farber Institute, Boston, MA, USA), INTOv1 and INTOv2 [8] and the nasopharyngeal carcinoma KB (ATCC). Total RNA from surgical samples was obtained from five patients at different stages of disease [9]. RNA from normal human tissues were purchased from Clontech (Palo Alto, CA, USA).

2.2. Plasmids

The constructs with (KBPE1-3-chloramphenicol acetyltransferase (CAT)) or without (P1PD-CAT) exon 3 were described previously [4]. The #4/6 ECO construct was obtained by PCR amplification of the KBPE1-3 plasmid with the forward primer named #22 (5′-GAGCTCAAGCTTGGCAAGGGGAGTGTAGAGCAG-3′), that anneals in exon 1, and a reverse primer that anneals in the 5′ CAT sequence (5′-CTCCTACCCGGGGCAACTGACTGACTGAAATG-CCTC-3′); digestion of the PCR product with *Hin*dIII (contained in forward primer) and *Eco*RI and cloning in the pCAT basic vector. #4/6 ECO construct was digested with *Bam*HI, blunt-ended and religated to generate #4/6 BAM construct. #4/6 XBA construct was obtained by PCR amplification of the KBPE1-3 plasmid with the forward primer #22 and reverse primer that anneal in the 3′ of exon 3 region (5′-AAGCCTTCTAGAGGGCCACGCCGGAGA-AA-3′).

2.3. RNase protection assay

RNA from cell lines was extracted using the RNeasy total RNA kit (Qiagen, Hilden, Germany). Total RNA from the normal tissues indicated (25 μg) or the KB and IGROV1 carcinoma cell lines (10 μg) was probed with the IG-1 and β-actin antisense riboprobes. The assay was performed essentially as described [10]. Comparison of the intensity of the high molecular weight bands with that of the splicing site (SS) protected fragment was performed with IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. RT-PCR assay

RT-PCR was performed on 1 μg of total RNA reverse transcribed

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Abbreviations: FR, folate receptor; CAT, chloramphenicol acetyltransferase; ORF, open reading frame; NCS, non-coding sequence; uORF, upstream ORF; SS, splicing site

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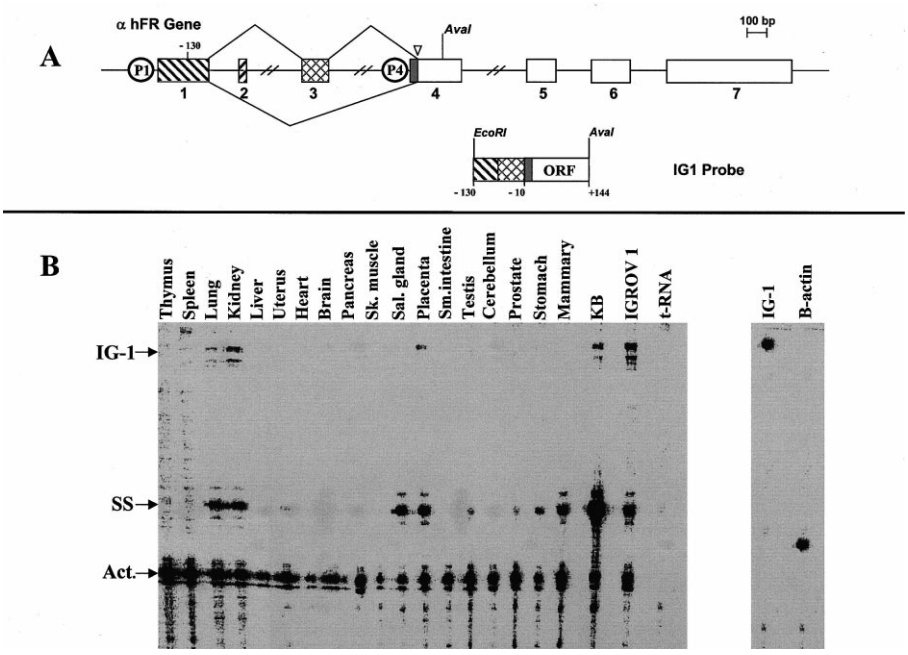

