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Expression Quantitative Trait Loci Analysis of 13 Genes in the Rat Prostate

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ABSTRACT

Differential expression of mRNA among animal strains is one of the mechanisms for their diversity. cDNA microarray analysis of the prostates of BUF/Nac (BUF) and ACI/N (ACI) rats, which show different susceptibility to prostate cancers, found 195 differentially expressed genes. To identify loci that control differential expression of 13 genes with diverse expression levels, their expression levels were measured by quantitative RT-PCR in 89 backcross rats, and expression quantitative trait locus (eQTL) analysis was performed. Nine genes [*Aldh1a1*, *Aldr1*, *Bmp6*, *Cdkn1a* (*p21*), *Cntn6*, *Ghr*, *Jund*, *Nupr1*, and RT1-M3] were controlled by *cis*-acting loci. *Cdkn1a*, a cell cycle regulator and a candidate for a prostate cancer susceptibility gene, was mapped to its own locus and had polymorphisms, including a 119-bp insertion in the 5′ upstream region in BUF rats. Four genes (*Kclr*, *Pbsn*, *Psat1*, and *Ptn*) were controlled by *trans*-acting loci. *Pbsn*, a prostate-specific gene on chromosome X, was controlled by a QTL on chromosome 8. Depending upon which gene that we selected from the genes widely used for normalization (*Actb*, *Gapd*, or *Ppia*), different QTL were mapped for *Kclr*, *Psat1*, and *Ptn*. Normalization using *Actb* most appropriately explained the expression levels in a congenic strain for chromosome 3. eQTL analysis with precise measurement of expression levels and appropriate normalization was shown to be effective for mapping loci that control gene expression *in vivo*.

THE diversity in gene expression is one of the mechanisms for diversity among individuals, such as susceptibility to common diseases. A differential gene expression could be due to a *cis*-acting polymorphism at the gene itself (Baier *et al.* 2000; Kuramoto *et al.* 2001) or due to a polymorphism of a gene upstream. Therefore, analysis of determinants of differential gene expression is important not only for studying the architecture of transcriptional regulation *in vivo*, but also for identifying causative genes for various phenotypes. However, it is often difficult to determine whether a differential gene expression is a cause or a consequence, in other words, whether it is due to a *cis*-acting or a *trans*-acting polymorphism.

A new approach to this issue is to treat mRNA expression levels as quantitative traits and to map quantitative trait loci (QTL) that control the expression levels *in vivo*. Using cDNA microarray technology, several studies performed comprehensive analysis of mRNA expression and QTL controlling the expression in budding yeast (Brem *et al.* 2002), eucalyptus (Kirst *et al.* 2005), mice (Schadt *et al.* 2003), rats (Hubner *et al.* 2005), and humans (Cheung *et al.* 2003; Schadt *et al.* 2003; Monks *et al.* 2004; Morley *et al.* 2004). The studies indicated that such an approach, expression QTL (eQTL) analysis, is effective for identifying the cause of the expression differences *in vivo* and can be applied to various genes. For generalized use of eQTL analysis, however, validation regarding whether a causative polymorphism is present in a mapped QTL and whether eQTL analysis is effective for genes with even low expression levels is necessary. The genes with low expression are not amenable to analysis by microarray. Expression analysis using only cDNA microarrays can mistake a sequence polymorphism in a probe sequence as an expression difference (Yamashita *et al.* 2003).

Here, we identified genes differentially expressed in the prostates of BUF/Nac (BUF) and ACI/N (ACI) rats that show different susceptibilities to prostate cancers (Isaacs 1984; Inaguma *et al.* 2003). Loci responsible for different prostate cancer susceptibility in rats were recently mapped (Yamashita *et al.* 2005), and if differentially expressed genes or their controlling genes are present in the mapped loci, they are considered to be good candidates. eQTL analysis was performed for 13 genes with diverse expression levels, and a putative causative polymorphism was identified for one of the *cis*-controlled genes, *Cdkn1a*. Effects of genes used for normalization (referred to as "control genes" in other studies) were analyzed at the same time using congenic rats.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AB194279, AB218281, AB218282, and AB218283.

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MATERIALS AND METHODS

Animals, prostate RNA, and genomic DNA samples: BUF/NacJcl (BUF), ACI/NJcl (ACI), and F344/Jcl (F344) rats were purchased from CLEA Japan (Tokyo). BN/Crj (BN) rats were purchased from Charles River Japan (Yokohama, Japan). F_1 rats were produced by mating female ACI rats with male BUF rats, and backcross rats were produced by mating female ACI rats with male F_1 rats. Two congenic strains, BUF.ACI-*Gcr2* and BUF.ACI-*Gcr3*, which had homozygous ACI chromosome 3 (*D3Rat56–D3Rat83*) and chromosome 16 (*D16Rat31–D16Arb1*), respectively, in the BUF background, were developed by the speed congenic method (Serreze *et al.* 1996). Specifically, in each generation of backcrossing, the male rat that had the most substituted loci after analysis of 171 loci was used to produce 30–100 progeny rats. At N5F12 and at N6F8, respectively, complete substitution of the ACI background by the BUF background was confirmed using the 171 genome-wide genetic markers. The congenic strains are deposited in The National Bio Resource Project for the Rat in Japan (http://www.anim.med.kyoto-u.ac.jp/NBR/home.htm).

