**Bex1**, a gene with increased expression in parthenogenetic embryos, is a member of a novel gene family on the mouse X chromosome

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Received November 9, 1998; Revised and Accepted January 8, 1999 GenBank accession nos AF097437-AF097440

Parthenogenetic and normal blastocysts were compared using differential display analysis as a means to identify new imprinted genes. A single gene was identified with increased expression in parthenogenetic blastocysts, suggesting it might be an imprinted gene expressed from the maternally inherited allele. The gene, named **Bex1** (brain expressed X-linked gene), maps near **Plp** on the mouse X chromosome and to Xq22 in humans. Database homology searches revealed two additional uncharacterized cDNAs similar to **Bex1** that were named **Bex2** and **Bex3**. Allele-specific expression analysis of **Bex1** using F1 blastocysts indicated an excess of transcript expressed from the maternally inherited allele compared with the paternally inherited allele. This excess level of transcript derived from the maternally inherited allele may be due to imprinted X inactivation of the paternally inherited allele in the extraembryonic lineages of female embryos rather than a result of genomic imprinting.

**RESULTS**

**Differential display analysis**

Differential display was used to compare the expression profiles of fully expanded parthenogenetic and normal blastocysts. A single, novel, differentially expressed transcript that we have named **Bex1** was identified. **Bex1** has up-regulated expression in parthenogenetic blastocysts compared with normal blastocysts and maps to the X chromosome. This report describes the cloning and characterization of **Bex1** including an investigation into its potentially imprinted nature. We also identified two other related genes that we have named **Bex2** and **Bex3**. The results are discussed with reference to genomic imprinting and X chromosome inactivation.

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Confirmation of differential expression and northern analysis

Semi-quantitative RT–PCR was used to confirm the differential expression of the gene as blastocysts could not provide enough RNA for a northern blot. Briefly, the novel gene was co-amplified with the β-actin gene and the X-linked Hprt gene from cDNA derived from pools of normal and parthenogenetic blastocysts. Replicate RT–PCRs were removed from the thermal cycler after 13, 16, 19 and 22 cycles and dot blotted in triplicate. Blots were then probed with the newly cloned novel transcript, β-actin or Hprt. The results in Figure 1a show that while β-actin and Hprt are amplified at comparable rates from pools of parthenogenetic and normal embryos, the novel cDNA shows greater amplification by at least six PCR cycles. Due to the exponential nature of PCR amplification, this equates to an ~64-fold difference in initial template levels, assuming that each amplification cycle is 100% efficient.

Further expression analysis of the novel cDNA was undertaken by northern blot analysis of multiple tissues from male and female 3-week-old mice. High expression of the 0.8 kb transcript can be seen in the brain, and detectable expression in the lung and gonads. Li, liver; Br, brain; H, heart; Ki, kidney; Sp, spleen; Lu, lung; In, intestine; M, skeletal muscle; O, ovary (mature); T3, testis (3 week); Tm, testis (mature).

cDNA sequence and genomic structure of the Bex1 gene

The original cDNA clone for Bex1 carried a 358 bp insert comprising the 3′ end of a transcript and including part of the poly(A) tail. The sequence of this clone was used to search the GenBank expressed sequence tag (EST) database and an EST clone with matching sequence was obtained from Genome Systems (12) and fully sequenced. The insert of this clone is 790 bp in length [not including poly(A) sequences], but 5′ RACE performed on ovary RNA extended the sequence a further 21 bp at the 5′ end (13).

Genomic lambda clones hybridizing positively to a Bex1 cDNA probe were isolated from a 129/SV genomic library. One of the genomic lambda clones was restriction mapped using PvuII, and the various fragments of the insert were subcloned into plasmid vectors. Sequencing and ordering of the subclones by comparison with the cDNA allowed the genomic structure of Bex1 to be determined (14). The intron and exon boundaries identified were found to contain consensus donor and acceptor sequences. The gene is comprised of three exons that span ~1.5 kb of genomic DNA. As well as EST clones identical to Bex1, the search of the GenBank mouse dbEST with the Bex1 sequence revealed multiple similar but not identical uncharacterized EST clones that could be assigned into two categories. Representative EST clones were obtained for each category (15,16) and fully sequenced. Northern analysis showed that both clones have an expression pattern similar to that of Bex1 (data not shown) and were named Bex2 and Bex3 (17,18). Figure 2a shows a comparison of the Bex1, -2 and -3 cDNA.