Fig. 1. RNase protection analysis of P1 promoter-driven αFR transcripts. A: Schematic representation of the FR gene showing the promoters, the intron/exon organization and the alternative splicing identified from the cDNA expression library. Exons are numbered and represented by boxes. A line represents introns and an arrowhead marks the common splice site upstream of ATG. The lower diagram represents the *Eco*RI/*Ava*I restriction fragment of the αFR cDNA clone #4/6 named IG1. B: Total RNA from the normal tissues or the KB and IGROV1 carcinoma cell lines was probed with the IG-1 and β-actin antisense riboprobes.

using 50 U of reverse transcriptase and a poly(dT) primer. The resulting cDNA (2 μl) was amplified using 2 U of Ampli *Taq* polymerase and 30-cycle PCR (95° 1 min, 58°C 1 min, 72° 1 min). The forward primer was #22 (see above) and the reverse primer was 5′-CCACCTTCTAGAGCCTGGCTTTTCCTTGTGGTG-3′, that anneals in exon 4. Total RNA extracted from KB and IGROV1 transfected cells was amplified with the following forward and reverse primers: 5′-CTCCTCTCCCAGGAACTGAA-3′, that anneals in the exon 1-exon 3 junction region; and 5′-TCCAGCTGAGCGGTCTGGTT-3′ specific for CAT-ORF. Quantification of ethidium bromine stained PCR products was performed with Eagleye software (Stratagene, La Jolla, CA, USA)

2.5. Cells transfection and CAT assay

Cells (1.2×10^5) were seeded in a 12 well plate and transfected the following day using the lipofectin method essentially as suggested by the manufacturer (Gibco BRL). CAT constructs (2.5 μg) were co-transfected with pCMV-Luc (0.05 μg) containing the luciferase gene downstream from a cytomegalovirus promoter (kindly provided from Dr. Maria Zajac-Kaye, Bethesda, MD, USA) to control for transfection efficiency. Luciferase and CAT activity were evaluated as described [11].

3. Results

3.1. Analysis of the αFR P1 mRNA expression

To establish the relative abundance of αFR P1-derived transcripts that include alternatively spliced exon 3, an RNase protection assay performed on a wide panel of normal tissues was compared to that obtained with two αFR overexpressing cell lines, IGROV1 ovarian carcinoma and KB nasopharyngeal carcinoma. Total RNAs were probed using an antisense riboprobe (IG1) that contains the 5′ NCS of cDNA clone #4/6 (130 bp) and initial 144 bp of the ORF up to the *Ava*I site and 10 bp between the common SS and the beginning of the ORF (154 bp) (Fig. 1A).

Major bands between 250 and 300 bp, representative of the

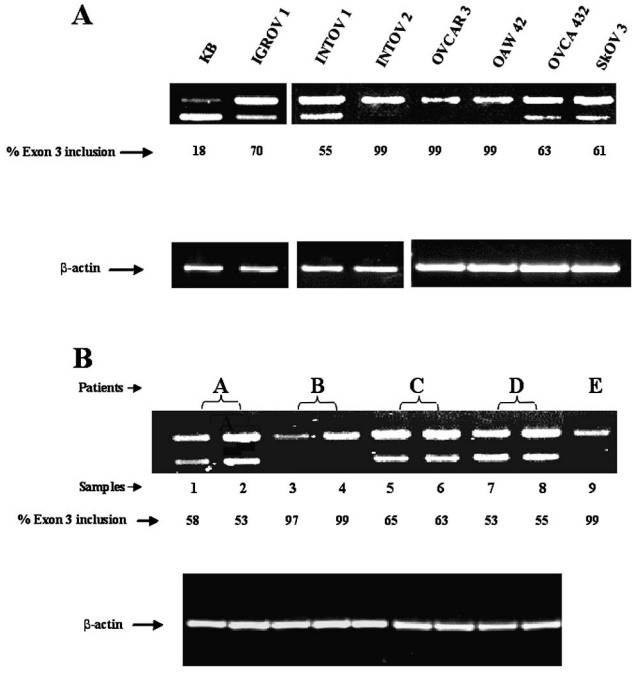

Fig. 2. Splicing pattern of P1 promoter-driven αFR transcripts by RT-PCR. A: Total RNA extracted from ovarian carcinoma cell lines and αFR-non-expressing carcinoma cell line KB. B: Ovarian tumor surgical specimens. The nine samples analyzed were derived from one borderline, three primary, and five metastatic tumors. Upper (305 bp) and lower (239 bp) bands represent the mRNA isoforms containing or excluding exon 3, respectively. The ratio between the upper and the lower bands was calculated as described in Section 2.