Total RNA was extracted from the entire prostate, including the ventral and lateral lobes, of male rats at 10 weeks of age using ISOGEN (NIPPON GENE, Tokyo). All the rats had been given 83 mg/liter *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG, Aldrich Chemical, Milwaukee) in drinking water for 2 weeks. RNA was purified using an RNeasy mini kit (QIAGEN, Valencia, CA), and the quality was examined by the ratio of S28 and S18 after running in a 1% agarose gel containing formalin. Genomic DNA was extracted from the tails of rats by an automated DNA extractor, GENEXTRACTOR TA-100 (Takara Shuzo, Kyoto, Japan).

Oligonucleotide microarray analysis: Equal amounts of RNA were pooled from three BUF and three ACI rats. cDNA microarray analysis was performed using GeneChip Rat Genome U34A (Affymetrix, Santa Clara, CA) as in our previous studies (Kuramoto *et al.* 2002; Abe *et al.* 2003; Yamashita *et al.* 2003). The signal intensities were normalized so that the average of all the genes on a GeneChip would be 500, and the data were processed using Affymetrix Microarray Suite version 5.0. Differentially expressed genes were selected by their 2-fold increase or a 0.5-fold decrease.

Quantitative RT-PCR: cDNA was synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) and oligo$(dT)_{12\text{-}18}$ primer (Invitrogen). Real-time PCR was performed using the iCycler iQ detection system (Bio-Rad Laboratories, Hercules, CA) with SYBR green PCR core reagents (Applied Biosystems, Foster City, CA). Samples of 89 backcross rats were simultaneously analyzed in a 96-well plate. The primers used are listed in supplementary Table S1 at http://www.genetics.org/supplemental/. By monitoring amplification curves of a test sample and samples that contained 10^1–10^6 molecules of the gene of interest, the number of target molecules in the test sample was analyzed. The number was normalized to that of *Actb, Gapd*, and *Ppia*, which are widely used as internal controls (Weisinger *et al.* 1999; Feroze-Merzoug *et al.* 2002; Lee *et al.* 2002).

Genotyping: A total of 146 microsatellite markers that distributed all the autosomes and spanned 1512 cM were used (supplementary Table S2 at http://www.genetics.org/supplemental/). The mean and median of intermarker distances were 12.0 and 12.3 cM, respectively. PCR was carried out using 20 ng of genomic DNA, and the products were electrophoresed in a 3 or 4% NuSieve GTG agarose gel.

The genotype of the *Cdkn1a* 5′ upstream region among rat strains was determined using an upper primer (5′-GCGC TGTTATTAGACATGA-3′) and a lower primer (5′-AGAGC CACGCACATCTATG-3′) that amplified −501 to −264 (transcription start site, 0). The genotype of *Cdkn1a* 3′ downstream was determined using an upper primer (5′-ATGTAGAACCAT TATTTAAGTCC-3′) and a lower primer (5′-GCGAGATGCGA GATGCAGATG-3′) (6292–6415).

Linkage analysis: A linkage map was constructed by MAPMAKER/EXP (version 3.0b) software (Lander *et al.* 1987) that was modified by the Fink project (http://fink.sourceforge.net/) and installed on Mac OSX. Interval mapping of QTL was performed using MAPMAKER/QTL (version 1.1b) software that was similarly installed. To obtain improved normality, expression levels measured by quantitative RT-PCR were logarithm transformed and treated as quantitative traits. Logarithm of odds (LOD) score values of 1.9 and 3.3 were considered as thresholds for "suggestive" and "significant" linkage, respectively (Lander and Kruglyak 1995). With these values, linkage was expected to occur 1 and 0.05 times, respectively, at random in a genome scan.

Sequencing analysis: Genomic DNAs of ACI and BUF rats were amplified by PCR using primers listed in supplementary Table S1 at http://www.genetics.org/supplemental/ and inserted into pGEM-T Easy Vector (Promega, Madison, WI). Cycle sequencing was performed using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and an ABI310 DNA sequencer (Applied Biosystems).

Luciferase reporter assay: 5′ regions of ACI (without insertion) and BUF *Cdkn1a* (with insertion) were amplified using an upper primer (5′-GCTGTCAAAAGAAGCTTGAACTCC-3′) and a lower primer (5′-ACACACGAAAGCTTGTGGGGACAC-3′). The PCR fragments were digested with *Hin*dIII and cloned in the pGL3-Basic vector (Promega). Along with a control for transfection efficiency (33 ng phRL-TK, Promega), 330 ng of pGL3-Basic, pGL3-Control (SV40 promoter and enhancer), pGL3-Cdkn1a-ACI, and pGL3-Cdkn1a-BUF was transiently transfected to rat fibroblast cell line 3Y1 cl-3 (Ushijima *et al.* 1994) and rat prostate cancer cell line AT6.1 (Dong *et al.* 1995). The cells were seeded at 5×10^4 cells per well (12-well plate) 24 hr ahead, and transfection was performed using 1 μl of FuGENE 6 transfection reagent (Roshe Diagnostics, Hague Road, IN). At 24 hr after transfection, cells were harvested, and luciferase activity was measured with a dual-luciferase reporter assay system (Promega) in Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Each transfection was performed in triplicate and experiments were repeated twice.