Deduced amino acid sequences of the Bex gene products

Analysis of the cDNA sequence of Bex1 indicated a putative open reading frame (ORF) in exon 3 with two potential start codons. The deduced sequence resulting from these two start codons yields proteins of either 116 or 128 amino acids. Similarly, Bex2 has a putative ORF with two potential start sites encoding a protein of 116 or 129 amino acids. Bex3 has an ORF with only one potential start site and encodes a protein of 124 amino acids. Database searching using the putative Bex amino acid sequences identified homology to a partially characterized human cDNA clone called pHGR74 (19) for which the most likely ORF had been determined (20). Figure 2b shows a comparison of the putative amino acid sequences for Bex1, Bex2, Bex3 and pHGR74. The pHGR74 sequence has greatest homology to Bex3.

The computer program ‘motif’ from the GCG package was used to search these putative amino acid sequences for protein motifs. The only recognizable sequence motif identified was a CAAX box at the C-terminal end of the protein. The CAAX box acts as a target for the post-translational modification of the protein by the addition of either a geranyl-geranyl or a farnesyl group to the cysteine residue. These modifications result in attachment of the protein to a cellular membrane (for a review, see ref. 21). Proteins containing CAAX boxes, such as Ras proteins, G proteins and nuclear lamins, are known to be subject to this kind of post-translational modification (22–25).

Mapping of the Bex genes

The Bex genes were mapped using the European interspecific backcross (EUCIB) (26). Sequence polymorphisms between Spretus and C57/BL6 mice were identified by direct sequencing of PCR products amplified from each of the Bex genes (see Materials and Methods). The EUCIB panel was screened by PCR for Bex1, -2 and -3, with 102, 97 and 98 animals typed,
Figure 2. (a) Alignment of the cDNA sequences of Bex1, -2 and -3. Regions of homology between the genes are boxed and potential start codons are in bold. For the Bex1 gene, exon 1 includes bp 1–122, exon 2, bp 123–199, and exon 3, bp 200–810. (b) Alignment of the putative amino acid sequences of Bex1, -2, -3 and pHGR74. Regions of homology between the sequences are boxed and the CAAX motif is in bold.

respectively. There were no recombinants between the three Bex genes for the animals typed, and analysis of the data by the UK Human Genome Mapping Project Resource Centre (HGMP-RC) placed the cluster at 53.77 cM on mouse chromosome X (for the EUCIB mouse X chromosome map, see http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html). For the backcross animals tested, Bex1, -2 and -3 showed two, four and five recombination events, respectively, with Plp that maps at 52.39 cM on the EUCIB map. The differences in numbers of recombinant animals occurred because slightly different groups of individual animals were typed for each gene. The corresponding region on the human X chromosome is Xq22, and the human cosm id (11) containing a sequence similar to Bex1 also maps to this location.

X inactivation status of Bex1

Bex1 was assayed for escape from X inactivation by using females carrying the T(X;16)16H translocation (T16H), as previously described (27–30). T16H females undergo total non-random inactivation of the paternally derived, normal X chromosome. As a result, expression from genes on the paternal X chromosome constitutes an escape from inactivation. Sequence polymorphisms, which could be used to identify the parental
allele of origin of transcripts, within the Bex1 cDNA were identified between the ‘Lab strain’ and PGK strain of mice (see Materials and Methods). [T16H×PGK]F1 12.5 and 14.5 d.p.c. embryos as well as [Lab strain×PGK]F1 12.5 and 14.5 d.p.c. control embryos were assayed for allele-specific expression of Bex1 by RT–PCR and direct sequencing of PCR products (Fig. 3a). In the RT–PCR product from the control [Lab strain×PGK]F1 embryo, sequencing detected both the maternal Lab strain allele (C) and the paternal PGK allele (T). This resulted in a base call of N in the sequence readout. In the [T16H×PGK]F1 embryos, expression of only the maternal Lab strain allele (C) can be detected, indicating that Bex1 is subject to normal X inactivation.

