Fine Mapping Reveals That Promotion Susceptibility Locus 1 (Ps1) Is a Compound Locus With Multiple Genes That Modify Susceptibility to Skin Tumor Development

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ABSTRACT Although it is well known that the majority of human cancers occur as the result of exposure to environmental carcinogens, it is clear that not all individuals exposed to a specific environmental carcinogen have the same risk of developing cancer. Considerable evidence indicates that common allelic variants of low-penetrance, tumor susceptibility genes are responsible for this interindividual variation in risk. We previously reported a skin tumor promotion susceptibility locus, Ps1, which maps to the distal portion of chromosome 9, that modified skin tumor promotion susceptibility in the mouse. Furthermore, Ps1 was shown to consist of at least two subloci (i.e., Ps1.1 and Ps1.2) and that glutathione S-transferase alpha 4 (Gsta4), which maps to Ps1.2, is a skin tumor promotion susceptibility gene. Finally, variants of human GSTA4 were found to be associated with risk of nonmelanoma skin cancer. In the current study, a combination of nested and contiguous C57BL/6 congenic mouse strains, each inheriting a different portion of the Ps1 locus from DBA/2, were tested for susceptibility to skin tumor promotion with 12-O-tetradecanoylphorbol-13-acetate. These analyses indicate that Ps1 is a compound locus with at least six genes, including Gsta4, that modify skin tumor promotion susceptibility. More than 550 protein-coding genes map within the Ps1 locus. Fine mapping of the Ps1 locus, along with two-strain haplotype analysis, gene expression analysis, and the identification of genes with amino acid variants, has produced a list of fewer than 25 candidate skin tumor promotion susceptibility genes.

KEYWORDS Ps1 skin tumor promotion susceptibility locus tetradecanoylphorbol acetate complex trait genetics compound locus

Human cancers can be divided into two major categories, familial cancers and sporadic cancers (reviewed in Angel et al. 2010). Familial cancers occur within affected families at a relatively high frequency and include a number of cancer syndromes that are caused by germ-line mutations of genes with strong effects that are directly involved in tumorigenesis. In contrast, the majority of human cancers are sporadic forms that result from exposure to environmental carcinogens (reviewed in Clavel 2007). Recent epidemiologic studies (Lichtenstein et al. 2000; Peto and Mack 2000; Pharoah et al. 2002), as well as studies that use animal models of cancer (reviewed in Angel et al. 2010), have shown that common allelic variants within the genome can act to modify relative risk of cancer development resulting from environmental carcinogen exposure.

Genes that modify susceptibility to tumor development (hereafter referred to as tumor susceptibility genes) are low-penetrance genes with modest effects on cancer susceptibility that are involved in DNA repair, immune response, carcinogen metabolism, cellular proliferation, differentiation, and death, as well as other cancer-related mechanisms.
The combined effects of multiple tumor susceptibility genes determine the overall susceptibility of an individual to the development of a particular type of cancer, with each gene acting to either increase or decrease susceptibility. Although the identification of specific genes that underlie tumor susceptibility loci has been difficult, genes that underlie several modifier loci, including Psl1.2 (GstA4), Mom1 (Pta2a), Pctr1 (Cdkn2a), Skts13 (Aurka), Skts14 (Tgfbi), Mies1 (Sipa1), and others have recently been identified and variants that affect both function and expression have been reported (reviewed in Angel et al. 2010). Importantly, polymorphisms in these genes have also been associated with cancer risk in humans, demonstrating the utility of using animal models to identify genes that modify susceptibility to cancer (reviewed in Angel et al. 2010).

The multistage model of mouse skin carcinogenesis is an excellent model for the study of human epithelial carcinogenesis, and genetic differences in susceptibility to multistage skin carcinogenesis have been known for many years (Boutwell 1976; Slaga 1984; Naito and Digiovanni 1989; Digiovanni 1992). Our laboratory, as well as others, has identified genetic loci that modify susceptibility to skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA), showing that susceptibility to skin tumor promotion is a multigenic trait (Nagase et al. 1995; Angel et al. 1997; Mock et al. 1998; Angel and Digiovanni 1999; Nagase et al. 1999; Angel et al. 2001; Peissel et al. 2001; Angel et al. 2003; Fujiwara et al. 2007). We previously mapped TPA promotion susceptibility loci to chromosomes (chr) 1 (Psl3), 2 (Psl2), 9 (Psl1), and 19 (Psl4) in genetic crosses of C57BL/6 with DBA/2 mice (Angel et al. 1997; Angel and Digiovanni 1999; Angel et al. 2001, 2003) and recently reported that Psl1 consists of at least two subloci, Psl1.1 and Psl1.2 (Abel et al. 2010). From these earlier studies, glutathione S-transferase alpha 4 (GstA4) was identified as a skin tumor promotion susceptibility gene that maps within the Psl1.2 locus (Abel et al. 2010). Furthermore, polymorphisms in GSTA4 were found to be associated with risk of developing nonmelanoma skin cancer in humans (Abel et al. 2010). In the current study, analyses of combinations of nested and contiguous subcongenic mouse strains suggest that at least two additional modifier genes underlie the Psl1.1 locus and that at least four modifier genes, including Gsta4, underlie the Psl1.2 locus, indicating that Psl1 is a compound quantitative trait locus (QTL). Additionally, inheritance of the DBA/2 allele of four of these loci (Psl1.1a, Psl1.2a, Psl1.2b, Gsta4) results in increased sensitivity whereas inheritance of the DBA/2 allele of two loci (Psl1.1b, Psl1.2c) results in decreased sensitivity to skin tumor promotion. Analysis of genes with expression or amino acid variants that map to regions of nonequivalency [chromosome regions that are not identical by descent (IBD)] within these loci has led to the identification of several attractive candidate skin tumor promotion susceptibility genes.

