Characterization of the c-specific promoter of the gene encoding human endothelin-converting enzyme-1 (ECE-1)

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Received 5 November 1999; received in revised form 23 December 1999

Edited by Ned Mantei

Abstract Human ECE-1 is expressed in four isoforms with different tissue distribution and its mRNA and protein levels are altered under certain pathophysiological conditions. To investigate the transcriptional regulation of ECE-1, we studied the regulatory region of ECE-1c, the major ECE-1 isoform. A genomic clone comprising the complete human ECE-1 gene including the putative ECE-1c-specific promoter was obtained. Up to 968 bp upstream of the putative c-specific translation initiation start codon and several serial deletion mutants were subcloned into a reporter vector and transfected into endothelial (BAEC, EA.hy926, ECV304) and epithelial (MDA MB435S, MCF7) cells, showing very strong promoter activity in comparison to the SV40 promoter and to the previously described ECE-1a and 1b promoters. Transfection of serial deletion mutants indicated two positive regulatory regions within the promoter (−142/−240 and −240/490) likely involved in binding GATA and ETS transcription factors. RNase protection assay (RPA) and 5′-RACE revealed multiple transcriptional start sites located at about −110, −140 and −350 bp. Site-directed mutagenesis demonstrated a crucial role for the E2F cis-element for basal ECE-1c promoter activity. Additionally, we found a correlation between isoform-specific ECE-1 mRNA levels and corresponding ECE-1a, 1b, 1c promoter activities.

Key words: Endothelin-converting enzyme 1; Alternative promoter; ETS; GATA; E2F; Initiator element (Inr)

1. Introduction

Endothelin-converting enzymes (ECEs) play a key role in the biosynthesis of endothelins because of their ability to generate endothelins by proteolytic cleavage of the biological inactive precursors big ET-1, big ET-2 and big ET-3. Three different ECEs (ECE-1, ECE-2 and ECE-3) have been described [1–3]. All of them constitute membrane-bound, phosphoramidon-sensitive metallo-proteases. ECE-1 mainly converts big ET-1 [1] and is synthesized for example in endothelial and vascular smooth muscle cells [4,5]. ECE-2 is predominantly expressed in the CNS and also preferentially cleaves big ET-1, whereas the recently isolated ECE-3 processes big ET-3, but not big ET-1. Human ECE-1 itself is expressed in at least three different isoforms (ECE-1a, ECE-1b and ECE-1c) [6–8] with similar enzyme kinetics but different subcellular localization [8–10] and different tissue distribution [5,11]: ECE-1c, which is mainly localized on the cell surface, is expressed among other tissues in lung, heart, kidney, liver and placenta and represents the major isoform with regard to the level of mRNA expression [8]. ECE-1b is localized mainly intracellularly which is consistent with the recently described di-leucine motif in its N-terminus [12], whereas ECE-1a is targeted to the cell membrane.

The genomic structure of ECE-1 is complex [5,8,11] (Fig. 1A): ECE-1c and 1b are specified by an isoform-specific exon that is spliced to exon 2 which is common to both isoforms. ECE-1a is transcribed from an alternative promoter located about 11 kb downstream of exon 2. The 5′-terminal part of exon 3 is the first ECE-1a-specific exon, whereas the 3′-terminal part of this exon is common to all known ECE-1 isoforms. Very recently a fourth ECE-1 isoform has been described [13]: The d-specific exon and its promoter are located in the small (about 200 bp) genomic region between exon 1b and exon 2.

The developmental significance of ECE-1 is demonstrated by studies of knockout mice [14]: Targeted disruption causes craniofacial and cardiovascular abnormalities in addition to congenital intestinal aganglionosis corresponding to human Hirschsprung's disease and an absence of epidermal melanocytes. The ontogenetic importance is further outlined by a patient with a loss of function mutation of the ECE-1 gene who shows similar symptoms [41].

There is also altered ECE-1 expression in adult pathophysiology, e.g. neointima formation after balloon injury [39,40] and pulmonary fibrosis [15]. But little is known regarding *isoform-specific* ECE-1 gene regulation in vivo and in vitro: Shao et al. [16] showed different regulation of ECE-1a, also termed ECE-1β [11,17], and ECE-1b (=1α) in a rat liver injury model and Corder et al. studied the expression of ECE-1a, 1b and 1c in stimulated bovine aortic endothelial cells [18]. In addition, we have recently demonstrated that ECE-1a and ECE-1b are regulated by alternative promoters and not by differential splicing and that members of the ETS family of transcription factors are involved in transcriptional regulation of ECE-1a expression ([5,19,20]; submitted data). To gain further insight into isoform-specific ECE-1 gene expression we have cloned and analyzed the promoter of the major isoform, ECE-1c.

2. Materials and methods

2.1. Cloning of the ECE-1c-specific promoter

Based on the sequence of human exon 1c we performed a nucleo-

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PII: S0014-5793(00)01086-3

tide database search (GenBank) and obtained a human genomic chromosome 1 clone (Acc. no. AL031728, GI 4491014) comprising the whole ECE-1 gene including the putative ECE-1c-specific promoter. Up to 968 bp of the 5′-genomic region upstream of the putative c-specific translation initiation start codon were subcloned into the luciferase reporter vector pGL3basic (Promega, Madison, USA) via a genomic PCR amplification using HotStarTaq (Qiagen, Hilden, Germany) and the following primers: 5′-GTTGTCCTGAAGTCCC-TGG-3′ (sense), 5′-AGCTCGCGTGCTCCGCC-3′ (antisense).