Single nucleotide primer extension (SNuPE) analysis of Bex1 allele-specific expression

Analysis of the relative abundance of expression from the maternally and paternally inherited Bex1 alleles in normal embryos was undertaken to determine whether the gene is subject to genomic imprinting. SNuPE (31) was used on pools of blastocysts to determine the ratio of Bex1 maternal and paternal allele expression. In addition, 12.5 and 14.5 d.p.c. embryos as well as brain, lung and gonad from 3-week-old females were collected. Both [Lab strain×PGK]F1 and [PGK×Lab strain]F1 samples were used. Figure 3b is an example of an SNuPE gel that shows the relative level of maternal and paternal Bex1 transcript in brain and in pools of blastocysts compared with Lab strain and PGK controls. Figure 3c shows the results of the SNuPE analysis for both crosses. The results are presented in graphical form as the percentage of total transcript that is expressed from the maternal allele.

Expression is seen from both maternal and paternal alleles in post-implantation embryonic samples and in adult tissues, indicating that Bex1 is not imprinted in these tissues. A trend is observable in the data such that the maternal allele is expressed at a level of <50% in [Lab strain×PGK]F1 samples and of >50% in [PGK×Lab strain]F1 samples. An explanation for this trend is that the Lab strain and PGK strains of mice carry different Xce alleles. Xce alleles act to modify the random nature of X inactivation in female mice (32). In female mice that are heterozygous for Xce alleles, the X chromosome bearing a ‘strong’ Xce allele will remain the active chromosome in a greater percentage of cells than the X chromosome bearing a ‘weak’ allele. The Lab strain carries either the Xce c allele while the PGK strain carries the ‘stronger’ Xce b allele (33). The stronger Xce b allele would bias allelic Bex1 expression, causing the observed higher expression from the PGK strain-derived allele.

The blastocyst samples do not follow the trend that is evident in all the other samples as they have a much greater level of transcript from the maternally inherited allele (∼90%) compared with that from the paternally derived allele (∼10%). The excess in expression from the maternally inherited allele is evident regardless of whether the cross is [Lab strain×PGK]F1 or [PGK×Lab strain]F1. If Bex1 were located on an autosome, this result would be consistent with Bex1 being subject to genomic imprinting, with the maternal allele being expressed preferentially. However, since Bex1 is X-linked, several other factors should be considered before interpreting this result. The first factor to consider is that a pool of blastocysts is of mixed sex (i.e. the pool has both XMY and XMX F embryos). Unlike the situation for autosomes, which are inherited in equal ratios from both parents, the ratio of maternally to paternally derived X chromosomes in a pool of mixed sex blastocysts is 2:1. If the level of expression was equal from all Bex1 alleles, we would expect twice the amount of maternally derived transcript compared with paternally derived transcript. In this scenario, if assayed by SNuPE, the maternal allele should represent 66.6% of total Bex1 transcript. The results of the SNuPE revealed the presence of a larger maternal bias (∼86 and 93% depending on the cross), indicating that something other than chromosome ratio is affecting the ratio of maternal to paternal Bex1 transcripts.

