Gene expression of vasoactive intestinal contractor/endothelin-2 in ovary, uterus and embryo: comprehensive gene expression profiles of the endothelin ligand–receptor system revealed by semi-quantitative reverse transcription-polymerase chain reaction analysis in adult mouse tissues and during late embryonic development

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ABSTRACT

Vasoactive intestinal contractor (VIC)/endothelin-2 (ET-2) is a 21 amino acid intestinal peptide characterized as a potent vasoactive and intestinal smooth muscle-contracting compound. To investigate the physiological roles of VIC/ET-2 further, we characterized the specificity of VIC gene expression relative to that of other members of the endothelin (ET) ligand–receptor system in adult mouse tissues and during embryonic development. Gene expression of ET-1, ET-3, ETA and ETB was ubiquitous in almost all tissues we examined while gene expression of VIC was localized to certain tissues. A high level of VIC gene expression was observed in ovary and uterus. The gene

expression of VIC, relative to that of glyceraldehyde-3-phosphate dehydrogenase, was approximately 2.0%, 0.4%, and 2.3% in ovary, uterus, and intestine respectively, and was approximately 1.6 and 7.1 times higher than that of ET-1 in ovary and intestine respectively. Thus, VIC may have some physiological role in adult ovary and uterus as well as intestine. In embryonic development, VIC gene expression sharply increased between 11 and 15 days post coitus and decreased after birth, suggesting an involvement in the later stages of embryonic development.

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INTRODUCTION

The endothelin (ET) system, composed of three ligands and two receptors, plays an important physiological role in mammals. ET family ligands, which include endothelin-1 (ET-1), vasoactive intestinal contractor (VIC)/endothelin-2 (ET-2), and endothelin-3 (ET-3), are peptides composed of 21 amino acid residues. These isopeptides have a variety of biological roles, including involvement in vasoconstriction (Yanagisawa *et al.* 1988), bronchial smooth muscle contraction (Mckay *et al.* 1991), and

cell growth (Lahav *et al.* 1996, Mazzocchi *et al.* 1997). The effects of these peptides are mediated by receptors (ETRs) that are classified into two subtypes, the endothelin A receptor (ETA) and the endothelin B receptor (ETB). Previous studies demonstrated that the expression of ET-1 or ET-3 and ETA or ETB mRNAs are co-localized in various tissues, suggesting that ET-1 and ET-3 function in an autocrine or paracrine manner (MacCumber *et al.* 1989, Huggins *et al.* 1993). ET-1 and ET-3 appear to play an important role in embryonic development. Targeted gene inactivation

experiments showed the ET-1/ETA pathway was essential for the development of tissues derived from cephalic and cardiac neural crest cells (Kurihara *et al.* 1994, 1995, Clouthier *et al.* 1998). ET-3 and ETB knockout mice exhibit spotted coat color and megacolon resulting from the absence of epidermal melanocyte and enteric neurons, showing the importance of the ET-3/ETB pathway in the development of tissues derived from vagal and trunk neural crest cells (Baynash *et al.* 1994, Hosoda *et al.* 1994).