2.2. Construction of deletion mutants

PCR reactions were performed with a common antisense primer in the 3′-terminal region of the putative c promoter region, directly 5′-terminal of the c-specific translation initiation codon, and several different sense primers located at positions −89, −142, −217, −240, −409, −490, −739, −885 (bp relative to the putative translation initiation codon of the ECE-1c isoform). PCR products were subcloned into the pGL3basic vector and the identities of the clones were confirmed by sequencing.

2.3. Further reporter gene constructs

ECE-1a/pGL3basic and ECE-1b/pGL3basic promoter vectors were constructed as previously published [5,19] and contain 1206 bp and 1278 bp respectively of the 5′-genomic regions. The TIE1/pGL3basic vector contains 0.8 kbp of the published mouse Tie1 promoter (Acc. no. S79346) [24].

2.4. Site-directed mutagenesis

Site-specific mutations were created using a two-step PCR based method as previously described [21]: In the first round two PCRs in parallel were performed: The first one with a wild-type 5′-sense primer (at position −491: 5′-CGGGTCACACTCCAGTGCA-3′) and a mutated antisense primer (5′-CGCGGCTTCGGAACCGAGTG-3′). The second one with a mutated sense primer (complementary to the mutated antisense primer; 5′-CACTCGGTTCCGAAGCCGCG-3′) and a non-mutated 3′-antisense primer (directly 5′-terminal of the c-specific translation initiation codon, 5′-AGCTCGCGTGCTCCGCC-3′). Reactions were performed using HotStarTaq (Qiagen, Hilden, Germany) and human genomic DNA as template. PCR products were purified via one QiaQuick column (Qiagen, Hilden, Germany) and served as template for a further PCR reaction using the wild-type 5′-sense primer and the wild-type 3′-antisense primer from the first round. The PCR product was subcloned into the luciferase reporter vector pGL3basic. Successful mutagenesis was confirmed by sequencing.

2.5. Cell culture

Bovine aortic endothelial cells (BAECs) were isolated and cultured as previously described [5,22,23]. The human endothelial cell lines ECV304 (ECACC, Salisbury, UK) and EA.hy926 (a generous gift from Dr. C.-J. Edgell) were cultured as previously described [5]. The human epithelial cell lines MCF7 (ATCC, Rockville, USA) and MDA MB435S (Cell Lines Service, Heidelberg, Germany) were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany; supplemented with penicillin/streptomycin (Bio-Whittacker, Verviers, Belgium), 10% fetal calf serum (Biochrom) and 1% L-glutamine (GibcoBRL, Life Technologies, Eggenstein, Germany)) and Dulbecco's modified Eagle's medium (Bio-Whittacker; supplemented with penicillin/streptomycin (Bio-Whittacker), 20% fetal calf serum (Biochrom) and 2% L-glutamine (GibcoBRL)), respectively. Calu-6 cells are purchased from ATCC (HTB-56).

2.6. Transfection experiments

Promoter luciferase constructs and control plasmids, pGL3 promoter (SV40 driven) and pGL3basic (promoterless) (Promega), were transfected using Lipofectine (GibcoBRL; for ECV304), Lipofectamine (GibcoBRL; for BAEC), Fugene-6 (Boehringer Mannheim; for EA.hy926 and MCF7) and Lipotaxi (Stratagene; for MDA MB435S) using 0.5–2 μg plasmid per well. Cotransfection with pSV-β-galactosidase control vector (0.5–1 μg/well; Promega) was carried out to standardize for transfection efficiency. Cells were harvested 48 h after beginning of the transfection procedure using reporter lysis buffer (Promega). Luciferase and galactosidase activities were measured in a Lumat LB 9501 (Laboratorium Prof. Berthold, Bad Wildbad, Germany) using luciferase assay substrate (Promega) and galacton/light emission accelerator (Tropix, Bedford, USA), respectively. A construct-specific transfection represents the mean value of six single transfections.

2.7. Generation of template constructs for RNase protection assay (RPA)

1. ECE-1 RPA: A 302 bp cDNA fragment of ECE-1c was generated by PCR using human fetal heart cDNA (transcribed from the respective poly-A^+ RNA) as template (sense: 5′-GGAGCTGCGC-GAAGCCGGGGCGG-3′; antisense: 5′-GACACAAGCTTCGC-TCAGGCACA-3′) and subcloned into pBluescript II KS(+) (Stratagene).
2. RPA analysis of transcriptional start points: A genomic DNA fragment (739 bp immediately upstream of the putative translation initiation codon of the ECE-1c isoform) was subcloned into pCR2.1 (Invitrogen) via genomic PCR. The identity of both subcloned fragments was confirmed by sequencing.