MATERIALS AND METHODS

Mice

C57BL/6 and DBA/2 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6.Psl1<sup>dsb</sup> congenic mouse strains (Figure 1, Table 1, and Table 2) were developed as previously described (Abel et al. 2010) and maintained by brother/sister mating. All mice were maintained in a specific pathogen-free environment in the vivarium at the University of Texas M.D. Anderson Cancer Center, Science Park—Research Division in accordance with institutional guidelines. Mice were housed five per plastic cage in an air-conditioned room at 24° ± 2°, had free access to food and water, and were exposed to a 12-hr light and dark cycle.

Reagents

TPA was purchased from LC Laboratories (Woburn, MA), and 7,12-dimethylbenza[a]anthracene (DMBA) was purchased from Sigma-Aldrich (St. Louis, MO).

Tumor experiments

Tumor experiments were carried out as previously described (Abel et al. 2009) with female mice. A standard area on the dorsal skin extending from the base of the tail to the base of the head was shaved on each mouse. Forty-eight hours later, mice were initiated by topical treatment of 100 nmol of DMBA in 0.2 mL of acetone on the shaved area. Two weeks later, mice were treated with either 6.8 or 13.6 nmol of the tumor promoter TPA twice weekly until the tumor response reached a plateau. Mice were checked weekly for the appearance of papillomas. Tumor multiplicity was calculated as the cumulative number of papillomas observed divided by the number of mice at risk when the first tumor was observed.

Candidate genes

The list of genes located under each QTL was downloaded from Ensembl, Build 37. A gene was considered as a candidate if the gene (1) was located within a region that differed in the haplotype of C57BL/6 and DBA/2; (2) had a nonsynonymous coding polymorphism between C57BL/6 and DBA/2 or (3) was differentially expressed in the epidermis of C57BL/6 and DBA/2.

Two-strain haplotype analysis

Single-nucleotide polymorphism (SNP) datasets of the C57BL/6 and DBA/2 inbred strains from the Imputed Diversity Array, Build 37 were used with the Mouse Strain Comparison Tool (The Center for Genome Dynamics at The Jackson Laboratory; http://cgd.jax.org/straincomparison/) to identify genetic intervals within the Psl1 region considered equivalent (i.e., IBD) between C57BL/6 and DBA/2, where there were at least 10 consecutive SNP calls that matched. Other regions of Psl1 were considered to be nonequivalent and genes within the nonequivalent regions were identified. Because of the high density of the SNPs and the nonuniformity of the SNP distribution within the genome, genes located within 10 Kb of the nonequivalent regions were also included.

Nonsynonymous coding polymorphisms

The dbSNP Build 128 available from the Mouse Genome Database (www.informatics.jax.org) was used to identify any nonsynonymous coding polymorphism between C57BL/6 and DBA/2. The potential functionality of these polymorphisms was evaluated using the Sorts Intolerant From Tolerant (SIFT) tool (http://sift.jcvi.org/) (Ng and Henikoff 2001). If the polymorphism was characterized as “damaging” or it led to a stop codon, the amino acid change was considered to be functional.

Gene expression analysis

For analysis of gene expression, the dorsal skin of female mice (7–9 wk of age) was shaved 48 hr before treatment. Mice (three mice per group) were treated twice weekly for 2 weeks topically with 0.2 mL of acetone (for controls) or TPA (6.8 nmol) in 0.2 mL of acetone. Mice were killed by cervical dislocation at various times after the final treatment and the treated skin was excised. Total RNA was extracted from the epidermis as previously described (Riggs et al. 2005). Expression ratios for individual genes were examined by microarray analyses (see Supporting Information, Table S1). Expression of all
genes found by microarray analyses to be differentially expressed 2-fold or greater was also analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR; see Table S2) to verify differential expression. In addition, expression of several genes thought to be potential candidate genes, based on their roles in carcinogenesis but not found to be differentially expressed by microarray analysis, were measured by qRT-PCR. Microarray analyses were conducted using Illumina Mouse WG-6 v2 BeadArray (Illumina, Inc., San Diego, CA). Target preparation, hybridization, and scanning were conducted by the DNA Discovery Core of the University of Tennessee Health Science Center of Genomics and Bioinformatics (University of Tennessee, Memphis) using methods suggested by the manufacturer. qRT-PCR analysis of gene expression for Hras1 was performed using primers and probe as previously described (Riggs et al. 2005). qRT-PCR analyses of all other genes used TaqMan gene expression assays (see Table S3) (Applied Biosystems, Foster City, CA). For relative quantification of expression, samples were normalized to the geometric means of the levels of Hras mRNA and 18S RNA, which were previously shown to be stable and useful as reference genes in this system (Riggs et al. 2005).