VIC is a mouse intestinal peptide characterized as a potent vasoactive and intestine-contracting compound (Ishida *et al.* 1989, Saida *et al.* 1989, Blank *et al.* 1991, Wollberg *et al.* 1991, Yoshinaga *et al.* 1992, Taniyama *et al.* 1993, Fang *et al.* 1994, Kan *et al.* 1994, de la Monte *et al.* 1995). ET-2 is a human-derived peptide characterized as a potent vasoconstrictor (Inoue *et al.* 1989). VIC differs from ET-2 in 1 amino acid of 21 residues and may be the mouse or rat counterpart of human ET-2 (Bloch *et al.* 1991). Throughout this manuscript we use the symbol VIC/ET-2 to represent either of these peptides. Structurally, VIC/ET-2 is highly homologous to ET-1. VIC differs from ET-1 in 3 amino acids of 21 residues, ET-2 differs from ET-1 in 2 amino acids. There are no obvious differences in antigenicity between VIC/ET-2 and ET-1, nor are there any differences in their selectivity for ETA or ETB receptors. These similarities make it difficult to analyze the specific biological activities for each ligand. Although the biological activities and physiological roles of ET-1 and ET-3 have been well documented thus far, little is known about the specific physiological role of VIC/ET-2. The tissue distribution of VIC/ET-2 mRNA, determined by RNase protection assay, in rat gastrointestinal tract has been reported (de la Monte *et al.* 1995). However few reports on the comprehensive tissue distribution of VIC/ET-2 mRNA in various adult tissues and throughout embryogenic development are available. Moreover, to our knowledge no comprehensive quantitative and comparative study of the gene expression of all members of the ET system has yet been reported. Thus, it is important to identify the specific tissues and developmental stages in which the VIC/ET-2 gene is preferentially expressed and to characterize the quantity of the VIC/ET-2 gene expression in comparison with other genes of the ET system, in an attempt to understand the specific physiological roles of VIC/ET-2 *in vivo*.

We established a semi-quantitative reverse transcription-linked polymerase chain reaction (RT-PCR) to analyze the gene expression levels of

all the ligands and receptors of the ET system. We then examined the expression levels of each gene quantitatively in tissues of adult mice and in embryos in the later stages of development using the RT-PCR method to characterize the specificity of VIC gene expression.

MATERIALS AND METHODS

Mice

Adult 9-week-old male as well as pregnant and non-pregnant adult 9-week-old female Slc:ICR mice were obtained from Japan Clea (Tokyo, Japan). Embryos at 11, 15, and 17 days post coitus (E11, E15, and E17) and neonates 1 day post partum (P1) were obtained from the pregnant mice. Our experimental procedures were in accordance with the Guidelines on Handling and Care of Laboratory Animals by the Universities Federation for Animal Welfare in the UK.

mRNA preparation

Immediately on induction of anesthesia the following tissues were removed: brain (cerebrum and cerebellum), heart, lung, stomach, intestine, liver, kidney, spleen, uterus, ovary, and testis. The excised tissues, whole embryos and neonates were washed well with phosphate-buffered saline (PBS(-)) on ice and weighed. One hundred milligrams of each tissue were immediately homogenized. Total RNAs were prepared from the homogenate using Isogen solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Poly(A)⁺ RNAs were obtained from the total RNAs by oligo(dT)-cellulose chromatography (Pharmacia, Uppsala, Sweden).

Cloning of cDNAs used as control templates

cDNAs of the three ligands (ET-1, VIC/ET-2, and ET-3) and two receptors (ETA and ETB) have been cloned previously in our laboratory by screening a cDNA library (Saida & Mitsui 1991, Kurama *et al.* 1996, and T Uchide & K Saida, unpublished data). The restriction fragments and PCR products were cloned using a pCR-Script SK+ Cloning Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol, and were sequenced using a DyeDeoxy Terminator Cycle Sequencing Kit and Model 373A DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). The cDNAs inserted in plasmids were used as quantitative controls to estimate the expression level of each gene.

TABLE 1. PCR conditions optimized for semi-quantitative cDNA amplification for the ET system and GAPDH

	Primers (sense/antisense, 5'→3')	No. of cycles	Product size (bp)
cDNA			
VIC	CTGCTGCTGTGTTAAAGACTGGCAAG AGGTTCTGTGCTCCCAAAAGTGTC	35	652
ET-1	AGCTGGTGGGAAGGAAGAACTACG GACAGTGCAGAAAGGTGAGGTAGACT	35	728
ET-3	CTTGTATGGGGGCGGATGACAAG TGCCTCGCTAACTTGCTCAGGC	35	679
ETA	CCCTTGATTACCGCCATTGAAA TGACAACCAAGCAGAAGACAGT	35	346
ETB	GGCTAGTGTGTTTTCAGAGGCTTG CAGAACCACAGAGACCACCCAAAT	35	822
GAPDH	TTTATTGACCTCAACTACATG GTGGCAGTGATGGCATGGAC	25	443

Annealing temperature at 60°C, AmpliTaq Gold DNA polymerase and template concentration at 5 ng single stranded cDNA/20 µl.