The second modifying factor that must be considered is imprinted X inactivation in the extraembryonic lineages within
the blastocyst. At the blastocyst stage of development, the trophectoderm and primitive endoderm are undergoing imprinted inactivation of the paternal X chromosome, whereas inner cell mass (ICM) cells retain both X chromosomes in the active state (34). As the ICM cell number is estimated to be only ~20% of the total cell number in the blastocyst (35), this would result in a further decrease in the amount of X-linked paternal transcript compared with maternal transcript. This has been demonstrated previously using SNUPE on single blastocysts, for the X-linked genes Hprt and Pgk1, where there was decreased expression of the paternal allele relative to the maternal allele (36). Taking these points into consideration, it is possible that Bex1 transcription from the paternal allele at the blastocyst stage may constitute as little as 10% of the total Bex1 transcripts as seen in our results. These data, combined with the lack of imprinting of Bex1 seen in post-implantation embryonic and adult tissues, do not support the contention that Bex1 is subject to genomic imprinting.

**DISCUSSION**

We have isolated a novel transcript in a differential display screen based on its increased expression in parthenogenetic blastocysts. Characterization of the transcript and the genomic sequence from which it is expressed revealed a small gene with three exons encoding a 129 or 116 amino acid protein with a CAAX box. Northern analysis revealed a high level of the transcript in the brain, and mapping localized the gene to the X chromosome. The gene was named Bex1, for brain expressed X-linked gene. Subsequently, through EST database homology searches, two transcripts highly similar to Bex1 were found. They were named Bex2 and Bex3. Like Bex1, these transcripts are highly expressed in the brain and originate from genes that map to the X chromosome. Recently, a cDNA with the same sequence as Bex1 was submitted to GenBank. The cDNA clone, named Rex-3, was isolated in a screen for genes showing reduced expression in response to retinoic acid-induced differentiation of F9 teratocarcinoma cells (37). Extensive characterization was not carried out; however, a northern blot confirmed expression of the mRNA in the brain and ovary.

We sought to determine the possible causes for the increased expression of Bex1 in parthenogenetic blastocysts compared with normal blastocysts. Possibilities considered were that Bex1 is expressed in only XX embryos and that Bex1 is expressed only from the maternally inherited allele. Although we were not able to ascertain sex-specific expression in single normal blastocysts, RT–PCR analysis of sexed post-implantation embryos (data not shown) and northern analysis of adult tissues did not reveal any overt sex-specific differences in expression. The bias in Bex1 expression from the maternally inherited allele at the blastocyst stage can be attributed to the combination of the 2:1 ratio of maternally to paternally derived X chromosomes in the pool of blastocysts and imprinted inactivation of the paternal X chromosome in the extraembryonic lineages of the blastocyst. Both these modifying factors would result in a reduction of the amount Bex1 transcript from the paternally inherited allele compared with that from the maternally inherited allele.

To determine whether it is the X chromosome linkage of Bex1 that resulted in its increased expression in parthenogenetic blastocysts compared with normal blastocysts, the nature of X inactivation in parthenogenetic blastocysts was considered. Parthenogenetic blastocysts have no paternally derived X chromosomes and, therefore, they are unable to undergo imprinted X inactivation. Parthenogenetic blastocysts have been found to express Xist, suggesting that another mechanism may take over and allow some kind of X inactivation from the blastocyst stage (38). This is supported by the demonstration of late replicating X chromosomes in parthenogenetic blastocysts, albeit at a reduced frequency compared with normal blastocysts (39). Factors contributing to the increased expression of Bex1 in parthenogenetic blastocysts compared with normal blastocysts are; (i) X inactivation is functional but possibly delayed in parthenogenetic blastocysts; and (ii) a population of parthenogenetic blastocysts has a greater number of X chromosomes than a population of normal blastocysts (and thus has higher expression of X-linked genes). The combination of these two factors would allow for a maximum of a 2-fold increase in Bex1 expression in parthenogenetic embryos. Clearly the difference estimated by semi-quantitative PCR is far greater. In addition, if the difference in expression were due only to the X-linked nature of Bex1, then we would expect other X-linked genes to share this expression profile. This large increase in expression in parthenogenetic embryos is not seen for the X-linked gene Hprt using our assay (Fig. 1a) even though it has been shown that Hprt is subject to imprinted X inactivation at the blastocyst stage (36). A partial explanation for this difference could be the closer proximity of Bex1 to the XIC compared with Hprt, thus resulting in greater repression of the paternal allele due to the ‘spreading’ nature of X inactivation in progress (36). The finding that expression of Rex-3/Bex1 is down-regulated upon differentiation of F9 cells by retinoic acid suggests a further explanation for the higher expression levels of Bex1 in parthenogenetic embryos. If, in vivo, this was reflected as a down-regulation of Rex-3/Bex1 expression as trophectoderm and primitive endoderm differentiate, then any delay in the development of parthenogenetic embryos would result in a higher level of Rex-3/Bex1 expression compared with normal embryos. Consistent with our results, other X-linked genes (e.g. Hprt), whose expression is not down-regulated upon differentiation, would not be subject to this effect. To minimize this, the blastocyst stage embryos used in the work reported here were selected visually on the basis of developmental stage rather than developmental time. However, it remains possible that the developmental defects of parthenogenetic embryos that become apparent at later stages of development are reflected in delayed changes in gene expression in the blastocyst.