2.8. RNase protection assay (RPA)

RPAs were performed with the RPA II kit and the MAXIscript T7 kit (both Ambion, Austin, TX, USA). Antisense RNA probes were synthesized from linearized plasmids (1 μg) in the presence of 5 μl [^{32}P]CTP (20 mCi/ml, Amersham) and ATP, GTP and UTP (each 500 μM) in a total volume of 20 μl using 20 U of T7 polymerase: The ECE-1c construct for mRNA expression analysis was linearized with *Xho*I yielding a transcript with a total length of 400 nt and a gene-specific length of 302 nt. The ECE-1c construct for analysis of transcriptional start points linearized with *Nhe*I resulted in an antisense RNA probe of 491 nt (total length)/394 nt (gene-specific length). The probes were gel purified by running the transcription reactions on a 5% denaturating polyacrylamide gel and eluting the appropriate bands overnight at room temperature in elution buffer (supplied with the kit). The actual RPA reaction was performed with about 3×10^5 cpm of the probe and approximately 30 μg of total RNA. After hybridization the digestion was carried out with RNase A (2.5 U) and RNase T1 (8 U) for 60 min at 37°C. Finally, the samples were denatured for 4 min at 95°C and separated by gel electrophoresis on a 5% denaturating polyacrylamide gel. Century marker template (Ambion) was used as size marker and transcribed according to the manufacturer's protocol. Gels were exposed on X-ray films (Kodak X-OMAT) or imaging plates (BAS-MP 2040S, Fuji) with subsequent scanning of the latter by a phosphoimager (BAS-1500, Fuji).

2.9. Rapid amplification of cDNA ends (RACE)

Reverse transcription (RT) was carried out using 5 μg DNase-treated total RNA, random hexamer primers (4 μM), RNasin (50 U; Promega) and Superscript RT (200 U; Gibco) at 37°C for 1 h in a 50 μl reaction. 10 μl of the resulting cDNA was tailed with dGTP (2 nmol/μl) using terminal transferase (0.4 U; New England Biolabs) at 37°C for 40 min. An aliquot of the tailed cDNA was used as a template in a PCR with a poly-C primer (15 nt) and an antisense primer in exon 2 (5′-CTGCAGGCCGTTGGGGTATGC-3′). Afterwards a second PCR with a nested antisense primer in exon 1c (5′-CATAGCTCGCGTGCTCCGCCCCGGCT-3′) was carried out. The resulting product was subcloned into the pCR2.1 vector (TA Cloning Kit, Invitrogen) and sequenced.

2.10. Sequence analysis

The sequence was analyzed for cis-elements using the computer program Transfac (version 3.5, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; mirrored at http://www.motif.genome.ad.jp; Heinemeyer, T., Chen, X., Karas, H. et al. (1999) Nucleic Acids Res. 27, 318–322).

2.11. Statistical analysis

A two-tailed *t*-test was performed and statistical significance was presumed at $P<0.05$.

3. Results

3.1. Cloning of the human ECE-1c promoter

To clone the ECE-1c-specific promoter a nucleotide sequence database (GenBank) was screened using the previously published exon 1c sequence as a 'probe' in analogy to a clas-

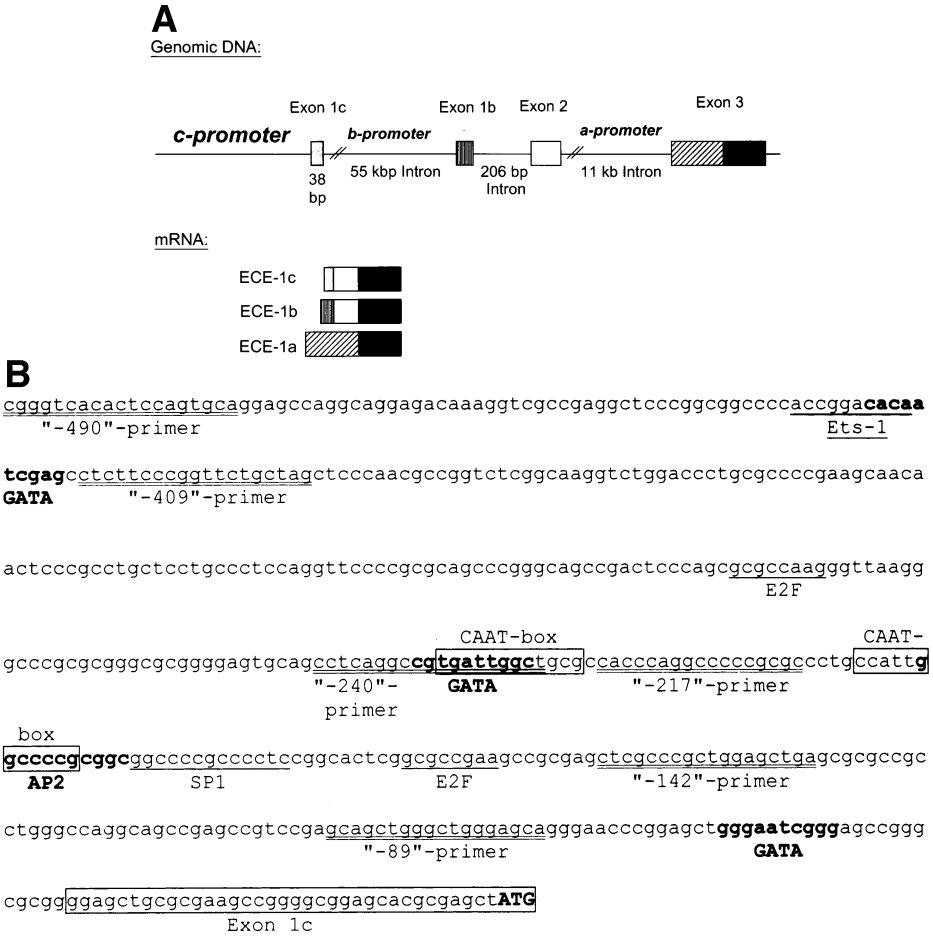

Fig. 1. A: Organization of the human ECE-1 gene. The genomic structure of the 5′-terminal part of the human ECE-1 gene with exon-intron boundaries and localizations of the alternative promoters is shown in the upper part. Exons 4 to 19, common to all isoforms, are not shown. ECE-1 isoforms (1a, 1b and 1c), transcribed by different promoters, are shown in the lower part. (Data compiled from Schweizer et al. (1998), Orzechowski et al. (1997) and unpublished experiments.) B: Human ECE-1c promoter sequence. Part of the genomic sequence (Acc. no. AL031728; GI 4972242) with the major regulatory regions of ECE-1c promoter. Primer positions are double underlined, cis-elements are indicated and exon 1c is marked by a box. The putative translational start codon of ECE-1c is represented in bold letters.