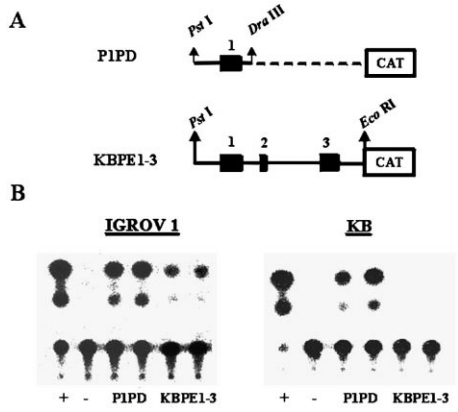

Fig. 3. Reporter CAT gene activity in IGROV1 and KB transfected cells. A: Chimeric αFR promoter-CAT constructs. B: A representative CAT reporter assay, with relevant positive (+) and negative (−) controls, analyzed on a TLC plate.

IG1 full length protected fragments, were evident in IGROV1 and KB cell lines, detectable in kidney and barely detectable in lung and placenta. Comparison of the intensity of the high molecular weight bands with that of the SS protected fragment indicated only weak protection of the KB RNA with the IG1 riboprobe (10% in KB vs. 47% in IGROV1) (Fig. 1B).

To compare the relative proportions of P1-derived αFR mRNA isoforms, an RT-PCR-based method was used. Analysis was performed on equal amounts of total RNA extracted from: a panel of ovarian carcinoma cell lines and surgical specimens and as control on IGROV1 and KB cells. The primers were designed to competitively amplify the mRNAs containing or lacking exon 3. All ovarian carcinoma cell lines and surgical specimens, irrespective to the stage of the disease, revealed a P1-derived mRNA that predominantly includes exon 3, ranging from 53 to 99% inclusion (Fig. 2), while KB cells only exhibited 18% exon 3 inclusion.

3.2. Analysis of expression specificity

To investigate on the inclusion of exon 3 in ovarian carcinoma, we transiently transfected IGROV1 and KB cells representing exon 3 inclusion and exclusion, respectively. Two CAT constructs were used: (i) P1PD, which contains the minimal P1 promoter region and exon 1; (ii) KBPE1-3, which contains the same P1PD sequence together with the genomic sequence that spans from exon 1 through exon 3 (Fig. 3A). While P1PD drove CAT expression in both cell lines, KBPE1-3 was only active in IGROV1 cells (Fig. 3B).

To define the region of the *Pst*I–*Eco*RI αFR genomic fragment regulating transcriptional and post-transcriptional events, deletions at the 5′ and 3′ end of the KBPE1-3 construct were generated and transiently transfected (Fig. 4). CAT activity was analyzed together with the splicing pattern of IGROV1 vs KB cell lines. The splicing pattern was assayed by RT-PCR as described in Section 2. KBPE1-3, even though less efficient than P1PD (see Fig. 3B), exhibited an 18-fold increase in specificity of expression in ovarian carcinoma cells as compared to nasopharyngeal carcinoma cells. Detectable levels of RT-PCR products were present in KB cells transfected with KBPE1-3, although no CAT activity was observed (see also Fig. 3B). Therefore, the lack of CAT translation might rest in the presence of the six upstream ORF (uORFs) in the part of downstream intron sequence of exon 3 contained in the KBPE1-3 construct [4]. Deletion at the 5′ end of exon 1, corresponding at the first internal transcriptional start site identified from an ovarian carcinoma cDNA expression library [7], was conducted to obtain the #4/6 ECO construct. This construct compared to KBPE1-3 shown an increased expression in both transfected cell lines (5-fold) with no loss in specificity (CAT activity ratio: 18-fold in IGROV1 vs. KB cells). The #4/6 BAM construct, in which the 3′ intronic region containing the uORFs was deleted, was very active in both cell lines and displayed a reduced specificity ratio (2.5-fold in IGROV1 vs KB cells). Both the #4/6 BAM and #4/6 ECO constructs, as well as the KBPE1-3, showed an imbalance between the amount of CAT transcripts and activity suggesting also a regulation of transcript stability and translation. Considering the activity of these constructs, #4/6 ECO appeared to maintain the highest specificity,