The Unigene map has placed a sequence-tagged site (STS) corresponding to the pHGR74 cDNA sequence on the human X chromosome between the markers DXS1231 and DXS1059. This is very close to the mapped position of the PLP gene at Xq22, which is the closest known gene to the Bex genes on the UK HGM X chromosome map for the mouse. The mapping of the highly related Bex genes as a cluster is suggestive of ancestral gene duplication events in this region of the X chromosome. It is interesting to draw parallels between the Bex genes and a recent report where another small gene, CXX1, as well as several pseudogenes were mapped in a cluster to Xq26 (40). Like the Bex genes, CXX1 encodes a small CAAX box motif-containing protein and, at present, there is no known function for CXX1. However, unlike the CXX1 sequences, all the Bex genes appear to encode full-length proteins, suggesting that they are multiple copies of similar functional genes rather than pseudogenes.

Bex3 is the most similar in sequence to pHGR74, suggesting that pHGR74 is the human homologue of Bex3. The pHGR74
cDNA was cloned originally due to its high expression in ovarian granulosa cells (20), and a recent publication (41) has found evidence for an imprinted locus on the mouse X chromosome that is associated with spontaneous ovarian granulosa cell carcinoma. The disease in the mouse has a parallel in humans in the form of spontaneous juvenile ovarian granulosa cell tumours. Although imprinting of Bex1 was not indicated in whole ovary from sexually mature female mice (see SNUPE results), ovarian granulosa cells were not specifically tested in our study. In addition, neither Bex2 nor Bex3 has been analysed for allele-specific expression. The androgen receptor (AR) gene was suggested as a candidate for the ovarian granulosa cell tumorigenesis locus based on its map location (41). Although it appears that the Bex genes do not map within the chromosomal region associated with this locus, a hypothesis of Bex gene involvement is intriguing given their appropriate expression patterns.

In addition to this possibility, a review of the literature on the human chromosomal region containing the Bex genes has revealed at least 16 diseases or syndromes, many involving hearing loss and mental retardation as symptoms, that map to this region. This is significant given the high levels of Bex gene expression in the brain. For some disorders, such as the MRX30 sub-type of non-syndromic X-linked mental retardation (42) and lissencephaly (LIS) (43), the genes responsible have been found. However, not all the genes responsible for neurological disorders mapping to this region have been identified (42), and screening these disorders for Bex gene involvement will be an important next step.

MATERIALS AND METHODS

PCR amplification

PCR amplification reactions were carried out using Dynazyme thermostable DNA polymerase (Finnzymes Oy, Finland) in a 50 µl reaction consisting of 10 pmol of each primer, 0.2 mM dNTPs, 2.5 U of Dynazyme and 1× Dynazyme reaction buffer. Standard cycles were 1 min at 94°C, 1 min at an annealing temperature appropriate for specific primers and 2 min at 72°C for 30 cycles, followed by a single step of 15 min at 72°C. Alternatively, amplification used AmpliTaq Gold (Perkin Elmer, Foster City, CA) in the appropriate buffer with the cycles modified to include a 12.5 min initial denaturation followed by 30 cycles with the annealing temperature set appropriately for the primer combination.