Design of primers and RT-PCR reaction

Oligonucleotide primer pairs specific for mouse genes of VIC, ET-1, ET-3, ETA, ETB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to amplify each specific band without producing any accessory bands. Optimized primer sequences and the predicted amplification products are listed in Table 1. The primers span an intron–exon boundary, so that each amplification product could be taken as a representative of tissue mRNA expression and not a contamination of genomic DNA.

cDNAs were generated using Poly(A)⁺ RNAs as templates in the reverse transcriptase (RT) reaction mixture, which contained 2.5 µM random primer, 1 unit/µl RNase inhibitor, 1 mM dNTP, 5 mM MgCl₂, 0.25 unit/µl RT, and RT reaction buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl), by incubating at 30 °C for 10 min and then at 42 °C for 30 min in a Thermal Cycler Model MP (Takara, Tokyo, Japan). After the incubation the reaction mixture was heated to 99 °C for 5 min. The RT product was amplified in a PCR reaction mixture (20 µl) containing 2.5 mM MgCl₂, 200 µM dNTP, 0.5 µM each of antisense and sense primers, 0.25 units of Ampli Taq Gold DNA polymerase (PE Applied Biosystems) and PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl). After preheating at 95 °C for 9 min, we performed the following sequential steps for 25 (GAPDH) or 35 cycles (ETs and ETRs): 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The final incubation was performed at 72 °C for 10 min. PCR products were run in 2% agarose gels, stained with ethidium bromide, and then photographed under UV illumination.

Semi-quantitative RT-PCR analysis; optimized PCR conditions

We employed a semi-quantitative RT-PCR method based on a previously described method (Ozawa *et al.* 1996, 1997) for analysis of gene expression of the ET system. The optimum number of amplification cycles and dilution levels of reverse-transcribed cDNA tissue samples used for semi-quantitative PCR were chosen within the linear range of the reaction (Table 1). We used AmpliTaq Gold (PE Applied Biosystems) polymerase to avoid producing accessory bands and to support sufficient amplification in later cycles, which were difficult in Taq polymerase. We optimized specific primers for eliminating the production of accessory bands. The optimized PCR conditions (Table 1) enabled us to estimate the starting template molecules over two to three orders of magnitude in the visible range of the ethidium bromide staining in agarose gel electrophoresis (Figs 1 and 2C), confirming that quantification of products was done during the linear phase of amplification.

Standard curves

We amplified various amounts of pre-determined standard cDNA templates simultaneously with tissue sample templates in separate tubes, and estimated the expression level of a particular gene by comparing the band intensity of sample templates with that of standard templates (Figs 1, 2C and 4A). Each standard template cDNA of the ET system (VIC, ET-1, ET-3, ETA and ETB) and GAPDH was serially (10^{-n} or 3×10^{-n}) diluted (lanes 1–8 in Fig. 1). The starting numbers of

serially diluted standard cDNA template molecules per each PCR reaction (20 µl) were: 2.4×10 (lane 1), 7.2×10 (lane 2), 2.4×10^2 (lane 3), 7.2×10^2 (lane 4), 2.4×10^3 (lane 5), 2.4×10^4 (lane 6), 2.4×10^5 (lane 7), 2.4×10^6 molecules (lane 8) for ET-1; 2.5×10 (lane 1), 7.5×10 (lane 2), 2.5×10^2 (lane 3), 7.5×10^2 (lane 4), 2.5×10^3 (lane 5), 2.5×10^4 (lane 6), 2.5×10^5 (lane 7), 2.5×10^6 molecules (lane 8) for VIC and ET-3; 2.8×10 (lane 1), 8.4×10 (lane 2), 2.8×10^2 (lane 3), 8.4×10^2 (lane 4), 2.8×10^3 (lane 5), 2.8×10^4 (lane 6), 2.8×10^5 (lane 7), 2.8×10^6 molecules (lane 8) for ETA; 2.0×10 (lane 1), 6.0×10 (lane 2), 2.0×10^2 (lane 3), 6.0×10^2 (lane 4), 2.0×10^3 (lane 5), 2.0×10^4 (lane 6), 2.0×10^5 (lane 7), 2.0×10^6 molecules (lane 8) for ETB; 2.7×10^3 (lane 1), 8.1×10^3 (lane 2), 2.7×10^4 (lane 3), 8.1×10^4 (lane 4), 2.7×10^5 (lane 5), 2.7×10^6 (lane 6), 2.7×10^7 (lane 7), 2.7×10^8 molecules (lane 8) for GAPDH. The starting number of molecules was calculated from the molecular weights (g/mole) of each PCR product (bp) and amounts (pg) of each serially diluted control cDNA. The intensities of the bands were analyzed by computer.