sical phage library screening. We obtained a human genomic clone (Acc. no. AL031728, GI 4491014) comprising the complete ECE-1 gene including the putative ECE-1c-specific promoter. Based on this cloned sequence it was possible to determine the previously unknown length of the intron between exon 1c and 1b, which totals 55 kbp (Fig. 1A). To further ensure that the genomic region upstream of the putative exon 1c indeed represents the ECE-1c-specific 5′-UTR, we performed RT/PCR using a sense primer located 142 nucleotides upstream of the ATG in exon 1c and an antisense primer located in exon 2 of the ECE-1 gene. Sequencing of the obtained product (data not shown) confirmed the existence of a transcript containing 5′-UTR and coding sequence of exon 1c directly attached to exon 2 (compare Fig. 1A).

3.2. Functional promoter analysis

Up to 968 bp upstream of the putative c-specific translation initiation start codon were synthesized by PCR using human genomic DNA as template and subcloned into the luciferase reporter vector pGL3basic. The full length construct (−968) and serial deletion mutants which were generated by genomic PCR using nested 5′-primers (at positions −89, −142, −240, −490, −739, −885) were transfected into primary cultured bovine aortic endothelial cells (BAECs) as well as the human endothelial cell line ECV304 (Fig. 2A). To assess the relative strength of ECE-1c promoter activity, an SV40 promoter driven pGL3 plasmid was transfected as a positive control (Fig. 2A). Maximum promoter activity was detected following transfection of mutant −490 in BAECs (372-fold pGL3basic, 4.8-fold of SV40 promoter) and of mutant −739 in ECV304 (111.8-fold pGL3basic, 8.9-fold of SV40 promoter). The two shortest promoter constructs (−89 and −142) showed only low relative luciferase activities (RLA) (3.5- and 5.5-fold pGL3basic in ECV304). Extension of the construct to position −240 resulted in a strong increase ($P < 0.01$) of RLA (49.5-fold pGL3basic in ECV304). Potential cis-acting elements identified in this region include consensus sequences for binding of the transcription factors E2F, SP1, AP2 and GATA (Fig. 1B). 5′-extension of the ECE-1c promoter to position −490 resulted in an additional increase ($P < 0.01$) in promoter activity (95.8-fold pGL3basic in ECV304). This region contains consensus sequences for GATA and ETS proteins (Fig. 1B). Further 5′-extension beyond position −490 (constructs −739, −885 and −968) did not show any significant changes in promoter activity. With regard to species it is of interest to note that the promoter constructs showed similar function in