Sequencing

All sequencing reactions on plasmids or directly on PCR products were done using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA) using the 17mer T7, T3 or SP6 sequencing primers or the androgen receptor (AR) primer used for cDNA synthesis and 2.5 µl of 5 M dNTPs

Mice

The ‘Lab strain’ mice used in this work were F1 progeny from a C57BL/6×CBA/Ca cross bred in-house. The C57BL/6Ros.Hprt1, Pgf1a congenic mice (herein referred to as PGK) originally derived by West et al. (44) were obtained from P.Tam (Children’s Medical Research Institute, Wentworthville, Australia). The [T16H×PGK]F1 mouse embryo cDNA samples were obtained from N. Brockdorff (MRC Clinical Sciences Centre, RPMS, London, UK).

Embryo collection and generation of parthenogenetic embryos

Female mice were superovulated at 4–6 weeks of age by injecting 5 IU of Foligon (Intervet International B.V., Boxmeer, Holland) on day 1 at 17:00 h followed by 5 IU of Chorulon (Intervet) on day 3 at 15:00 h. The mice were then either mated to stud males when fertilized embryos were to be collected or housed with other superovulated females when unfertilized oocytes were to be collected. Oocytes were collected by standard procedures, washed through several changes of M2 (Sigma, St Louis, MO) and carefully inspected to remove contaminating maternal cells. Embryos were then washed into M16 (Sigma) and cultured to the blastocyst stage. To generate parthenogenetic embryos, unfertilized oocytes were activated by incubation in M2 with 7% ethanol for 4.5 min, followed by incubation in M16 with 5 µg/ml cytochalasin B (Sigma) for 4 h at 37°C. The parthenogenetic embryos were then cultured in M16 to the blastocyst stage. Pools of 200 blastocysts were collected, snap frozen on dry ice and stored at –70°C.

Preparation of RNA

RNA was prepared by lysing pools of 200 or more embryos in a microfuge tube using 400 µl of RNAzolB reagent (Tel-Test, Friendswood, TX), after which 40 µl of chloroform was added. The samples were mixed by vigorous shaking and incubated on ice for 5 min. Phases were separated by centrifugation at 14 000 r.p.m. for 15 min at 4°C. Supernatant was removed and precipitated overnight at –20°C with an equal volume of isopropanol using 20 µg of glycogen (Life Technologies, Gaithersburg, MD) as carrier. Precipitated RNAs were pelleted by centrifugation at 14 000 r.p.m. for 15 min at 4°C and washed with 1 ml of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water. RNA pellets were resuspended in 5 µl of DEPC-treated water per 200 blastocysts and stored at –70°C. RNA was prepared from tissue and larger embryos by the suggested RNAzolB protocol.

Differential display

The differential display method was performed essentially as described by Liang and Pardee (9). Total RNA equivalent to 100 blastocysts was reverse transcribed with the primers (dT)12 VA, (dT)12 VC or (dT)12 VG (Operon Technologies, Alameda, CA), in a 20 µl reaction using Superscript II (Life Technologies) as per the manufacturer’s instructions. cDNA was stored at –70°C until use. cDNAs were amplified in the presence of [α-33P]dATP (10 mCi/ml; Amersham Pharmacia-Biotech, Amersham, UK). Amplification of cDNAs was carried out as follows: each 20 µl mixture contained 1 µl of the reverse transcription reaction, 2 µl of 10× reaction buffer (Finnzymes Oy), 2.5 µl of 20 µM dNTPs (Promega), 0.3 µl of [α-33P]dATP, 0.1 µl of Dynazyme (Finnzymes Oy), 5 µl of 10 µM oligo(dT) primer used for cDNA synthesis and 2.5 µl of 5 M random 10mer from the kits OPK or OPX (Operon Technologies). The following PCR cycles were used: one cycle of 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 42°C for 1 min, 72°C for 30 s followed by 5 min at 72°C.
Samples were removed from oil, 10 µl of formamide loading dye was added, then 6 µl was run on a 6% denaturing polyacrylamide gel. The gel was dried without fixation and bands were visualized with overnight autoradiography using Kodak Biomax MR film.