**Statistical analysis**

Statistical analyses of differences between tumor multiplicity for each mouse strain were evaluated using the Mann–Whitney U-test included in the Prism 5 software package (GraphPad Software, La Jolla, CA). A one-tailed test was used for analyses between tumor multiplicities of
C57BL/6 and all other strains. A two-tailed test was used for analyses between tumor multiplicities at any two congenic strains. The Fisher's combined probability test was used to evaluate the combined results of independent experiments (Table 1). Comparisons of normalized mRNA levels were evaluated using a standard two-sided Student's t-test. A P-value of 0.05 or less was considered statistically significant.

**RESULTS**

As noted in the Introduction, the skin tumor promotion susceptibility locus, Psl1, maps to the distal half of chr 9 (Angel et al. 1997, 2001, 2010; Angel and Digiovanni 1999; Abel et al. 2010). We recently reported that when initiated with 2.5 μmol of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and promoted twice weekly with 13.6 nmol of TPA for 36 weeks, the congenic mouse strain, C57BL/6. Psl1Adba, which inherited a 59.1-Mb region of chr 9 (distal to nmol of TPA for 36 weeks, the congenic mouse strain, C57BL/6.

Ps1Bdba and C57BL/6.Psl1Edba subcongenic strains (Figure 1) initiated with 2.5 μmol of MNNG and promoted twice weekly with 13.6 nmol of TPA suggested that Psl1 consisted of at least two subloci, Psl1.1 and Psl1.2, that modify susceptibility to skin tumor promotion (Abel et al. 2010). To further refine the map location of these subloci, additional interval specific C57BL/6.Psl1dba subcongenic mouse strains were developed (Figure 1, Table 1, and Table 2) and tested for sensitivity to skin tumor promotion by TPA.

**Fine mapping of the Psl1.1 sublocus**

To fine map the Psl1.1 sublocus, the response of interval specific subcongenic mouse strains (Table 1 and Figure 1A) to skin tumor promotion with TPA was examined via a standard protocol. Mice were initiated with 100 nmol of DMBA and promoted twice weekly with 6.8 or 13.6 nmol of TPA until the tumor response reached a plateau (Table 1). C57BL/6.Psl1Gdba, C57BL/6.Psl1Edba, and C57BL/6. Psl1E6dba were significantly more sensitive than C57BL/6 mice to skin tumor promotion by TPA whereas the tumor response of C57BL/6. Psl1E6dba was similar to that of C57BL/6 (Table 1). These data are consistent with other studies in which mice were initiated with MNNG.

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**Table 1 Tumor response of C57BL/6.Psl1.1 subcongenic strains**

| Substrain | Introgressed Region | Length, Mb | 6.8 nmol TPA | 13.6 nmol TPA |
|-----------|---------------------|------------|-------------|-------------|
|           |                     |            | Tumor Multiplicity | N | P<sub>v</sub> | Tumor Multiplicity | N | P<sub>v</sub> | Combined P<sub>v</sub> |
| A         | D9Mit316 - telomere | 59.0       | 2.4 ± 1.6 | 30 | 0.0179 | 8.6 ± 3.2 | 29 | <0.0001 | <0.0001 |
| E         | Chst2 - telomere    | 29.2       | 1.8 ± 1.5 | 30 | 0.3314 | 4.4 ± 2.8 | 30 | 0.3686 | >0.1   |
| E6        | D9Mit12 - D9Mit53   | 4.9        | 3.2 ± 2.1 | 30 | 0.0005 | ND         | ND | ND      | ND     |
| G         | D9Mit346 - telomere | 4.9        | 3.0 ± 2.1 | 30 | 0.0017 | 5.6 ± 2.7 | 30 | 0.0015 | 0.0005 |
| G5        | D9Mit346 - D9Mit310 | 5.4        | 2.4 ± 1.5 | 29 | 0.0054 | ND         | ND | ND      | ND     |
| H         | D9Mit337 - D9Mit75  | 1.1        | 2.0 ± 1.6 | 29 | 0.2151 | 5.7 ± 3.4 | 30 | 0.1123 | >0.1   |
| H3        | D9Mit303 - D9Mit75  | 4.60       | 1.7 ± 2.0 | 30 | 0.0020 | 2.0 ± 1.3 | 30 | 0.0001 | 0.09    |
| H4        | D9Mit337 - D9Mit75  | 5.40       | 2.0 ± 1.3 | 30 | 0.0001 | 1.3 ± 1.4 | 30 | 0.09    | 0.001   |
| H6        | D9Mit316 - Gsta4    | 2.00       | 1.3 ± 1.1 | 30 | 0.0020 | 2.1 ± 1.8 | 30 | 0.001   | 0.01    |
| H7        | D9Mit337 - Gsta4    | 10.60      | 0.8 ± 1.1 | 30 | 0.005   | 3.2 ± 1.8 | 30 | 0.0001 | 0.09    |
| C57BL/6   |                     |            |            |     |         |            |     |         |         |

DMBA, 7,12-dimethylben[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate, ND, not done.