Specificity analysis of PCR products: Southern hybridization and restriction enzyme digestion

The specificity of each band was determined by Southern hybridization and restriction enzyme digestion to produce bands corresponding to expected fragment lengths. PCR products separated in agarose gels were transferred onto nylon membranes (Boehringer Mannheim, Mannheim, Germany). The membranes were hybridized with each specific probe; cloned cDNA was labeled with digoxigenin-dUTP by random priming method (DIG DNA Labeling kit, Boehringer Mannheim). The protocols of hybridization, washing and chemiluminescence detection were as previously described (Engler-Blum *et al.* 1993). Each PCR product was digested with a restriction enzyme appropriate for producing fragments of expected lengths for identifying the specificity of PCR products.

Gene expression rate

Using the relative intensity of bands (Fig. 1) and the linear range of the standard curve (as shown in Fig. 2C), we estimated the number of starting cDNA molecules in each sample tissue solution. The gene expression rate for each member of the ET system was defined as the ratio of the number of the cDNA molecules of the ET system to the number of

GAPDH cDNA molecules multiplied by 100. GAPDH exists in all tissues at a constant level of expression and was used as an internal control for normalizing the differences in quality and quantity of reverse-transcribed cDNAs of mRNAs extracted from each tissue (el-Husseini *et al.* 1994). The gene expression rate was calculated using the following formula:

$$[\text{no. of molecules (ETs or ETRs)}/\text{no. of molecules (GAPDH)}] \times 100.$$

Statistical analysis was carried out by ANOVA using Statview statistical software (Abacus Concepts, Berkeley, CA, USA). Data are presented as means $\pm$ s.d.

RESULTS

Establishment of a semi-quantitative RT-PCR analysis for the ET system: standard curve and gene expression rate

To detect and characterize the gene expression of VIC, we established a semi-quantitative RT-PCR analysis and calculated the gene expression rate for each member of the ET system (VIC, ET-1, ET-3, ETA and ETB) as described below and in Materials and Methods. To ensure that the quantification of products is done within the linear range of the PCR amplification to avoid the plateau effect, we first determined the optimized PCR conditions (primers, number of cycles, polymerase, annealing temperature, template concentration), as shown in Table 1. Then, under the optimized conditions, we amplified the ET system and GAPDH samples along with corresponding standards at pre-determined concentrations, which we used to determine the starting concentrations of the samples, and RT-PCR products were analyzed by electrophoresis (Fig. 1). To confirm reproducibility, RT-PCR experiments were repeated three times. Figure 1, representative of the three trials, shows the expression of the ET system and GAPDH mRNA in each tissue. To confirm the specificity of amplification, the RT-PCR products (Fig. 1) were analyzed by Southern blot hybridization and restriction enzyme digestion for each gene. Each specific probe was hybridized to produce bands with relative intensities parallel to those bands produced by ethidium bromide staining (Fig. 1). Southern hybridization produced only the signal bands; no accessory bands were evident (Fig. 2A). Digestion of the PCR products of each gene with restriction enzymes produced fragments of expected lengths (as in Fig. 2B), confirming the identity of the PCR products. Next, we plotted the starting number of serially diluted standard template cDNA against the relative intensity of the