RESULTS

Genes differentially expressed between BUF and ACI prostates: We first analyzed expression levels of 8800 probe sets on GeneChip rat genome U34A in the prostate of BUF and ACI rats. A total of 153 probes (134 genes) showed higher expression levels at twofold or more in the BUF prostates, and 72 probes (61 genes) showed higher expression levels in the ACI prostates (supplementary Table S3 at http://www.genetics.org/supplemental/). From these 195 genes, we selected 12 genes with high and low expression levels (*Aldh1a1*, *Aldr1*, *Bmp6*, *Cntn6*, *Ghr*, *Jund*, *Nupr1*, *Pbsn*, *Psat1*, *Ptn*, RT1-M3, and *Sv2b*), including *Pbsn*. We also added *Cdkn1a* (*p21*) and *Kclr*, which we had previously identified as differentially expressed, although these were classified as "no change" by GeneChip analysis due to their low expression levels. The expression levels of the 14 genes in the prostate were not altered by MNNG administration (data not shown).

By quantitative RT-PCR analysis, differential expression at 1.5-fold or more was confirmed for 13 of the 14 genes (*Sv2b* was excluded) (Table 1). Since sequence polymorphisms in PCR products could produce apparent expression differences, we further confirmed the absence of sequence polymorphisms. For all the 13 genes, no differences in the melting temperatures of the PCR products were observed between BUF and ACI rats. Further, for *Cdkn1a* and *Jund*, quantitative RT-PCR was performed using different regions of the genes, and results were in good accordance (data not shown).

eQTL analyses: Expression levels of the 13 genes were measured by quantitative RT-PCR in 89 ACI × (ACI × BUF)F_1 backcross rats and normalized to that of *Actb* (β-*actin*). By linkage analysis, expression levels of 9 genes (*Aldh1a1*, *Aldr1*, *Bmp6*, *Cdkn1a* (*p21*), *Cntn6*, *Ghr*, *Jund*, *Nupr1*, RT1-M3) were mapped to their own loci with relatively high LOD scores (4.0–78.0) (Table 2). In contrast, expression levels of 4 genes (*Kclr*, *Pbsn*, *Psat1*, *Ptn*) were mapped to loci different from their own with LOD scores between 2.2 and 5.0 (Table 2). The expression level of even *Cdkn1a*, which had very low expression, was mapped to its own locus (chromosome 20) with a LOD score of 37.5. The expression level of *Pbsn*, a prostate-specific gene on chromosome X with abundant expression, was mapped to a locus on chromosome 8 with a LOD score of 4.0. By quantitative RT-PCR using different aliquots of the same samples, reproducibility of mapping was confirmed for *Jund*, which had very low expression, and *Pbsn*.

Identification of *Cdkn1a* polymorphisms and their effects in mRNA expression: *Cdkn1a* (*p21*) was expressed at a higher level in BUF rats than in ACI rats, and classified as a *cis*-controlled gene. To identify a causative polymorphism for the differential expression, we sequenced the 5′ upstream region, exon 1, exon 2, intron 2, exon 3, and the 3′ downstream region (−3374–181 and 5378–6400, transcription start site, 1). A 119-bp insertion in the 5′ upstream region (at −477) in BUF rats (accession no. AB194279) and a 14-bp repeat number difference (ACI = 2, BUF = 3) in the 3′ downstream region (6345, accession no. AB218281∼3) were found. Six other polymorphisms were found in the 5′ upstream region and in intron 2 (supplementary Table S4 at http://www.genetics.org/supplemental/).

The 119-bp insertion in the 5′ upstream region consisted of a 15-bp duplication, a 85-bp rat identifier (ID) sequence (one of short interspersed nuclear elements in the rat) (Daniels and Deininger 1985), and a 19-bp poly(A) in an antisense direction with *Cdkn1a*. A putative p53-binding site was present in the 15-bp duplication. Among five rat strains [BUF, ACI, F344, BN, and (ACI × BUF)F_1], the presence of these polymorphisms and high *Cdkn1a* expression in the prostate were associated (Figure 1A). BUF and F344, which showed higher *Cdkn1a* expressions among five strains of rats, had the same sequences.

The effect of this insertion was further analyzed by a luciferase reporter assay using DNA fragments between −555 and +135 with and without the insertion. In the rat fibroblast cell line 3Y1 cl-3, the DNA fragment without the insertion showed a 35-fold increase of luciferase activity compared with the control vector without the DNA fragment, demonstrating its promoter activity. The 119-bp insertion resulted in a significant increase (1.6-fold) of the transcriptional activity (Figure 1B). A similar result (1.5-fold increase) was obtained using a rat prostate cancer cell line, AT6.1.