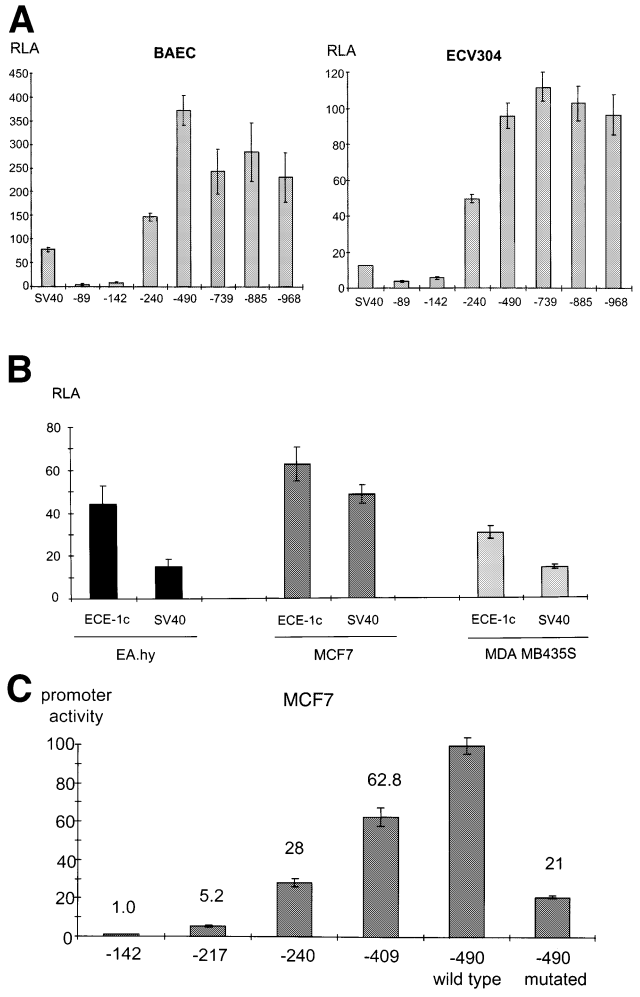

Fig. 2. A: Promoter activity of serial deletion mutants in bovine aortic endothelial cells and ECV304 cells. Relative luciferase activity (RLA) is the ratio of the luciferase/galactosidase mean values of each construct related to the promoterless reporter plasmid pGL3basic [Luc (Construct)/Gal(SV40-βGal)/Luc (pGL3basic)/Gal(SV40-βGal)]. Constructs were transfected into BAEC (left) and ECV304 (right). An SV40 promoter driven pGL3 vector ('SV40') served as control. Standard deviations related to Luc (pGL3basic)/Gal(SV40-βGal) are indicated. B: Cell-specific activity of the ECE-1c promoter. ECE-1c promoter construct −739 and an SV40 driven pGL3 vector were transfected into EA.hy926, MCF7 and MDA MB435S cells. RLA: Relative luciferase activity. C: Transfection of ECE-1c promoter constructs. The ECE-1c reporter constructs −142, −217, −240, −409, −490 (wild-type) and −490 (mutated at the E2F cis-element at position −154) were transfected into MCF7 cells. The RLA of the wild-type −490 construct (non-mutated) was set to 100.

the human endothelial cell line ECV304 and in primary cultured BAECs (Fig. 2A).

To assess a possible cell-specific regulation of the ECE-1c promoter mutant −739 was additionally transfected into the human endothelial cell line EA.hy926 and into the human epithelial cell lines MDA MB435S and MCF7 (Fig. 2B). The ECE-1c promoter construct showed strong reporter activity irrespective of endothelial or epithelial cellular origin.

To examine the role of the GATA consensus site at position −226 two deletion mutants (−217 and −240) were generated which differed by the GATA consensus sequence only. Deletion mutant −240, which contains the GATA consensus sequence, was 5.4-fold more active than mutant −217 (Fig. 2C). To determine the importance of the GATA and ETS consensus sites at positions −415 and −422 respectively, deletion mutant −409 was created: 5′-extension to position −490 increased promoter activity by the factor 1.6 relative to −409 ($P < 0.01$) (Fig. 2C).

3.3. Determination of transcriptional initiation points

Transcriptional start sites of ECE-1c mRNA expression were determined by two independent methods, RNase protection assay (RPA) and 5′-RACE reaction. An antisense RNA probe, which was based on genomic sequence, was used to map the ECE-1c-specific start sites in several different cell types (Fig. 3A): Protected fragments approximately sized 110 bp (appearing as a double band), 140 bp and 350 bp (less intensive) were detected in the RPA, corresponding to transcriptional start sites at approximate positions −110, −140 and −350 respectively, relative to the 'ATG' in exon 1c. Electrophoresis of the 3′-nested 5′-RACE products, using

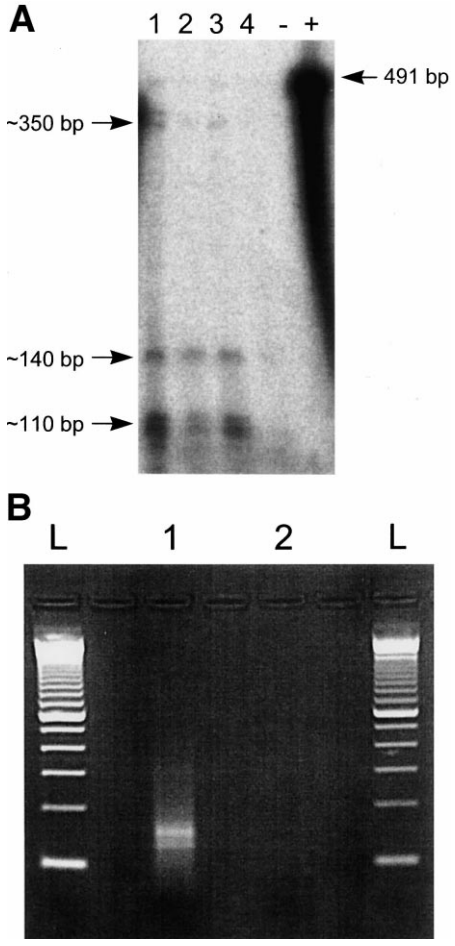

Fig. 3. A: Analysis of transcriptional start points by RNase protection assay (RPA). Total RNA was hybridized with an antisense RNA probe (total length 491 nt; specific length 394 nt (5′ of the 'ATG' in exon 1c)). Lane 1, HUVEC (40 μg); lane 2, Calu-6 (40 μg); lane 3, EA.hy926 (40 μg); lane 4, MCF7 (23 μg); lanes −/+, yeast RNA with/without RNase. B: Analysis of transcriptional start points by 5′-RACE. 5′-RACE was performed using HUVEC mRNA and an antisense primer in exon 1c. Reaction products were separated on agarose gel. Lane 1, 5′-RACE; lane 2, negative control; lane 'L', 100 bp ladder.