FIGURE 1. RT-PCR amplification of mRNA in tissues (Cl-St) and the standard (1–8) for semi-quantitative analysis - expression of the ET system and GAPDH genes in adult mouse tissues. Gene expression of the ET system (VIC, ET-1, ET-3, ETA and ETB) and GAPDH were examined by RT-PCR under optimized conditions (Table 1) using as templates mRNAs extracted from mouse tissues (Cl-St). Gene expression rates were estimated by comparison with amplified standard templates (1–8). Tissue samples are cerebellum (Cl), cerebrum (Cr), testis (Te), ovary (Ov), uterus (Ut), lung (Lu), heart (He), spleen (Sp), kidney (Ki), liver (Li), intestine (In), and stomach (St). In the lane used as a blank (Bl) (negative control) no template amplification was performed. Each standard template (1–8) cDNA, with a pre-determined starting concentration, was serially diluted and amplified by PCR under the same conditions (Table 1) as, and simultaneously with, the tissue samples. The relative intensity of bands (1–8) and the starting numbers of each serially diluted standard template were used to draw standard curves, as shown in Fig. 2C. The starting number of standard template per PCR reaction is described in Materials and Methods. The relative intensity of bands (Cl-St) was used to estimate the starting number of each cDNA molecule in the sample RT solution, as shown in Fig. 2C. Expression of the GAPDH gene was used as an internal normalizing control for calculating the gene expression rate (see Fig. 3).

bands (Fig. 1) and drew standard curves of relative intensity versus number of molecules, as shown in Fig. 2C. The standard curves showed linearity over two to three orders of magnitude (Fig. 2C). The relative intensities of the sample bands were also confirmed to be within the linear range of the reaction (Fig. 2C). Similarly, we were also able to confirm the identity of the PCR products and the linearity of the standard curves for ET-1, ET-3, ETA, ETB and GAPDH (T Uchide & K Saida, unpublished data).

Using the linear range of the standard curve we estimated the number of starting molecules of template cDNAs in the reverse-transcribed sample solution, and calculated the gene expression rate by dividing the number of cDNA molecules by the number of GAPDH cDNA molecules for normalization and multiplying by 100. Using the gene expression rate, we characterized the specificity of VIC gene expression, as shown in expression profiles of the ET system genes in Fig. 3 and Fig. 4B.

Profile of VIC gene expression in adult tissues: gene expression rates of the ET system

To gain insight into the physiological role of VIC in adult mouse tissues, we characterized the specificity of VIC gene expression by comparing gene expression rates of the ET system, normalized by GAPDH. Figure 3 shows the expression profile of the ET system genes estimated by gene expression rates. As compared with ET-1, ET-3, ETA and ETB, the expression of VIC was rather tissue-specific. A high level of VIC gene expression was observed in ovary and uterus (Fig. 3) as well as in intestine as previously described (Saida *et al.* 1989). Low expression was observed in stomach, lung, testis, cerebellum and cerebrum (Fig. 3) and very little cDNA was detected in heart and kidney (Fig. 2A). A high ET-1 gene expression rate, greater than one, was detected in lung, uterus, and ovary. A high level of ET-3 gene expression was detected in lung and uterus. The gene expression rate of ETB was ten

times higher than that of ETA in cerebrum, cerebellum, and kidney. In other tissues, the ETA gene was more abundantly expressed. Except for VIC, our semi-quantitative observations essentially confirm previous observations of ET system gene expression reported in adult rat tissues (Sakurai *et al.* 1990, 1991, Firth & Ratcliffe 1992, Shiba *et al.* 1992, de la Monte *et al.* 1995).