CHIMERIC CONSTRUCTS	RELATIVE CAT ACTIVITY		IGROV1/KB ratio	
	IGROV1	KB	CAT activity	mRNA transcript°
KBPE 1-3 — CAT	0.150*	0.008*	18.75	1.8
#4/6 ECO — CAT	0.780*	0.043*	18.30	1.6
#4/6 BAM — CAT	3.400**	1.350**	2.52	1.8
#4/6 XBA — CAT	4.550*	4.25**	1.07	1.0

°Note that the total amount of RT-PCR products are comparable within the different constructs

Fig. 4. Analysis of 5′ and 3′ deleted P1–CAT constructs. IGROV1 and KB cells were transiently transfected with the chimeric CAT constructs shown on the left. RT-PCR and CAT activity were analyzed as described in Section 2. CAT activities relative to that of the P1PD construct are mean of duplicate determinations in three independent experiments; standard deviations were in the range * ± 0.01–0.3 and ** ± 0.6–0.9.

although net gene expression was lower than in #4/6 BAM. A further 3′ deletion (#4/6 XBA construct), that removes the 5′ splice site of exon 3 together with the last 35 bp of the downstream intron, resulted in the vanishing of post-transcriptional specificity and higher reporter activity. Interestingly, the lack of the entire intron region of #4/6 XBA construct arose in an equal amount of mature RNA in both cell lines.

4. Discussion

In the present study we have pointed out that the P1-driven exon 3 containing mRNAs are more abundant in ovarian αFR-expressing carcinoma cells than in the non-ovarian αFR-expressing tumor cells (KB) and normal tissues. Thus, we provided evidence that post-transcriptional events enhance the specificity of the P1 promoter-driven gene expression in ovarian carcinoma. Furthermore, we demonstrated that this specificity is mainly related to the presence of the 5′ splice site and 35 bp intron region downstream of exon 3.

We have showed that only ovarian carcinoma cells are able to efficiently utilize the 3′ and 5′ consensus splice sites of exon 3 in the P1-derived pre-mRNA maturation process. Moreover the results that we obtained with the construct that lacks the 5′ splice site of exon 3 (#4/6 XBA), imply the presence of at least one regulatory element in this region responsible for ovarian exon 3 inclusion efficiency. Indeed this construct displays the same reporter activity in both ovarian (IGROV1) and non-ovarian (KB) cell lines (see Fig. 4). Note that the intron III stretch of 35 bp, deleted from the construct #4/6 BAM to generate the construct #4/6 XBA, contains at least two consensus elements: (i) the sequence GAGCAG homologous with SR binding site to SC35 [12],(ii) a G triplet sequence. The G triplet is an element frequently found in close proximity to the 5′ splice site and has been demonstrated to be of predictive value for identification of exons in sequenced genomic DNA [13,14]. A recent study provides the first experimental evidence that the α-globin G-rich elements interact directly with the RNA component of the U1 snRNP, suggesting that the recognition of such sequence by splicing factor(s) may play an important role in 5′ splice site selection in a large number of pre-mRNAs [15].

In the same intron we also observed the presence of six uORFs that shut off the translation of the messenger in KB but not in IGROV1 cells as demonstrated by the absence of CAT activity but not of CAT mRNA in the KB cells transfected with KBPE1-3 construct (see Fig. 3). Our data are in keeping with the previous evidence that uORFs inhibit mRNA translation [16] and that the inhibition can be exerted in a cell specific manner [17,18]. We also hypothesize in the 5′ terminus of KBPE1-3 construct the presence of a negative regulator of transcription in KB cells.

Ovarian carcinoma is the major cause of death from gynelogical cancer. At diagnosis, the disease is often already disseminated into the peritoneal cavity, and 28–35% of patients survive overall no longer than 5 years [19]. New therapeutic target specific modalities on ovarian carcinomas are deeply investigated: some are based on the targeting ability of monoclonal antibodies, others on the selective gene expression of tumor-associated antigens [20,21]. A major limiting step of gene therapy approach remains the delivery of toxic agents specifically to the tumor cells while sparing the surrounding normal cells. At present, few tumor or tissue specific promoters have been identified [22] and some await an in vivo demonstration of suitability. Our results point to the promise of exploiting αFR P1 promoter region and additionally the alternative splicing of exon 3 region in developing a specific gene therapy strategy against ovarian carcinoma. Indeed, beside the low level of P1-derived exon 3 containing αFR mRNA detected in critical normal tissues such as lung and kidney, the confinement of ovarian carcinoma within the peritoneal cavity suggest the sparing of these tissues during such therapy.

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