<sup>a</sup> Mice were initiated with 100 nmol DMBA and promoted twice weekly with 6.8 nmol TPA for 40 wks or 13.6 nmol TPA for 35 wks.

<sup>b</sup> Tumors/mouse ± SD.

<sup>c</sup> Number of mice at risk.

<sup>d</sup> Significance level of tumor multiplicity compared to C57BL/6. Not adjusted for multiple testing.

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**Table 2 Tumor response of C57BL/6.Psl1.2 subcongenic strains**

| Substrain | Introgressed Region | Length, Mb | Tumor Multiplicity<sup>b</sup> | N<sup>c</sup> | P<sup>d</sup> |
|-----------|---------------------|------------|-------------------------------|------------|-----------|
| A         | D9Mit316 - telomere | 59.0       | 2.0 ± 1.9                     | 30         | 0.005     |
| B         | D9Mit316 - Chst2    | 30.60      | 0.8 ± 0.7                     | 26         | >0.1      |
| B4        | D9Mit316 - D9Mit239 | 28.40      | 1.5 ± 1.6                     | 30         | 0.04      |
| B9        | D9Mit316 - D9Mit196 | 20.80      | 1.5 ± 1.6                     | 30         | 0.03      |
| BH1       | D9Mit263 - D9Mit307 | 7.60       | 1.9 ± 1.6                     | 30         | 0.0006    |
| H         | D9Mit316 - Gsta4    | 13.10      | 1.5 ± 1.2                     | 30         | 0.0072    |
| 1H        | D9Mit316 - D9Mit302 | 2.00       | 1.3 ± 1.1                     | 30         | 0.02      |
| H2        | D9Mit316 - D9Mit260 | 3.70       | 2.3 ± 1.7                     | 17         | 0.0012    |
| H3        | D9Mit303 - D9Mit75  | 4.60       | 1.7 ± 2.0                     | 30         | 0.002     |
| H4        | D9Mit337 - D9Mit75  | 5.40       | 2.0 ± 1.3                     | 30         | 0.0001    |
| H6        | D9Mit166 - Gsta4    | 5.00       | 1.3 ± 1.4                     | 30         | 0.09      |
| H7        | D9Mit337 - Gsta4    | 10.60      | 2.1 ± 1.8                     | 30         | 0.001     |
| C57BL/6   |                     |            | 0.8 ± 1.1                     | 30         | 0.001     |

DMBA, 7,12-dimethylben[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate.

<sup>a</sup> Mice were initiated with 100 nmol DMBA and promoted twice weekly with 6.8 nmol TPA for 36 wks.

<sup>b</sup> Tumors/mouse ± SD.

<sup>c</sup> Number of mice at risk.

<sup>d</sup> Significance level of tumor multiplicity compared to C57BL/6. Not adjusted for multiple testing.
(J. M. Angel and J. DiGiovanni, unpublished data). These results suggest that one or more genes mapping to a 3.2-Mb region of chr 9 between D9Mit115 and D9Mit53 modifies susceptibility to skin tumor promotion by TPA. This locus has been designated Psl1.1a (Figure 1A). The observation that C57BL/6.Psl1G5dba mice were significantly more sensitive than C57BL/6 mice (Table 1) to skin tumor promotion by TPA is consistent with the mapping of a locus to this region. Inheritance of Psl1.1a from DBA/2 results in increased susceptibility to skin tumor promotion.

C57BL/6.Psl1E6bma mice, which are homozygous for the DBA/2 allele of Psl1.1a (Figure 1A), had a tumor response similar to that of C57BL/6 when initiated with 100 nmol of DMBA and promoted twice weekly with either 6.8 or 13.6 nmol TPA (Table 1). The observations that C57BL/6.Psl1E6 mice are sensitive whereas C57BL/6.Psl1E mice are resistant to skin tumor promotion (Table 1) suggest that one or more genes mapping to a 5.5-Mb region of chr 9 between D9Mit112 and D9Mit346 modify susceptibility to skin tumor promotion by TPA. This locus has been designated Psl1.1b (Figure 1A), and inheritance of this locus from DBA/2 results in decreased susceptibility to skin tumor promotion by TPA.

**Fine mapping of the Psl1.2 sublocus**

To fine map the Psl1.2 sublocus, additional interval specific subcongenic strains (Table 2, Figure 1B) were analyzed for their susceptibility to skin tumor promotion with TPA. Again, mice were initiated with 100 nmol of DMBA and promoted twice weekly with 6.8 nmol of TPA until the tumor response reached a plateau (Table 2). The observations that C57BL/6.Psl1BH1daa mice were more sensitive than C57BL/6 mice (Table 2) and that C57BL/6.Psl1H6bma mice had a tumor response similar to C57BL/6 (Table 2) are consistent with the conclusion that Gsta4 is a tumor promotion susceptibility gene as recently reported (Abel et al. 2010).