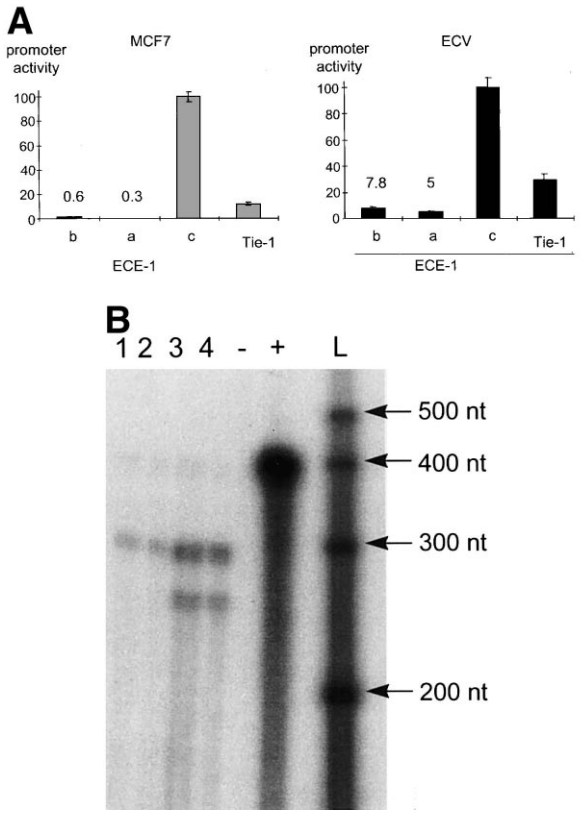

Fig. 4. A: ECE-1 isoform-specific promoter activity. Luciferase promoter constructs of human ECE-1a (−1206/pGL3basic), 1b (−1278/pGL3basic) and 1c (−490/pGL3basic) were transfected into MCF7 (left) and ECV304 cells (right). The RLA of the ECE-1c construct was equated to 100. The transfection of a mouse TIE1/pGL3basic vector served as control. B: ECE-1 isoform-specific gene expression. mRNA levels of human ECE-1a, 1b and 1c in ECV304 and MCF7 cells were determined by RNase protection assay (RPA): Total RNA was hybridized with an antisense RNA probe (total length 400 nt). A protected length of 302 nt corresponds to ECE-1c, whereas ECE-1b is represented by a band of 264 nt. ECE-1a would be expected at 177 nt. Lanes 1 and 2, MCF7 (30 μg); lanes 3 and 4, ECV304 (30 μg); lanes −/+, yeast RNA with/without RNase; lane L, molecular weight marker.

an oligo-C primer and an antisense primer immediately 5′ of the putative translational start codon in exon 1c, showed a double band of about 130 bp (Fig. 3B). This corresponds to transcript-specific fragments about 20 bp shorter. Subcloning and sequencing of the RACE products indicated specificity of the fragments and 5′-ends at positions −101 and −106 bp relative to the 'ATG', respectively.

To gain further insight into the mechanisms of *basal* transcriptional regulation of the ECE-1c promoter, the consensus sequence for Inr-associated protein E2F at position −154 (Fig. 1B) was mutated by site-directed mutagenesis (5′-gcgccgaa-3′ to 5′-gTTccgaa-3′), which resulted in a dramatic decrease in reporter activity by 79% (Fig. 2C).

3.4. *Isoform-specific ECE-1 promoter activities correspond with transcript levels*

To assess the ECE-1c promoter activity in comparison to the previously characterized ECE-1a (also termed 1β) and ECE-1b (=1α) promoters [5,19], isoform-specific promoter functions were analyzed in different cell types (Fig. 4A): MCF7 cells showed only marginal activities of the ECE-1a and ECE-1b promoters in comparison to the ECE-1c promoter (Fig. 4A, left). In contrast, the ECE-1a and ECE-1b promoters were active in ECV304 cells and reached 5% and 7.8% of ECE-1c promoter activity respectively (Fig. 4A, right); the difference in promoter activity, between ECE-1a and 1b is significant ($P < 0.01$). Additionally the mouse Tie1 promoter was transfected into both cell types to compare the ECE-1 promoters with another well characterized (almost endothelium-specific [22]) promoter, showing 11.8% activity of ECE-1c promoter in MCF7 and 29.2% in ECV304. A possible correlation of isoform-specific ECE-1 promoter activities and corresponding mRNA levels was addressed by ECE-1-specific RPA on MCF7 and ECV304 mRNA (Fig. 4B): MCF7 cells express ECE-1c, but no ECE-1a or 1b, whereas ECV304 cells express ECE-1c, some ECE-1b and no detectable ECE-1a.

4. Discussion

Here, we report cloning and basal characterization of the human ECE-1c-specific promoter localized approximately 55 kbp upstream of the recently cloned ECE-1b promoter [5,11]. Based on GenBank database searches, the 55 kbp sized intron does not contain any other known gene. The ECE-1c promoter shows extremely strong reporter activity in endothelial and epithelial cells in comparison to the ECE-1a and ECE-1b promoters as well as the recently published ECE-1d promoter [13]. Strong c-specific promoter activity is consistent with ECE-1c as the major ECE-1 isoform regarding mRNA levels [8,13].

The c-specific promoter lacks a TATA-box, consistent with multiple transcriptional start sites mapped by RPA in epithelial and endothelial cells at approximately 110 bp, 140 bp and 350 bp upstream the translational start codon. The two adjacent transcriptional start points identified by RPA at about −110 were further confirmed by 5′-RACE. Regarding the start sites at about −110 it is important to note that there is a non-canonical CAAT-box at −190, in appropriate distance of about 75 bp. There is also a consensus sequence for the E2F binding at about −150. This protein has been implicated in cell proliferation control [25] and, importantly, in mediating activation of initiator elements (Inrs) [26–28]. Inrs are located around the transcriptional start point and can determine the start site in promoters without a TATA-box. Therefore, it is likely that this E2F consensus element in the TATA-box less ECE-1c promoter participates in transcription initiation at −140. This hypothesis is strongly supported by site-directed mutagenesis of the E2F consensus which dramatically decreased ECE-1c promoter activity.