For statistical analysis of gene expression rates of the ET system in ovary, uterus and intestine, where VIC expression was observed more abundantly than in other tissues, semi-quantitative RT-PCR was independently carried out using mRNA extracted

from three individual mice. The gene expression rates of VIC were 2.0, 0.4, and 2.3 in ovary, uterus and intestine respectively, as shown in Table 2. The gene expression rates of VIC in ovary and intestine were significantly ($P<0.01$) higher than those of ET-1 or ET-3. The gene expression rate of VIC was 1.6 and 7.1 times higher than that of ET-1 in ovary and intestine respectively. In ovary, gene expression rate of ETA was significantly ($P<0.01$) higher than that of ETB.

Profile of VIC gene expression during embryonic development: gene expression rates of the ET system

To gain insight into the physiological role of VIC during late embryonic development, we characterized the specificity of VIC gene expression by comparing the normalized gene expression rates. The gene expression of VIC, ET-1, ET-3, ETA, ETB, and GAPDH in whole embryos and neonates throughout prenatal development (E11, E15, E17, and P1) is shown in Fig. 4A. A diagram of the gene expression rates, showing the expression profile of the ET system, is presented in Fig. 4B. We detected VIC gene expression, along with the expression of other ET family ligand and receptor genes, during late embryonic development. VIC showed a sharp

FIGURE 2. Specificity analysis of RT-PCR products and standard curve for estimating cDNA molecules of VIC. (A) Specificity analysis by Southern blot hybridization. Electrophoresed bands of RT-PCR products of VIC shown in Fig. 1 (652 bp) were transferred onto nylon membranes and hybridized with a digoxigenin-labeled VIC probe. Relative intensities of hybridized bands, detected by chemiluminescence, correspond to intensities of bands produced by ethidium bromide staining (Fig. 1). Abbreviations of tissues as in Fig. 1. (B) Specificity analysis by restriction enzyme digestion. RT-PCR products of VIC were digested with BamHI. Digested (lane a) and undigested (lane b) samples were electrophoresed. Two fragments of predicted sizes (390 bp and 262 bp) were obtained, confirming the identity of the RT-PCR products (Fig. 1). (C) Linear region of the standard curve for estimating the starting number of cDNA molecules of VIC in tissues. Standard curve (○) for the quantification of VIC in tissue samples was drawn by plotting the starting number of standard template versus the relative intensity (RI) of each standard band (Fig. 1). Because the relative intensities of all bands (●) obtained from tissue samples (Fig. 1) was confirmed to be within the linear region (shaded area) of the standard curve, the starting number of cDNA reverse-transcribed from tissue samples was estimated using the linear region. The relative intensities of bands (Fig. 1) were analyzed by computer.

FIGURE 3. Expression profile of the ET system genes in adult mouse tissues - gene expression rates normalized by GAPDH. Gene expression rates were calculated as the ratios of the starting numbers of cDNA molecules of the ET system (VIC, ET-1, ET-3, ETA, ETB) to that of GAPDH. Abbreviations of tissues as in Fig. 1. ND, not detected.

increase between E11 and E15 and a decrease after birth similar to the changes observed in ET-1 expression. ET-3 and ETB gene expression rates showed a comparable rise and fall. Levels of ETA gene expression remained constant throughout the period examined. In contrast, the other genes showed peaked patterns. Expression of the receptor genes was found to be at higher levels than that of the ligand genes during late embryonic development. The gene expression rate of ETA was

approximately ten times greater than that of ETB throughout this period, as it is in adult liver, heart, uterus, ovary, and testis (Fig. 3).

DISCUSSION

A number of reports on the distribution of gene expression of each member of the ET system in various tissues have been published (Yanagisawa

TABLE 2. Statistical analysis of gene expression rates for the ET system. The results are the means ± s.d. of three independent experiments

	VIC	ET-1	ET-3	ETA	ETB	VIC:ET-1
Tissue						
Ovary	2.01 ± 0.26*	1.22 ± 0.31	0.46 ± 0.13	24.4 ± 4.66**	2.42 ± 1.86	1.6
Uterus	0.36 ± 0.09	1.12 ± 0.29	0.93 ± 0.38	13.9 ± 7.14	2.40 ± 2.15	0.3
Intestine	2.28 ± 0.80*	0.32 ± 0.02	0.60 ± 0.04	0.08 ± 0.04	0.07 ± 0.05	7.1