C57BL/6.Psl1H1abaa mice were also significantly more sensitive than C57BL/6 mice to skin tumor promotion by TPA. These data are consistent with other studies in which mice were initiated with MNNNG (J. M. Angel and J. DiGiovanni, unpublished data) suggesting that one or more genes mapping proximal to Gsta4 modify susceptibility to skin tumor promotion. Additional subcongenic strains were analyzed for skin tumor promotion susceptibility to further delimit this region. Both C57BL/6.Psl1H1abaa and C57BL/6.Psl1H3abaa had similar tumor responses that were significantly greater than that of C57BL/6 (Table 2). Furthermore, C57BL/6.Psl1H2abaa mice were significantly more sensitive to skin tumor promotion by TPA than either C57BL/6.Psl1H1abaa (P = 0.02) or C57BL/6.Psl1H3abaa (P = 0.055) mice and had a tumor response similar to the combined response of C57BL/6.Psl1H1abaa and C57BL/6.Psl1H3abaa (data not shown). Taken together, these data suggest that at least two genes that modify susceptibility to skin tumor promotion by TPA map between D9Mit316 and D9Mit260. One locus, Psl1.2a (Figure 1B), maps to a region of chr 9 between D9Mit316 and D9Mit302 whereas the second locus, Psl1.2b (Figure 1B), maps to a region of chr 9 between D9Mit303 and D9Mit260. The observations that C57BL/6.Psl1H4abaa and C57BL/6.Psl1H4abaa mice were also significantly more sensitive than C57BL/6 mice to skin tumor promotion (Table 2) suggest that the map location of Psl1.2b can be narrowed to a region of chr 9 between D9Mit337 and D9Mit320 (Figure 1B). However, the possibility that the sensitivity of these two strains is due to a third skin tumor promotion susceptibility locus mapping between D9Mit260 and D9Mit75 cannot be ruled out at the present time. Inheritance of either Psl1.2a or Psl1.2b from DBA/2 results in increased susceptibility to skin tumor promotion by TPA.

The tumor response of C57BL/6.Psl1Babaa mice was similar to that of C57BL/6 mice when initiated with 100 nmol of DMBA and promoted twice weekly with 6.8 nmol of TPA (Table 2). The observation that C57BL/6.Psl1Babaa mice were relatively resistant to skin tumor promotion by TPA, even though they inherited the susceptibility alleles of Psl1.2a, Psl1.2b, and Gsta4 from DBA/2, suggests that a fourth gene mapping distal to D9Mit306 causes a decrease in skin tumor promotion susceptibility when inherited from DBA/2. To further narrow the map location of this gene, the tumor response of two additional subcongenic mouse strains, C57BL/6.Psl1B4abaa and C57BL/6.Psl1B9bma (Table 2, Figure 1B), was examined. Both strains were significantly more sensitive than C57BL/6 mice to skin tumor promotion by TPA (Table 2), suggesting that a resistance locus maps to a region of chr 9 between D9Mit238 and Chst2. This locus has been designated Psl1.2c (Figure 1B).

**Characterization of the Psl1.1a locus**

Potential candidate genes can be identified with haplotype mapping, which takes advantage of the fact that laboratory strains of mice were derived from the same founders and share regions of the genome that are IBD (Wiltsshire et al. 2003). Haplotype blocks in which the two parental strains are not IBD are the most probable location for causal genetic variation (Wade et al. 2002). Two-strain haplotype analysis, comparing C57BL/6 and DBA/2 haplotypes as described in the Materials and Methods section, were conducted to identify regions of the Psl1 locus that are not IBD (nonequivalent), and genes mapping to these regions were identified. Of 24 protein-coding genes that map to Psl1.1a, 15 map to regions of nonequivalence. In addition, one microRNA gene (Mir2136) and one long intergenic non-coding RNA (Gm17708) map to the Psl1.1a locus, both in regions of nonequivalence.

Different alleles of a candidate gene that underlie a QTL must display either an expression or functional variant. Twenty-two of the 24 protein-coding genes mapping to the Psl1.1a locus were analyzed by microarray and/or qRT-PCR for mRNA levels in the epidermis of TPA-treated C57BL/6 and DBA/2 mice 6 hr after the last of twice-weekly treatments for 2 wk. Nine of these protein-coding genes that map to regions of nonequivalence were found to be expressed above background (see Table S1 and Table S2), and three of these genes, chemokine (C–C motif) receptor-like 1 (Ccr1), receptor-like tyrosine kinase (Ryk), and receptor-like tyrosine kinase 3 (Ryk3), were differentially expressed in the epidermis of C57BL/6 compared with DBA/2 mice 6 hr after the final TPA treatment (Figure 1A and Table 3). Four other genes examined by qRT-PCR were found to be differentially expressed at other time points, with mRNA levels being greater in the epidermis of C57BL/6 compared with DBA/2 mice for angiotensin-like 2 (Amot2) and kynureninase (Kynase), and greater in TPA-treated DBA/2 compared with C57BL/6 epidermis for transmembrane protein 108 (Tmem108) and Eph receptor B1 (Ephb1) (data not shown).

| Locus | Map Location | Gene Symbol | C57BL/DBA* Microarray qRT-PCR |
|-------|--------------|-------------|-------------------------------|
| Psl1.1a | 102737250 | Ryk | 1.0 | 2.4 |
| Psl1.1a | 10000468 | Ccr1 | 1.4 | 5.5 |
| Psl1.1a | 104190570 | Acpp | 3.5 | 5.2 |
| Psl1.1b | 99210386 | Esyt3 | 2.8 | 4.6 |
| Psl1.2a | 66870400 | Tpm1 | 2.0 | 6.4 |

qRT-PCR, quantitative reverse transcription polymerase chain reaction. *Mice were treated twice weekly for 2 wk with 6.8 nmol of TPA in 0.2 mL of acetone and killed 6 hr after the final treatment.