Effect of a gene used for normalization and usefulness of *Actb*: Expression levels of each gene were normalized using three genes, *Actb*, *Gapd*, and *Ppia*, to correct variations in RNA quality among individual rats. Although all three genes are widely used for normalization by many researchers, different loci were mapped for *Kclr*, *Psat1*, and *Ptn*, depending upon the gene used for normalization (Table 2). For example, the main QTL that controlled the expression of *Kclr* was mapped on chromosome 3 by normalization with *Actb* (LOD score: 3.8) and on chromosome 8 with either *Gapd* (2.8) or *Ppia* (2.5). The main QTL that controlled expression of *Psat1* was mapped on chromosome 3 with *Actb* (3.1), on chromosome 5 with *Gapd* (2.8), and on chromosome 12 near *Planh* with *Ppia* (2.2). Expression levels of *Actb*, *Gapd*, and *Ppia* were quantified twice. Each gene, whose expression level was quantified once, was normalized using the two values, and eQTL analysis was performed (supplementary Table S5 at http://www.genetics.org/supplemental/). Between the two quantifications, QTL mapped with LOD scores >3.0 were always reproducible, while those mapped with LOD scores <3.0 were occasionally not.

To clarify an appropriate gene for normalization among rat strains, two congenic rat strains were used. BUF.ACI-*Gcr2* (Gcr2) had an ACI-derived genome around *D3Rat98* in the BUF background, and BUF.ACI-*Gcr3* (Gcr3) had an ACI-derived genome around *D16Rat17* and *D16Rat18* in the BUF background (Figure 2, A and B). Expression levels of five genes (*Cdkn1a*, *Jund*, *Kclr*, *Pbsn*, and *Psat1*) were analyzed in BUF, ACI, Gcr2, and Gcr3. If no controlling genes were on chromosome 3 and chromosome 16, Gcr2 and Gcr3 were expected to show expression levels equivalent to BUF rats. This was observed for *Cdkn1a* and *Pbsn* (Figure 2, C and F). In the case of *Jund*, whose QTL was mapped on chromosome 16, Gcr3 showed an expression level equivalent to that in ACI rats (Figure 2D). This confirmed that a locus on chromosome 16 controlled the expression of *Jund*. In the case of *Kclr* and *Psat1*, for which different controlling QTL were mapped, depending upon the gene used for normalization, Gcr2 showed expression levels close to those in ACI rats using any of the three genes for normalization (Figure 2, E and G). This showed that loci on chromosome 3 controlled the expression levels of *Kclr* and *Psat1* and that the effect was most clearly detected using *Actb* for normalization (Table 2).

TABLE 1

GeneChip and real-time RT-PCR analyses in the prostate of rats

	GeneChip result			Real-time result						
	BUF *vs.* ACI:	Signal		Copy number/β-actin ($\times 10^4$, mean ±SD)						
Probe	Signal log ratio change	BUF	ACI	BUF ($n = 3$)	ACI ($n = 3$)	Backcross ($n = 89$)	Gene symbol	Chromosomal location	UniGene ID	Gene title
AF001898_at	3.5 I	5520	470	885.4 ± 235.5	97.0 ± 11.2	120.7 ± 79.4	*Aldh1a1*	Chr:1q51	Rn.6132	Aldehyde dehydrogenase family 1, member A1
M60322_g_at	2.4 I	144	21	32.4 ± 10.4	10.2 ± 4.1	17.4 ± 6.0	*Aldr1*	Chr:4q22	Rn.107801	Aldehyde reductase 1 (low Km aldose reductase)
U66298_at	6.8 I	996	5	448.4 ± 216.8	2.7 ± 0.1	102.7 ± 109.6	*Bmp6*	Chr:17p12	Rn.40476	Bone morphogenetic protein 6
X58830_at	4.1 I	2856	140							
L41275cds_s_at	1.5 NC	50	18	17.9 ± 2.1	5.8 ± 2.3	8.9 ± 9.6	*Cdkn1a*	Chr:20p12	Rn.10089	Cyclin-dependent kinase inhibitor 1A (p21)
D87248_at	2.9 I	515	65	177.4 ± 44.9	35.2 ± 10.0	47.9 ± 26.6	*Cntn6*	Chr:4q41	Rn.10644	Contactin 6
Z83757mRNA_at	3.9 I	73	4	25.5 ± 11.8	5.2 ± 1.8	8.9 ± 4.1	*Ghr*	Chr:2q16	Rn.2178	Growth hormone receptor
Z83757mRNA_g_at	4.1 I	58	2							
D26307cds_at	−3.5 D	23	296	6.0 ± 3.1	39.1 ± 5.3	23.6 ± 13.0	*Jund*	Chr:16p14	Rn.46225	Jun D proto-oncogene
M55532_at	4.1 NC	66	3	0.94 ± 0.58	0.30 ± 0.05	0.61 ± 0.25	*Kclr*	Chr:4q34	Rn.9886	Kupffer cell receptor
AF014503_at	1.9 I	3061	903	2280 ± 1394	442 ± 123	584 ± 303	*Nupr1*	Chr:1q36	Rn.11182	Nuclear protein 1 (p8)
M27156_at	2.2 I	16461	2990	19580 ± 10480	1310 ± 580	5440 ± 6140	*Pbsn*	Chr:Xq22	Rn.9862	Probasin
M27156_g_at	2.5 I	10249	1563							
rc_AI102868_g_at	2.1 I	969	156	227.7 ± 94.1	28.9 ± 5.1	74.8 ± 42.2	*Psat1*	Chr:1q43	Rn.100813	Phosphoserine aminotransferase 1
rc_AI230228_at	1.8 I	474	130							
rc_AI102795_at	1.8 I	324	134	391.5 ± 220.1	95.7 ± 11.0	141.1 ± 55.7	*Ptn*	Chr:4q22	Rn.1653	Pleiotrophin
U16025_g_at	2.4 I	50	12	35.9 ± 20.8	20.8 ± 2.8	23.5 ± 14.0	*RT1-M3*	Chr:20p12	Rn.92606	RT1 class Ib, locus M3
L10362_at	2.9 I	47	7	0.49 ± 0.21	0.50 ± 0.22		*Sv2b*	Chr:1q31	Rn.58137	Synaptic vesicle glycoprotein 2 b