Focusing on consensus sites for regulatory transcription factors in contrast to basal ones, reporter gene activities of serial deletion mutants, especially the differences for −217 vs. −240 and for −409 vs. −490, support the idea that the GATA consensus sequence at −226 and the overlapping GATA and ETS consensus sites at −415 and −422 respectively, are involved in binding GATA and, regarding the upstream region, GATA or ETS transcription factors. GATA proteins (reviewed in [29–31]) constitute a family of zinc-finger transcription factors that can be subdivided into two subfamilies binding to the GAT(A/T) motif [32,33]. GATA-1, 2, 3 are mainly expressed in hematopoietic cells, whereas GATA-4, 5, 6 show overlapping expression in the developing and adult heart as well as in the gut. The importance of GATA

proteins for cardiac development is demonstrated by antisense experiments [34] and the GATA-4 knockout which shows a cardia bifida phenotype [35,36]. GATA factors are also implicated in *human* congenital heart defects [37] and in neointima formation after balloon injury [38], and these are pathophysiological conditions in which an involvement of ECE-1 was shown [39–41]. Additionally, the preproendothelin-1 gene itself was the first non-erythroid target gene regulated in endothelial cells by GATA-2 [42,43]. Furthermore there are consensus sites for GATA proteins in the ECE-1a and 1b promoters [5]. In this context, a transcriptional regulation of ECE-1c by GATA factors in heart and endothelium appears conceivable.

An alternative explanation regarding the regulatory region between −217 and −240 involves the CAAT-box which overlaps the GATA site (Fig. 1B). This box is in appropriate distance to the start site at −140, but to our knowledge no interactions between CAAT-boxes and Inr elements or E2F proteins have been described. Gel-shift experiments would be necessary to clarify the role of GATA and E2F proteins in ECE-1c regulation. As mentioned above, transcription factors of the ETS family are also candidates for ECE-1c promoter binding. ETS family members are expressed in endothelial cells [44,45] and play a role in angiogenesis [46], neointima formation [47,48] and endocardial cushion development [49,50]. We previously reported data which suggest that the ECE-1a promoter is regulated by ETS proteins in cultured endothelial cells [20]. Whether this is also the case for the ECE-1c promoter has to be further elucidated.

Another significant result is the correlation between isoform-specific ECE-1 promoter activities and corresponding mRNA levels in ECV304 and MCF7 cells (Fig. 4A, B): MCF7 cells only express ECE-1c mRNA consistent with neglectable promoter activities of the other isoforms. ECV304 cells additionally express ECE-1b mRNA and show a significant b-specific promoter activity. The relative strong ECE-1b signal in the RPA in comparison to the b-specific promoter activity (both related to ECE-1c) could be explained by the fact that the protected fragment of 264 nt in our RPA not only represents ECE-1b but also the recently published ECE-1d [13] (the protected exon 2 is common to both isoforms). These data indicate that the ECE-1 isoforms are regulated on transcriptional level and that the promoter assays mirror tissue mRNA expression.

This work in conjunction with the previously published ECE-1 promoter studies [5,13,19] demonstrates that ECE-1 isoforms are not generated by differential splicing of one common precursor but rather by alternative promoters. In general, alternative promoters have been implicated in spatial (cell-specific) and temporal (developmental) gene expression. In addition they can contribute to different subcellular localizations and different quantities of isoforms [51,52]. All that applies to ECE-1: The isoforms show different cellular distributions, different subcellular localizations and seem to be subjected to developmentally controlled gene expression ([8,13] and unpublished observations).

In conclusion, this work provides first insights into the transcriptional regulation of the major ECE-1 isoform, which may contribute to a better understanding of the regulation of the endothelin system in developmental and under pathophysiological conditions.

Acknowledgements: H.F.-K. is a recipient of a grant of the Deutsche Herzstiftung, Frankfurt. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to M.P. and H.-D.O. (MP 332/4-1). The excellent technical assistance of Mrs. C. Meissner and Mrs. B. Schwaneberg is greatly acknowledged.