Table 3 Potential candidate genes differentially expressed in the epidermis of TPA-treated C57BL/6 vs. DBA/2 mice
These genes may also be candidate promotion susceptibility genes. Interestingly, Rab6h, which maps within a region of IBD, was also differentially expressed in the epidermis of TPA-treated C57BL/6 compared with DBA/2 mice (data not shown), suggesting that expression of this gene may be regulated in trans.

Comparison of DNA sequences between C57BL/6 and DBA/2 identified nonsynonymous SNPs for three protein-coding genes and one unclassified noncoding RNA gene, Gm5627, mapping to the Psl1.1a locus. Two of these genes, nephronophthisis 3 (adolescent) (Nphp3, Figure 1A) and acyl-Coenzyme A dehydrogenase family member 11 (Acad11, Figure 1A), which were found to be expressed in the epidermis by qRT-PCR (see Table S2), were considered to be potential candidate genes. The Nphp3 SNP was predicted to be tolerated in the SIFT database (Table 4).

Characterization of the Psl1.1b locus

Of 49 protein-coding genes that map to Psl1.1b, 35 map to regions of nonequivalency. Thirty-nine of the 49 protein-coding genes mapping to the Psl1.1b locus were analyzed by microarray and/or qRT-PCR for mRNA levels in the epidermis of TPA-treated C57BL/6 and DBA/2 mice 6 hr after the last of twice weekly treatments for 2 wk. Twenty-one genes that map to regions of nonequivalency were found to be expressed above background (see Table S1 and Table S2). mRNA levels of one protein-coding gene, extended synaptotagmin-like protein 3 (Eys3, Figure 1A), were found to be >2-fold greater in the epidermis of C57BL/6 than DBA/2 mice (Table 3). Therefore, Eys3 was considered to be a potential candidate gene.

Comparison of C57BL/6 and DBA/2 sequences identified six protein-coding genes with nonsynonymous SNPs mapping to the Psl1.1b locus. One of these genes was not expressed above background when examined by microarray. The remaining five genes (Table 4 and Figure 1A) were considered to be potential candidate genes. SNPs for three genes were found in the SIFT database (Table 4) and the nonsynonymous SNP for one of these genes, armadillo repeat containing 8 (Armc8, Table 4), was predicted to be damaging.

Characterization of the Psl1.2a locus

Of 36 protein-coding genes that map to Psl1.2a, 25 map to regions of nonequivalency. Four unclassified noncoding RNA genes map to the Psl1.2a locus, all in regions of nonequivalency. Thirty-three of the 36 protein-coding genes mapping to the Psl1.2a locus were analyzed by microarray and/or qRT-PCR for mRNA levels in the epidermis of TPA-treated C57BL/6 and DBA/2 mice 6 hr after the last of twice weekly treatments for 2 wk. Of those genes, 20 were expressed above background (see Table S1 and Table S2). mRNA levels of one protein-coding gene, tropomyosin 1, alpha (Tpm1, Figure 1B), were found to be >2-fold greater in the epidermis of C57BL/6 than DBA/2 mice (Table 3). Therefore, Tpm1 was considered to be a potential candidate gene.

Comparison of the DNA sequences of C57BL/6 and DBA/2 identified eight protein-coding genes with nonsynonymous SNPs that map to the Psl1.2a locus. One of these genes was determined by microarray analysis to not be expressed above background. The remaining genes (Table 4 and Figure 1B) were considered to be candidate genes. Nonsynonymous SNPs found in four of these genes were found in the SIFT database and all four were predicted to be tolerated (Table 4).

Characterization of the Psl1.2b locus

Of 12 protein-coding genes that map to Psl1.2b, seven map to regions of nonequivalency. One microRNA and two noncoding RNA genes map to the Psl1.2b locus and are found in regions of nonequivalency. Nine of the 12 protein-coding genes mapping to the Psl1.2b locus were analyzed by microarray for mRNA levels in the epidermis of TPA-treated C57BL/6 and DBA/2 mice 6 hr after the last of twice weekly treatments for 2 wk. Of those genes, four mapped to regions of nonequivalency and were expressed above background (see Table S1 and Table S2). mRNA levels of Tpm1 were found to be >2-fold greater in the epidermis of C57BL/6 than DBA/2 mice (Table 3). Tpm1 maps to a region of overlap for the Psl1.2a and Psl1.2b loci (Figure 1B) and was considered a potential candidate gene for either locus.