TABLE 2

LOD score of linkage analyses using gene expression as QTL (LOD >1.9)

Gene/ normalization	Peak 1 chromosome (cM)	Closest markers	LOD score	Variance explained (%)	Peak 2 chromosome (cM)	Closest markers	LOD score	Variance explained (%)	Peak 3 chromosome (cM)	Closest markers	LOD score	Variance explained (%)
Aldh1a1 (Chr:1q51)												
Actb	1 (115)	*D1Rat70*	23.2	77.7	3 (76)	*D3Rat10*	2.6	14.1	19 (59)	*D19Rat4*	2.1	10.5
Gapd	1 (117)	*D1Rat298*	22.4	75.8	3 (80)	*D3Rat10*	2.9	15.3	19 (57)	*D19Rat4*	2.0	10.9
Ppia	1 (117)	*D1Rat298*	24.1	76.0	3 (85)	*D3Rat10*	2.7	15.6	19 (59)	*D19Rat4*	2.4	11.8
Aldr1 (Chr:4q22)												
Actb	4 (25)	*D4Rat15*	16.5	60.6								
Gapd	4 (27)	*D4Rat30*	16.5	60.4								
Ppia	4 (31)	*D4Rat30*	9.8	40.1								
Bmp6 (Chr:17p12)												
Actb	17 (6)	*D17Rat15*	65.9	97.1								
Gapd	17 (6)	*D17Rat15*	69.9	97.6								
Ppia	17 (6)	*D17Rat15*	78.0	98.4								
Cdkn1a (Chr:20p12)												
Actb	20 (10)	*D20Rat3*	37.5	87.6								
Gapd	20 (10)	*D20Rat3*	39.4	88.8								
Ppia	20 (10)	*D20Rat3*	42.2	90.3	10 (6)	*D10Rat44*	2.1	11.0				
Cntn6 (Chr:4q41)												
Actb	4 (62)	*Ampp*	6.5	31.3	3 (110)	*D3Rat3*	2.4	12.0	12 (0)	*D12Rat58*	2.0	9.7
Gapd	4 (62)	*Ampp*	7.2	34.4	3 (110)	*D3Rat3*	3.2	15.5				
Ppia	4 (60)	*Ampp*	7.6	33.6	3 (107)	*D3Rat3*	4.6	24.6				
Ghr (Chr:2q16)												
Actb	2 (22)	*D2Rat75*	4.4	26.3								
Gapd	2 (20)	*D2Rat75*	6.9	39.2								
Ppia	2 (18)	*D2Rat298*	6.2	33.2								
Jund (Chr:16p14)												
Actb	16 (9)	*D16Rat18*	6.0	29.3								
Gapd	16 (15)	*D16Rat17*	6.6	31.6								
Ppia	16 (15)	*D16Rat17*	5.4	27.5								
(trial2)												
Actb	16 (18)	*D16Rat17*	4.2	20.4								
Gapd	16 (18)	*D16Rat17*	5.1	24.7	12 (4)	*D12Rat58*	2.2	12.2				
Ppia	16 (15)	*D16Rat17*	3.6	19.1								
Kclr (Chr:4q34)												
Actb	3 (27)	*D3Rat98*	3.8	17.8	8 (20)	*D8Rat47*	2.3	12.3	18 (18)	*Adrb*	2.3	11.2
Gapd	8 (19)	*D8Rat49*	2.8	13.7	3 (29)	*D3Rat98*	2.8	14.7	5 (110)	*D5Rat50*	2.2	11.7
Ppia	1 (33)	*D1Rat10*	2.3	12.7	8 (18)	*D8Rat49*	2.2	13.3	18 (16)	*Adrb*	2.1	12.6
Nupr1 (Chr:1q36)												
Actb	1 (104)	*D1Rat57*	7.8	38.5	16 (42)	*D16Rat15*	2.1	10.6	3 (27)	*D3Rat98*	2.1	10.3
Gapd	1 (104)	*D1Rat57*	9.0	42.8	2 (100)	*D2Rat65*	2.0	11.4	5 (110)	*D5Rat50*	1.9	9.6
Ppia	1 (104)	*D1Rat57*	10.4	47.2	16 (42)	*D16Rat15*	2.4	11.9	2 (92)	*D2Rat65*	2.1	12.9
Pbsn (Chr:Xq22)												
Actb	8 (42)	*D8Rat44*	4.0	21.0	11 (38)	*D11Mit8*	2.8	14.2				
Gapd	8 (44)	*D8Rat44*	4.7	24.7	11 (38)	*D11Mit8*	2.4	11.9				
Ppia	8 (42)	*D8Rat44*	5.0	26.2	11 (38)	*D11Mit8*	2.3	11.5				
(trial2)												
Actb	8 (42)	*D8Rat44*	4.5	25.6	11 (25)	*D11Rat63*	2.9	21.4	15 (0)	*D15Rat69*	2.1	11.0
Gapd	8 (44)	*D8Rat44*	5.2	29.3	11 (38)	*D11Mit8*	2.2	12.1	15 (0)	*D15Rat69*	2.1	11.0
Ppia	8 (44)	*D8Rat44*	5.1	28.9	14 (41)	*D14Rat18*	2.2	11.7	11 (38)	*D11Mit8*	2.1	11.7
Psat1 (Chr:1q43)												
Actb	3 (27)	*D3Rat98*	3.1	15.1	2 (52)	*D2Rat183*	2.5	14.4	5 (110)	*D5Mgh9*	2.0	9.9
Gapd	5 (110)	*D5Mgh9*	2.8	13.6	2 (52)	*D2Rat183*	2.7	15.2	3 (27)	*D3Rat98*	2.3	11.5
Ppia	12 (30)	*Planh*	2.2	12.0	1 (115)	*D1Rat70*	2.1	11.6	2 (56)	*D2Rat183*	2.1	13.6
Ptn (Chr:4q22)												
Actb	3 (43)	*D3Rat40*	3.1	16.8	8 (77)	*D8Rat10*	2.0	11.4				
Gapd	3 (45)	*D3Rat40*	2.3	13.1	10 (49)	*D10Rat195*	2.2	11.3	8 (81)	*D8Rat10*	1.9	10.5
Ppia	20 (43)	*D20Rat29*	2.6	12.5								
RT1-M3 (Chr:20p12)												
Actb	20 (16)	*D20Rat5*	2.3	11.3								
Gapd	20 (16)	*D20Rat5*	2.3	11.1								
Ppia	20 (16)	*D20Rat5*	2.9	13.9								