Cloning of differential cDNAs

Fluorescent stickers on the differential display gel allowed the autoradiogram to be aligned with the dried gel. The differential band of interest was excised from the gel using a sterile scalpel blade and placed in a microfuge tube with 200 µl of sterile water. The tube was vortexed vigorously and the contents boiled for 10 min to facilitate elution of DNA from the gel slice. The tube was then centrifuged for 15 min, the supernatant removed to a clean tube and ethanol precipitated in the presence of glycogen. The pellet was resuspended in 20 µl of sterile water, and 1–5 µl of this was used as template in a PCR using the original differential display primers and standard amplification conditions with an annealing temperature of 42°C. PCR products of the correct size were gel purified using the Qiagel Quick Gel Extraction kit (Qiagen, Chatsworth, CA), end-polished using T4 polymerase (NEB, Beverley, MA) and cloned into the EcoRV site of pZER (Clontech, Palo Alto, CA).

Semi-quantitative PCR

Primers used for Bex1 were PBCX11F2 (5′-ccg gag cgg act cat gtt ctc aac cag gta ctc cac-3′) and PBCX11R (5′-tag aag cca ccg ccc gag cac cag g-3′). These primers amplify a 266 bp product from cDNA and genomic DNA template; however, reactions without reverse transcription were included in this experiment to control for DNA contamination. The primers used for β-actin, i.e. β-actin 5′ (5′-gtg cgc ccc act gca gac gac g-3′) and β-actin 3′ (5′-ctc att acct tct tca aag cag gcc gta ctc-3′), were primers designed for the human sequence (45) which also work for the mouse sequence. These primers amplify a product of 539 bp from cDNA only. The primers used for Hprt were HprtRNAR (5′-gtag gcc ata tcc gac aac aac c-3′) and HprtRNAR (5′-ctc ccc gcc cag ctc ctc-3′). These primers amplify a 552 bp product from cDNA only. PCR Mismatchmixes were set up and cDNA made from normal or parthenogenetic blastocystcs was added, after which the mismatchmix was aliquotted to 25 µl per tube. Each 25 µl had the following amounts of reagents: 2.5 µl of 10× PCR buffer, 0.83 µl of each of 2.5 mM primers for Bex1, β-actin and Hprt, 0.5 µl of 10 mM dNTPs and 0.25 µl of Dynazyme polymerase. A program of 22 cycles with an annealing temperature of 54°C was set and tubes were removed from the thermal cycler after 13, 16, 19 and 22 cycles. Equal amounts of each PCR were then dot-blotted in triplicate onto Zeta-Probe GT membrane (Bio-Rad, Hercules, CA). Blots were then probed with either Bex1, β-actin or Hprt PCR products labelled with [32P]dATP (Amerham) using the Rediprime kit (Amerham). The results were visualized using a phosphorimager.

Northern analysis

RNA was prepared from multiple mouse tissues as described earlier, and 10 µg of total RNA per lane was run on a 1% agarose, phosphate-buffered gel with glyoxal added to the loading mix. The gel was blotted onto Genescreen (NEN, Boston, MA). The blot was then probed with the fragment of Bex1 cDNA cloned during differential display.

Library screening and genomic subclones

A 129/SV mouse genomic library (Stratagene, La Jolla, CA) was screened by standard methods using the fragment of Bex1 cDNA cloned during differential display as a probe. Positive plaques were screened by PCR using the Bex1 primers PBCX11F2 and PBCX11R. A representative clone with the correct product was purified using standard procedures. DNA from the lambda was either digested with PstI and cloned into the PstI site of the pZER vector for internal fragments, or digested with PstI–SalI and cloned into a PstI–XhoI-digested pZER vector. Subclones were then sequenced and ordered by comparison with the known Bex1 sequence.