Comparison of the DNA sequences of C57BL/6 and DBA/2 identified two protein-coding genes with nonsynonymous SNPs that map to the Psl1.2b locus. Microarray analysis indicated that neither of these genes was expressed above background in the epidermis of acetone- or TPA-treated C57BL/6 or DBA/2 mice 6 hr after the last of four treatments.

Characterization of the Psl1.2c locus

Three protein-coding genes map to the Psl1.2c locus, and two of these genes map to a region of nonequivalency. Neither of these genes was differentially expressed in the epidermis after the last of four treatments with TPA (see Table S1), and no nonsynonymous SNPs have been reported for either gene.

DISCUSSION

The skin tumor promotion susceptibility locus, Psl1, was previously mapped to a 59.1-Mb region of mouse chr 9 (Angel et al. 1997, 2001, 2010; Angel and Digiovanni 1999) and the glutathione S-transferase gene, Gsta4, was shown to underlie at least some of the effect of this locus on skin tumor promotion susceptibility in mice (Abel et al. 2010). In addition, Gsta4 has been shown to be a modifier of susceptibility to nonmelanoma skin cancer in humans (Abel et al. 2010). As described in the current study, analyses of skin tumor promotion susceptibility using interval specific subcongenic mouse strains indicate that Psl1 is a compound locus made up of a cluster of subloci (see Figure 1), similar to other QTL affecting complex traits such as blood pressure (reviewed in Rapp and Joe 2012), diabetes (Granhall et al. 2006), body composition (Diament and Warden 2004; Farber and Medrano 2007; Ishikawa et al. 2007; Prevorsek et al. 2010), and cancer

| Table 4 Potential candidate genes with nonsynonymous SNPs that map to Psl1 subloci |
|----------------|-----------------|-----------------|-----------------|
| Locus | Map Location | Gene Symbol | SIFT |
| Psl1.1a | 103904874 | Nphp3 | T |
| Psl1.1a | 103966033 | Acad11 | NF |
| Psl1.1b | 96585842 | Zbtb38 | T |
| Psl1.1b | 99210386 | Eys3 | NF |
| Psl1.1b | 99356662 | Nme9 | T |
| Psl1.1b | 99378810 | Armc8 | Damaging |
| Psl1.1b | 99530014 | Dzip1I | NF |
| Psl1.2a | 65142102 | Clpx | NF |
| Psl1.2a | 65283589 | Mtfmt | T |
| Psl1.2a | 65515808 | Oat2 | NF |
| Psl1.2a | 65676733 | Trip4 | T |
| Psl1.2a | 65935934 | Sna1 | NF |
| Psl1.2a | 66198257 | Herc1 | T |
| Psl1.2a | 66561493 | Car12 | T |

SNP, single-nucleotide polymorphism; SIFT, Sorts Intolerant From Tolerant; T, tolerated; NF, not found in database.
Inheritance of Psll.1a, Psll.1b, Psll.2a, Psll.2b, and Gsto6 from DBA/2 results in increased susceptibility to skin tumor promotion whereas inheritance of Psll.1b and Psll.2c from DBA/2 results in decreased susceptibility. Although the skin tumor susceptibility locus, Skts6, was mapped to the distal half of chr 9 using crosses of Mus spretus with NIH/Ola mice (Nagase et al. 1999), it is not possible to determine from the current studies whether any of the Psll subloci and Skts6 are the same.

Of the more than 550 protein-coding genes that map within the Psll locus, fewer than 25 genes that fulfill the criteria for candidate skin tumor promotion susceptibility genes were identified in the current study (summarized in Table 3 and Table 4). For example, the receptor-like tyrosine kinase, Ryk, maps to the Psll.1a locus and is expressed 2.6-fold greater in the epidermis of TPA-treated C57BL/6 compared with DBA/2 mice (Table 3). The receptor encoded by Ryk functions as a co-receptor with Fzd1 for Wnt ligands and binds to Dishevelled, through which it activates the canonical Wnt Pathway (Lu et al. 2004). A genetic variant of Ryk has been associated with risk of breast cancer in humans (Wang et al. 2010), supporting the hypothesis that Ryk may be a candidate tumor promotion susceptibility gene.

Ccr1 (CCX-CKR) also maps to the Psll.1a locus and is expressed at greater levels in TPA-treated epidermis of C57BL/6 compared with DBA/2 mice (Table 3). Ccr1 is a atypical chemokine receptor that lacks a signaling domain. Overexpression of Ccr1 inhibited proliferation and migration of human breast cancer cells both in vitro and in vivo (Feng et al. 2009), supporting the hypothesis that Ccr1 is a cancer susceptibility gene. The CCR1 protein selectively binds the homeostatic CC-chemokines CCL19, CCL21, and CCL25 (Gosling et al. 2000; Townsend and Nibbs 2002; Comerford et al. 2010). Binding to CCR1 results in internalization and degradation of these ligands (Comerford et al. 2006), which attenuates the immune response (Heinzel et al. 2007). Recent reports have shown that the CCR1 ligands promote proliferation and prevent apoptosis (Wang et al. 2005; Li et al. 2009; Shen et al. 2009; Johnson et al. 2010; Xu et al. 2011, 2012), suggesting that increased expression of Ccr1 may reduce tumor response through chemokine depletion. This idea is further supported by the observation of a significantly higher rate of tumor growth in wild-type BALB/c mice compared to plt mice, which lack CCL19 and CCL21, implanted with a syngeneic squamous cell carcinoma cell line (Mburu et al. 2006).