Numbers in parentheses are the distances in centimorgans from the first marker used on the chromosome. Underlining indicates a significant result (LOD score >4.3)

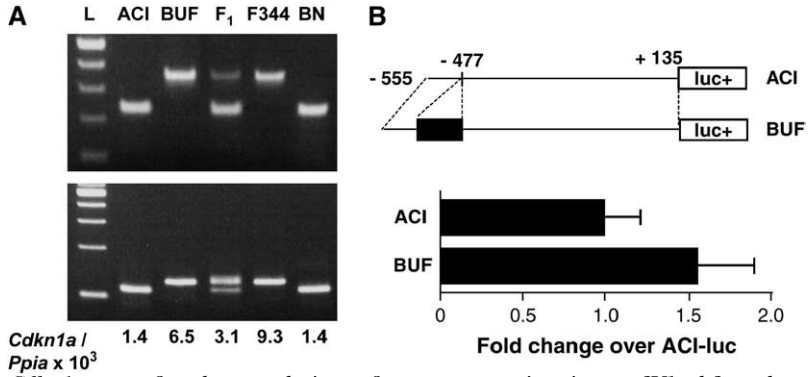

FIGURE 1.—(A) Genotypes of *Cdkn1a* among five rat strains and correlation between genotypes and expression levels. (Top) The genotype 5′ upstream of the *Cdkn1a* gene (amplify −501 to −264, transcription start site, 0). The presence of insertions consisting of rat ID sequence were detected. (Bottom) The genotype 3′ downstream of the *Cdkn1a* gene (amplify 6292–6415). The PCR products were run in a 3.0% agarose gel. F_1, (ACI × BUF)F_1; L, 100-bp DNA ladder. Expression levels of the *Cdkn1a* gene were analyzed by quantitative RT-PCR. Concordance between the genotypes and expression level was observed. (B) Effect of the insertion 5′ upstream of the *Cdkn1a* gene for the regulation of gene expression in rat 3Y1 cl-3 evaluated by luciferase reporter assay. DNA fragments of the rat Cdkn1a 5′ region in ACI (without insertion) and in BUF (with insertion) were cloned in the pGL3-basic vector. The values of promoter activity were calculated on the basis of the activity observed upon cotransfection with the phRL-TK vector and expressed as the ratio to the promoter activity of ACI. Bars represent means +SD.