Semi-quantitative RT-PCR was independently carried out using mRNAs extracted from three individual mice (*n*=3) for statistical analysis of the gene expression rates of the ET system in mouse ovary, uterus, and intestine.
*Gene expression rates of VIC in ovary and intestine were significantly (*P*<0.01) higher than those of ET-1 or ET-3. **Gene expression rate of ETA in ovary was significantly (*P*<0.01) higher than that of ETB. VIC:ET-1 is the ratio of gene expression rates of VIC and ET-1.

et al. 1988, Bloch *et al.* 1989, MacCumber *et al.* 1989, Matsumoto *et al.* 1989, Saida *et al.* 1989, Arai *et al.* 1990, Nunez *et al.* 1990, Sakurai *et al.* 1990, 1991, Firth & Ratcliffe 1992, Shiba *et al.* 1992). However, different analytical methods (Northern blot analysis, RNase protection assay, immunological detection methods, and RT-PCR), different species (rat, human, and pig), and different stages of animal development have been presented in these reports. Moreover, none of these reports has dealt with both ligands and receptors of the ET system in a single paper. Hence, it was difficult to compare gene expression quantitatively among different tissues or to compare different genes in a single tissue.

In this paper, we analyzed the distribution of the gene expression of all members in the ET system by establishing a semi-quantitative RT-PCR method. RT-PCR is useful because it is highly sensitive and therefore requires only negligible quantities of tissue, thus allowing analysis of even the smallest organs. However, we must also consider spurious or artefactual detection caused by the high sensitivity of the RT-PCR method. The confirmation of the identity of RT-PCR products is essential. Southern blot analysis and restriction enzyme digestion allow us to confirm the identity of the products (Fig. 2A and 2B). The PCR conditions including the optimization of primers (Table 1) may be useful in analyzing the expression profile of the mouse ET system in pathophysiological as well as physiological states. The semi-quantitative normalized gene expression rate allows a comparative and comprehensive analysis of gene expression, enabling us to characterize the expression profiles of a number of genes in different tissues and different developmental stages.

We previously identified the VIC gene and examined its expression in mouse tissues by Northern blot analysis (Saida *et al.* 1989, Saida & Mitsui 1991). VIC gene expression was detected only in intestine and was not observed in other tissues. In this paper, we present a new insight: that

the mouse VIC/ET-2 gene is expressed abundantly in ovary as well as intestine, moderately in uterus, slightly in stomach, lung, testis, cerebrum, and cerebellum, and insignificantly in kidney and heart (Figs 2A and 3). The observation that the ratio of VIC/ET-2 gene expression to that of ET-1 is 7.1 in intestine and 1.6 in ovary (Table 2) suggests that VIC/ET-2 may play a greater physiological role in these tissues than ET-1, if it is accepted that VIC/ET-2 and ET-1 have the same physiological activities and that peptide concentrations are parallel to mRNA concentrations. Previous reports showed that VIC/ET-2 gene expression was detected in human kidney (Bloch *et al.* 1991, Karet & Davenport 1996). RNase protection assay in rat demonstrated a high level of VIC/ET-2 expression in both small and large intestine, a low level in stomach and heart, but none in lung, liver, spleen, kidney, testis and brain (Firth & Ratcliffe 1992). de la Monte *et al.* (1995) investigated the expression of rat VIC/ET-2 gene using the RNase protection assay and were able to detect high levels of VIC/ET-2 mRNA in colon and relatively lower levels in small intestine and stomach. O'Reilly *et al.* (1993) detected the expression and alternative splicing of the ET-2 gene in human uterus. Thus, our expression profile of the VIC/ET-2 gene in mouse tissues agrees with the previous data published on rat and human. Our data adds new information, namely that the ovary and embryo also express high levels of VIC/ET-2 mRNAs, suggesting that VIC/ET-2 may have some reproductive and developmental functions and that the VIC/ET-2 gene may also be expressed in rat and human ovary and embryo.