Another potential candidate tumor promotion susceptibility gene, Zbtb38 (zinc finger and BTB domain containing 38), maps to a region of nonequivalency within the Psll.1b locus and has an amino acid variant between C57BL/6 and DBA/2 (Table 4). The protein encoded by Zbtb38 is a member of the Kaiso-like family of methyl-CpG-binding proteins. It is a BTB-zinc finger transcriptional repressor that interacts with the transcriptional co-repressor C-terminal binding proteins (Sasai et al. 2005). C-terminal binding protein is thought to be involved in development and carcinogenesis (reviewed in Chinnadurai 2003). The encoded protein with a serine at amino acid 117, whereas the DBA/2 allele encodes a protein with a proline at this position. This conserved amino acid variant maps within the phospholipid-binding-motif termed the phox homology (PX) domain, which is involved in localizing the ZBTB38 protein to early endosomes (Worby and Dixon 2002). These observations suggest that Zbtb38 may modify skin tumor promotion susceptibility through altered regulation of a-catenin degradation.

Sorting nexin 1 (Snx1) is a potential candidate promotion susceptibility gene for the Psll.2a locus (Table 4). SNX1 is a putative tumor suppressor gene (Nguyen et al. 2006), and the encoded protein is a member of a large family of endocytic proteins that help determine the fate of internalized receptors as the receptors reach the early endosome (Worby and Dixon 2002). It plays a role in targeting ligand-activated epidermal growth factor receptor (EGFR) to the lysosomes for degradation after endocytosis from the cell surface and release from the Golgi (Kurten et al. 1996). Small interfering RNA knockdown of SNX1 levels in tumor cell lines resulted in increased proliferation, decreased apoptosis, decreased anoikis, increased EGFR phosphorylation after EGF stimulation, and increased downstream signaling (Nguyen et al. 2006; Nishimura et al. 2012). These results suggest that Snx1/Snx1 may be involved in carcinogenesis by regulating ligand-induced EGFR phosphorylation and suggest a critical function for Snx1/Snx1 in the maintenance of tightly regulated EGFR-mediated signaling. The C57BL/6 allele of Snx1 encodes a protein with a serine at amino acid 117, whereas the DBA/2 allele encodes a protein with a proline at this position. This conserved amino acid variant maps within the phospholipid-binding-motif termed the phox homology (PX) domain, which is involved in localizing the SNX1 protein to early endosomes (Worby and Dixon 2002). A human variant of SNX1 with a point mutation (K214A) in the PX domain was incapable of binding 3-phosphoinositides and localized to the cytosol rather than to early endosomes (Cozier et al. 2002). Furthermore, the K214A variant could not stimulate EGFR degradation whereas the wild-type SNX1 did, indicating that the PX domain-dependent/early endosomal association of SNX1 is important for its ability to regulate the targeting of internalized EGFR for lysosomal degradation (Cozier et al. 2002). Additional studies will be required to determine whether the amino acid variant at position 117 in the mouse SNX1 protein has a similar effect and if this variant plays a role in skin tumor promotion susceptibility.

A recent study found that loss of expression of bone morphogenetic protein 5 (Bmp5), which maps to distal chr 9, was associated with reduction in size and number of clonogenic keratinocyte stem cells and an increase in sensitivity to skin tumor promotion (Kangsamaskin and Morris 2011) suggesting that Bmp5 may underlie a portion of the effect of Psll on skin tumor promotion susceptibility. However, Bmp5 does not map to any of the Psll subloci identified in the present study (Figure 1B). Furthermore, the congenic mouse strain C57BL/6.Psll1H6dis, which has a tumor response similar to C57BL/6 when initiated with 100 nmol of DMBA and promoted twice weekly with 6.8 nmol of TPA (Table 2), inherited the DBA/2 allele of Bmp5 (Figure 1B), indicating that Bmp5 does not affect skin tumor promotion susceptibility in genetic crosses of C57BL/6 with DBA/2 mice.
In conclusion, at least six genes mapping to the Psl1 locus on distal chr 9 modify susceptibility to skin tumor promotion by TPA. Previous studies have shown that one gene that resides in this region (Gsta4) is a modifier of skin tumor promotion susceptibility (Abel et al. 2010). Other genes mapping to regions of nonequivalency within the Psl1 subloci are either either expressed in the epidermis of resistant C57BL/6 vs. sensitive DBA/2 mice treated with TPA or have amino acid variants between the two strains of mice. Several of these genes have been associated with cancer risk in humans or play a role in signaling pathways associated with carcinogenesis, making them excellent candidate skin tumor promotion susceptibility genes. Additional studies will be required to confirm that these genes are modifiers of skin tumor promotion susceptibility.

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