Mapping

DNA from the C57BL6 and Spretus strains of mice was provided by HGMP-RC. The primers used to amplify the three genes were as follows: Bex1, CL132F (5′-atg gac tcc aac gta cca gg-3′) and EST10R (5′-ggt ccc cat gtc atct gtc g-3′); Bex2, EST101F (5′-gag tcc aat gga cca gg-3′) and EST10R (see above); and Bex3, EST183F (5′-aac cga gat ggt gca c-3′) and EST182R (5′-ggc aca tca cac tga tgt g-3′). The sequence polymorphisms identified in the amplified products and used for mapping were as follows: Bex1, cDNA position 417 C57BL6 (G) and Spretus (T); Bex2, cDNA position 257 C57BL6 (G) and Spretus (T); cDNA position 348 C57BL6 (G) and Spretus (A), cDNA position 456 C57BL6 (C) and Spretus (T); Bex3, cDNA position 289 C57BL6 (A) and Spretus (C), cDNA position 603 C57BL6 (C) and Spretus (T). Interspecific backcross random panel 1 was then obtained from HGMP-RC and data were obtained on the recombinant status of the mice by PCR amplification and direct sequencing. Sequences were scored according to whether the C57BL6 allele, Spretus allele or both were present. A further panel of 82 animals selected by HGMP-RC was also typed for each gene.

X inactivation assay

The Bex1 primers EST21F (5′-ccg aac gaa gcc ccc cgg cgg ctc ctc ctc-3′) and PBCX11R (5′-tag aag cca ccg ccc gag cac cag gta ctc-3′) were used to amplify a 524 bp product from cDNAs derived from the parental laboratory and PGK mouse strains. The polymorphisms identified in Bex1 and used in the X inactivation assays are as follows: cDNA position 257 Lab strain (A) and PGK (G), cDNA position 348 Lab strain (A) and PGK (G), and cDNA position 456 Lab strain (C) and PGK (T). cDNA was made from [T16H×PGK]F1 and control [Lab strain×PGK]F1 female embryos at either 12.5 and 14.5 d.p.c. Embryos were sexed by the co-amplification of Bex1×-actin, i.e. β-actin 5′ (5′-gtg ggg cgc ccc act gca gac gac g-3′) and Bex1×-actin 3′ (5′-ctc ctt aat gtc acg cac gat-3′). Dynazyme polymerase was used for the single round of extension that consisted of 96 × 3 min, 61°C for 1 min and 72°C for 1 min. Each 10 µl SNuPE

SNuPE assay

The SnuPE assay was done as previously described (31). The primers used for the Bex1 gene were as shown for the X inactivation assay. The primer immediately 5′ to the sequence polymorphism was Bex1F (5′-gag gtc gca cca cag gta ctc-3′). Dynazyme polymerase was used for the single round of extension that consisted of 96°C for 3 min, 61°C for 1 min and 72°C for 1 min. Each 10 µl SnuPE
reaction contained 10 ng of template, 1 μl of 10× Dynazyme buffer, 0.25 μl of Dynazyme (2 U/μl), 10 pmol of Bex1F and 0.2 μl of either [33P]dATP or [33P]dGTP (10 μCi/μl; NEN).

5′ RACE

5′ RACE was performed essentially as described (47). The sequence of the 5′ anchor primer was (5′)-ggc cac gcg tcg act agt acg ggi igg-3′. The Bex1 gene-specific primers for hemi-nested PCR were PBCX11R2 (5′-act ctc atc ctc ccc tgg-3′) for first round amplification at 53°C annealing and EST21R (5′-tct ctc ctt ctc ctc ttt c-3′) for second round amplification at 55°C. The source RNA was derived from Lab strain mouse ovary.

ACKNOWLEDGEMENTS

We wish to thank Dr Neil Brockdorff and Dr Andy Greenfield for help with the escape from X inactivation assays and Maree Knight for help with the 5′ RACE assays. This project was supported by NH and MRC project grant no. 950958.

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