Cellular distribution of the VIC/ET-2 gene expression in rat gastrointestinal tract was characterized by de la Monte *et al.* (1995) using *in situ* hybridization. Specific expression signals were primarily observed in intestinal lamina propria cells, suggesting that this peptide has a role in local villous motility in intestine. However, no information on gene expression or the physiological roles

of VIC/ET-2 in uterus and ovary has been reported. In contrast, there have been several reports on the physiological roles of ET-1 in uterus and ovary. Expression of the ET-1 gene was detected in human and porcine granulosa cells (Kubota *et al.* 1994, Magini *et al.* 1996). The peptide is considered to play an important role in granulosa cell function. Furthermore, ET-1 in rat uterus has a pivotal role

in controlling bleeding from the placental bed through myometrial contraction in the early postpartum period (Kajihara *et al.* 1996). Based on our knowledge of the function of ET-1, we may speculate that VIC/ET-2 could regulate physiological events in the uterus and ovary in cooperation with ET-1. Studies are currently under way in our laboratory using *in situ* hybridization/histochemistry of the VIC/ET-2 gene with specific VIC/ET-2 probes that identify VIC/ET-2 expressing cells in an effort to understand this gene's physiological role in the intestine, ovary, and uterus.

The ET system plays a very important role in embryonic development. The targeted inactivation of the ET system genes demonstrates that ET-1/ETA and ET-3/ETB are involved in neural crest-derived cell differentiation in the mouse embryo at the early stages of development. ET-3 and ETB knockout experiments demonstrate that the ET-3/ETB signaling pathway is essential for the development of enteric neurons and melanocytes (Baynash *et al.* 1994, Hosoda *et al.* 1994). Our findings (Fig. 4B) show similar expression patterns of embryonic development for ET-3 and ETB, suggesting that these genes contribute to embryonic development even in the later stages (after E11). Additionally, it has been reported that ET receptors are already widely distributed on cells not originating from neural crest cells (Brand *et al.* 1998). The ET system might also play an important role in the proliferation and differentiation of embryonic cells other than those derived from neural crest cells during the later stages of embryonic development after E11. An earlier report (Chan *et al.* 1995) demonstrated that the expression of ET-1 becomes increasingly stronger in heart, lung, eye, ear, stomach, and intestine towards the later stages of development (after E11.5). Our experiment supports this finding that ET-1 expression is high at E15 and E17. The VIC/ET-2 gene is expressed sequentially and simultaneously with ETA and ETB receptor genes in late embryonic development

FIGURE 4. Detection of VIC gene expression during late embryonic development. (A) Gene expression of the ET system and GAPDH during late embryonic development. RT-PCR was performed using mRNAs extracted from embryos 11, 15 and 17 days post coitus (E11, E15, E17) and neonates 1 day post partum (P1). (B) Expression profile of the ET system gene during late embryonic development. Gene expression rates of VIC (●), ET-1 (■), ET-3 (▲), ETA (□) and ETB (○) were calculated using the linear region of the standard curve (as shown in Fig. 2C) and the relative intensities of bands (Fig. 4A) as described in Materials and Methods.

(Fig. 4B), suggesting its involvement in a variety of developmental processes in embryos. In our laboratory, *in situ* hybridization/histochemistry studies of the VIC/ET-2 gene using specific VIC/ET-2 probes are under way to identify the specific VIC/ET-2 expressing cells throughout embryonic development.

Conclusion

We showed the comprehensive expression profiles of ET system genes in adult mouse tissue and during late embryonic development, using gene expression rate analyzed by semi-quantitative RT-PCR. In the profiles, we identified relatively high expression of the VIC/ET-2 gene in ovary, uterus, and embryo as previously unidentified expression sites, suggesting that VIC/ET-2 may have some reproductive and developmental functions. Further investigations, including the use of gene disruption, are under way to identify the specific physiological roles of VIC/ET-2 in adult intestine, uterus, ovary, and during embryonic development.

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