DISCUSSION

Among the 195 genes differentially expressed in the prostates of ACI and BUF rats, eQTL analyses were performed for 13 genes selected for their wide range of expression levels and possible involvement in prostate carcinogenesis. Nine genes were *cis*-controlled, and 4 genes were *trans*-controlled.

Among the *cis*-controlled genes, a tumor-suppressor gene, *Cdkn1a*, was expressed 3.1-fold higher in BUF rats. We identified eight polymorphisms, including a 119-bp insertion in the 5′ upstream region, but not in the p53-binding tetramer (EL-DEIRY *et al.* 1995) or in a repressor in its 3′ untranslated region (RISHI *et al.* 1997). The 119-bp insertion contained the rat ID, which can act as a *cis*-acting positive regulator or enhancer (MCKINNON *et al.* 1986; OSBOURN *et al.* 1995), and a putative p53-binding sequence. However, our transient reporter gene assays demonstrated that the insertion upregulates the *Cdkn1a* expression only at 1.5- to 1.6-fold. The luciferase activities of the *Cdkn1a* promoter of both ACI and BUF rats exceeded even that of pGL3-Control, which has a strong promoter activity of SV40 promoter and enhancer. The *Cdkn1a* promoter activity is known to be induced by cellular stress (PARK *et al.* 2002), which is caused by transfection itself, and the difference between ACI and BUF rats could have been attenuated. That a polymorphism(s) outside the regions that we sequenced is also responsible for the differential expression still remains a possibility.

ACI is susceptible to prostate carcinogenesis, and Wister, the original strain of BUF, is resistant (ISAACS 1984; INAGUMA *et al.* 2003). By linkage mapping with prostate cancers, we have recently mapped *Pcr1* (chromosome 2), *Pcr2* (chromosome 1), *Pcs1* (chromosome 19), and *Pcs2* (chromosome 20) (YAMASHITA *et al.* 2005). Among the 195 differentially expressed genes, 7 were located on these loci, and, if *cis*-controlled, they are good candidates for prostate cancer susceptibility genes. Especially, *Cdkn1a* was on *Pcs2* and *cis*-controlled and was a good candidate for it. An oncogenic transcription factor, *Jund* (5.1-fold higher in ACI rats), and a putative transcription factor, *Nupr1* (*p8* in human; 5.2-fold higher in BUF rats), were also *cis*-controlled, but were not within the four prostate cancer susceptibility loci.

Among the *trans*-controlled genes, *Pbsn* was expressed 15-fold higher in BUF rats and was shown to be controlled mainly by a locus on chromosome 8. *Pbsn* is known to be specifically expressed in the prostate and is preferred as a promoter for prostate-specific expression of transgenes (GREENBERG *et al.* 1995; ASAMOTO *et al.* 2001). A polymorphic prostate-specific transcription factor is expected to be present on rat chromosome 8. Both *Kclr* and *Psat1* were *trans*-controlled by QTL(s) in the same region on chromosome 3, and possibility that both genes are controlled by the same polymorphism was suggested. The *trans*-linkages show lower significance and explain less expression variance than do the *cis*-linkages. This pattern has been observed in another eQTL study (SCHADT *et al.* 2003).

eQTL analysis was effective for genes with various expression levels when quantitative RT-PCR was used. Even for *Cdkn1a*, whose difference in expression level was difficult to detect using a microarray, the controlling locus was mapped with high LOD scores. To achieve precise analysis, melting temperatures of PCR products were confirmed to be the same between BUF and ACI rats since a polymorphism in a PCR product can potentially affect PCR efficiency. The expression levels were highly reproducible between two independent measurements, giving correlation coefficients of 0.78 and 0.92 for *Jund* and *Pbsn*, respectively.

Selection of a gene for normalization was important for quantitative RT-PCR since different QTL were mapped depending upon the gene, especially for *trans*-controlled genes with low expression. To determine an appropriate gene for normalization, we used congenic rats that had ACI-derived chromosome 3 in the BUF background (Gcr2). Expression levels of *Kclr* and *Psat1* in Gcr2 were similar to that in ACI using any of the three genes for normalization, showing that their expression levels were controlled by a locus on