References

[1] Xu, D. and Yanagisawa, M. (1994) Cell 78, 473–485.
[2] Emoto, N. and Yanagisawa, M. (1995) J. Biol. Chem. 270 (25), 15262–15268.
[3] Hasegawa, H. and Hiki, K. et al. (1998) FEBS Lett. 428, 304–308.
[4] Takahashi, M., Fukuda, K., Shimada, K., Barnes, K. and Turner, A.J. et al. (1995) Biochem. J. 311, 657–665.
[5] Orzechowski, H.-D., Richter, C.-M. and Funke-Kaiser, H. et al. (1997) J. Mol. Med. 75, 512–521.
[6] Shimada, K. et al. (1995) Biochem. Biophys. Res. Commun. 207 (2), 807–812.
[7] Schmidt, M. and Kröger, B. et al. (1994) FEBS Lett. 356, 238–240.
[8] Schweizer, A. and Valdenaire, O. et al. (1997) Biochem. J. 328, 871–877.
[9] Russel, F.D., Skepper, J.N. and Davenport, A.P. (1998) Circ. Res. 83, 314–321.
[10] Parnet, C., Le Moullec, J.-M., Cousin, M.-A., Guédin, D., Corvol, P. and Pinet, F. (1997) Hypertension 30, 837–844.
[11] Valdenaire, O. et al. (1995) J. Biol. Chem. 270, 29794–29798.
[12] Cailler, F. and Zappula, J.P. et al. (1999) Biochem. J. 341, 119–126.
[13] Valdenaire, O., Lepailleur-Enouf, D. and Egidy, G. et al. (1999) Eur. J. Biochem. 264, 341–349.
[14] Yanagisawa, H. and Yanagisawa, M. et al. (1998) Development 125, 825–836.
[15] Saleh, D., Furukawa, K. and Tsao, M.-S. et al. (1997) Am. J. Respir. Cell. Mol. Biol. 16, 187–193.
[16] Shao, B., Yan, W. and Rockey, D.C. (1999) J. Biol. Chem. 274, 3228–3234.
[17] Shimada, K. and Takahashi, M. et al. (1995) FEBS Lett. 371, 140–144.
[18] Corder, R. and Barker, S. (1999) J. Cardiovasc. Pharmacol. 33, 671–677.
[19] Funke-Kaiser, H., Orzechowski, H.-D., Richter, M. and Paul, M. (1998) J. Cardiovasc. Pharmacol. 31 (Suppl. 1), S7–S9.
[20] Orzechowski, H.-D., Günther, A., Menzel, S., Funke-Kaiser, H., Richter, M., Bohnemeier, H. and Paul, M. (1998) J. Cardiovasc. Pharmacol. 31 (Suppl. 1), S55–S57.
[21] Higuchi, R. et al. (1988) Nucleic Acids Res. 16, 7351–7367.
[22] Campbell, J.H. and Campbell, G.R. (1993) Clin. Sci. 85, 501–513.
[23] Gordon, R.B., Sussman, I.I. and Hatcher, V.B. (1983) In Vitro 19, 661–671.
[24] Korhonen, J., Lahtinen, I. and Halmekytö, M. et al. (1995) Blood 86, 1828–1835.
[25] Nevins, J.R. (1992) Science 258 (5081), 424–429.
[26] Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B. and Smale, S.T. (1994) Mol. Cell. Biol. 14, 116–127.
[27] Roeder, R.G. (1996) TIBS 21, 327–334.
[28] Novina, C.D. and Roy, A.L. (1996) TIG 12, 351–355.
[29] Charron, F. and Nemer, M. (1999) Semin. Cell. Dev. Biol. 10, 85–91.
[30] Evans, T. (1997) Trends Cardiovasc. Med. 7, 75–83.
[31] Simon, M.C. (1995) Nat. Genet. 11, 9–11.
[32] Merika, M. and Orkin, S.H. (1993) Mol. Cell. Biol. 13, 3999–4010.
[33] Sakai, Y., Nakagawa, R., Sato, R. and Maeda, M. (1998) Biochem. Biophys. Res. Commun. 250, 682–688.
[34] Jiang, Y. and Tarzami, S. et al. (1998) Dev. Genet. 22, 263–277.
[35] Kuo, C.T. and Morrisey, E.E. et al. (1997) Genes Dev. 11, 1048–1060.
[36] Molkentin, J.D., Lin, Q., Duncan, S.A. and Olson, E.N. (1997) Genes Dev. 11, 1061–1072.
[37] Pehlivan, T., Pober, B.R. and Bruckner, M. et al. (1999) Am. J. Med. Genet. 83, 201–206.

[38] Mano, T. and Luo, Z. et al. (1999) Circ. Res. 84, 647–654.
[39] Wang, X. and Douglas, S.A. et al. (1996) Circ. Res. 78, 322–328.
[40] Minammino, T., Kurihara, H.K., Takahashi, M. and Shimada, K. et al. (1997) Circulation 95, 221–230.
[41] Hofstra, R.M.W., Valdenaire, O. and Arch, E. et al. (1999) Am. J. Hum. Genet. 64, 304–308.
[42] Lee, M.-E., Temizer, D.H., Clifford, J.A. and Quertermous, T. (1991) J. Biol. Chem. 266, 16188–16192.
[43] Dorfman, D.M., Wilson, D.B., Bruns, G.A.P. and Orkin, S.H. (1992) J. Biol. Chem. 267, 1279–1285.
[44] Iljin, K., Dube, A. and Kontusaari, S. et al. (1999) FASEB J. 13, 377–386.
[45] Dube, A., Akbarali, Y., Sato, T.N., Libermann, T.A. and Oettgen, P. (1999) Circ. Res. 84, 1177–1185.
[46] Wernert, N., Raes, M.B. and Lassalle, P. et al. (1992) Am. J. Pathol. 140, 119–127.
[47] Tanaka, K. and Oda, N. et al. (1998) J. Cell. Physiol. 176, 235–244.
[48] Hultgardh-Nilsson, A., Cercek, B., Wang, J.W. and Naito, S. et al. (1996) Circ. Res. 78, 589–595.
[49] Majka, S.M. and McGuire, P.G. (1997) Mech. Dev. 68, 127–137.
[50] Macias, D., Perez-Pomares, J.M. and Garcia-Garrido, L. et al. (1998) Anat. Embryol. (Berl.) 198, 307–315.
[51] Ayoubi, T.A.Y. and van de Ven, W.J.M. (1996) FASEB J. 10, 453–460.
[52] Schibler, U. and Sierra, F. (1987) Annu. Rev. Genet. 21, 237–257.