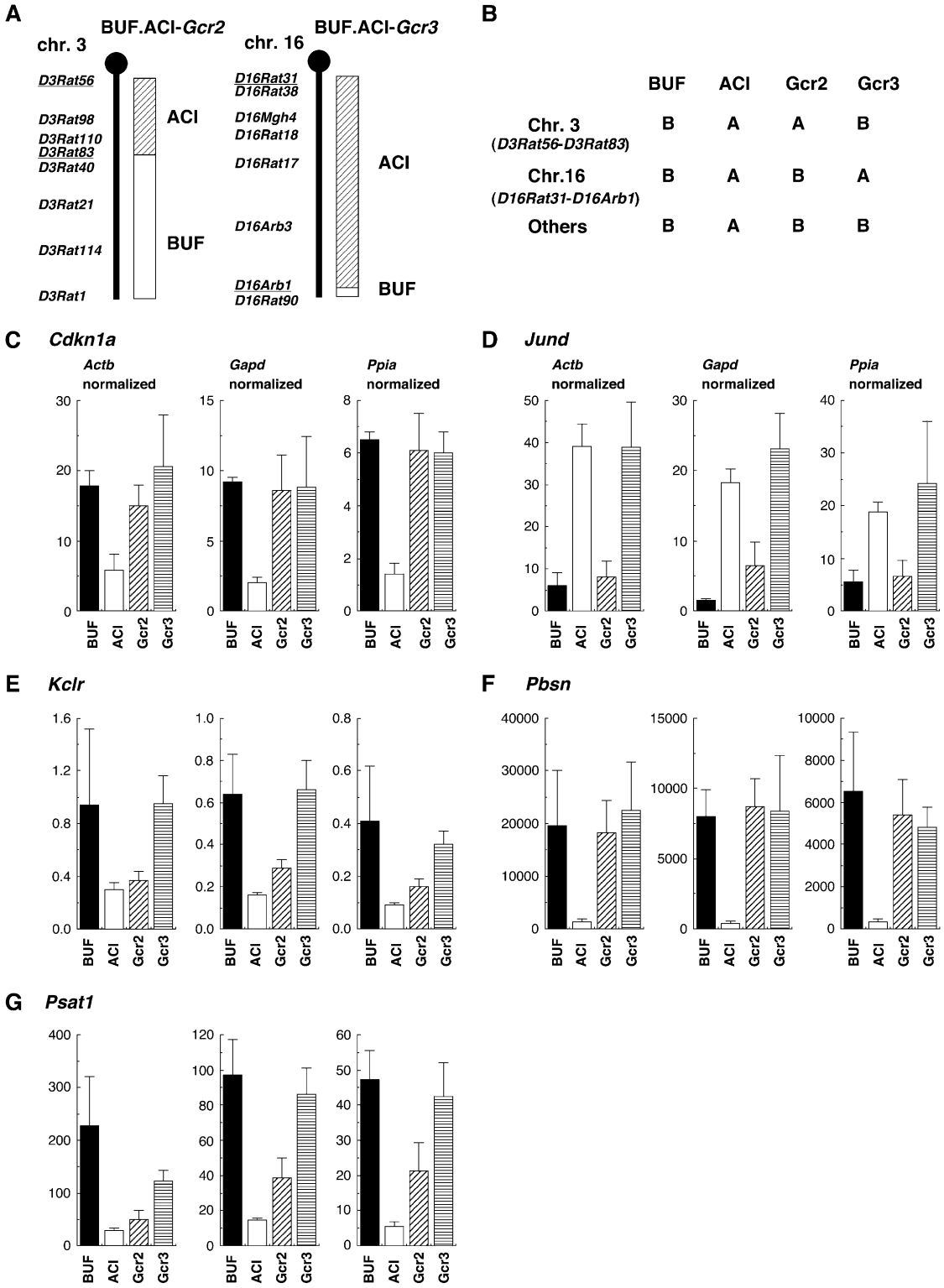

FIGURE 2.—(A) Genetic map of chromosome 3 of BUF.ACI-*Gcr2* (Gcr2) and chromosome 16 of BUF.ACI-*Gcr3* (Gcr3). Gcr2 and Gcr3 had homozygous ACI chromosome 3 (*D3Rat56–D3Rat83*) and chromosome 16 (*D16Rat31–D16Arb1*), respectively, in the BUF background. (B) Summary of genetic backgrounds of BUF, ACI, Gcr2, and Gcr3 in various chromosomes. (C–G) mRNA expression levels of *Cdkn1a*, *Jund*, *Kclr*, *Pbsn*, and *Psat1* in BUF, ACI, Gcr2, and Gcr3. For each strain, three rats were analyzed, and the number of molecules was normalized to each of *Actb*, *Gapd*, and *Ppia* (means +SD; *Actb* normalized, $\times 10^4$; *Gapd* and *Ppia* normalized, $\times 10^3$).

chromosome 3. However, this effect of chromosome 3 was detected only when *Actb* was used for normalization. This indicated that expression levels of even genes for normalization had variations among rat strains. Although it was suggested that the variation of *Actb* was smaller than those of *Gapd* and *Ppia* among different strains of rats, analysis of more *trans*-controlled loci seems necessary. In contrast, for comparison between different tissues or different conditions in the same strain, *Ppia* was reported to give the most reproducible results (Weisinger *et al.* 1999; Feroze-Merzoug *et al.* 2002; Yamashita *et al.* 2004). Careful selection of a gene for normalization seems important.

In conclusion, we showed eQTL analysis for the rat prostate, the effectiveness of eQTL analysis for genes with a broad range of expression levels using quantitative RT-PCR, and an appropriate gene for normalization.

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