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At least four genes and sex are associated with susceptibility to urethane-induced pulmonary adenomas in mice

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Summary

Susceptibility to urethane-induced lung adenomas in mice has a polygenic mode of inheritance, with no obvious discontinuity in lung tumour counts among 37 AXB recombinant inbred strains. However, mean tumour counts were markedly higher in strains carrying the A/J allele at the *Kras2* and *H2* complex than in those carrying the C57BL/6 allele. In 162 F_2 hybrids and small numbers of both backcrosses between strain A/J (susceptible) and C57BL/6 (resistant) mice, five factors influencing susceptibility were identified. Variation due to the 'major' *Kras2* locus (chromosome 6) accounted for 60% of the total variation. 'Minor' loci linked to microsatellite markers *Tnfb* (in the *H2* complex), *D9Mit11* and *D19Mit16* (on chromosomes 17, 9 and 19, respectively) accounted for a further 13% of the variation, and males had more tumours than females with sex differences accounting for 2% of the variation. No significant association with 32 other loci was detected. On a square-root transformed scale, heterozygotes at all marker loci were of intermediate susceptibility compared with homozygotes. The three minor loci and sex only affected lung tumour counts when at least one susceptible *Kras2* allele was present.

1. Introduction

Inbred strains of mice differ widely in their susceptibility to the induction of lung adenomas when treated with carcinogens, with strain A mice being the prototype of a susceptible strain and C57BL/6 being resistant. Strains can also have an intermediate level of susceptibility, suggesting a polygenic mode of inheritance. Mouse lung tumours are particularly useful as a model of carcinogenesis, as sensitivity can be quantified by counting the number of tumours on the surface of the lung, providing a more sensitive end point than the qualitative presence or absence that is necessary with many other models of carcinogenesis. The strain A lung tumour model has been proposed as an assay for chemical carcinogenicity (Stoner & Shimkin,1985), though Maronpot *et al.* (1983) found that it correlated poorly with combined rat and mouse long-term carcinogenesis bioassays. A clearer understanding of the mechanisms of lung tumorigenesis in mice may be useful in the interpretation of carcinogenesis bioassays.

Studies on the mode of inheritance of susceptibility have been reviewed by Malkinson (1991). Early work by Bloom & Falconer (1964) suggested that there were relatively few genes governing susceptibility, and they concluded that there was at least one major gene, although they were unable to map it using genetic tools available at that time. More recently, Malkinson *et al.* (1985) used the AXB and BXA recombinant inbred strains and suggested that at least three 'Pas' (pulmonary adenoma susceptibility) genes must be involved. Ryan *et al.* (1987) showed that a genetic polymorphism at the *Kras2* locus on chromosome 6 was strongly associated with susceptibility in these recombinant inbred strains, a finding which was supported by Gariboldi *et al.* (1993) who concluded that a gene on chromosome 6, identical to, or closely linked with *Kras2*, was the only gene associated with variation in sensitivity in a cross between strain A and resistant C3H/He mice. The *Kras2* locus is the cellular homologue of the transforming gene of the Kirsten sarcoma virus, v-Ki-*ras*, and is homologous to the KRAS2 gene in humans (Green, 1989, p. 197). As most lung tumours have *Kras2* mutations (You *et al.* 1989; Ohmori *et al.* 1992), it is reasonable to conclude that the polymorphism at the *Kras2* locus itself represents one of the *Pas* loci.

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Congenic mouse strains with different *H2* haplotypes also vary in the incidence and number of spontaneous and chemically induced lung tumours (Smith & Walford, 1976; Faraldo *et al.* 1979; den Engelse *et al.* 1981; Miyashita *et al.* 1989), suggesting that the *H2* region on chromosome 17 contains a second *Pas* gene. The identity of this gene has not been established, though Oomen *et al.* (1989) suggested that it may be associated with the glucocorticoid receptor gene that is encoded in this region. They found, for example, that administration of glucocorticoid hormone to mice treated with ENU *in utero* reduced the number of lung tumours in adults. Similarly, Droms *et al.* (1988) found that dexamethasone reduced tumour numbers in adult mice treated with urethane. The sex of the mouse is usually assumed to have little influence on tumour number, and Miyashita *et al.* (1989) concluded that '... no effect of the genes on the sex chromosome has yet been described'.

The recent development of a wide range of convenient microsatellite genetic markers in the mouse (Love *et al.* 1990; Dietrich *et al.* 1992) has made it possible to study the mode of inheritance of polygenic characters in more detail (Lander & Botstein, 1989). The aim of this study was to quantify any association between susceptibility to urethane-induced lung adenomas and defined genetic markers in mice as a first step in identifying genes controlling this polygenically inherited character.

2. Materials and methods

(i) *Re-analysis of AXB and BXA data*

Data on the lung tumour number, and the *Kras2* allele and *H2* haplotype in the AXB recombinant inbred strains were taken from Malkinson *et al.* (1985) and Marshall *et al.* (1992), and re-analysed to quantify the relative importance of *Kras2* and *H2*.

(ii) *Animals*

Strains A/JOlaHsd (strain A/J), C57BL/6JOlaHsd (strain B6) and the F_1 hybrid between these two strains were supplied by Harlan UK Ltd. The mice were of 'SPF' status, and were maintained in plastic isolators with a filtered air supply to minimize possible exposure to pathogens. They were maintained at 19–21 °C, were housed 3–5 per box (depending on age), fed standard mouse diet and were supplied with water ad lib. F_2 hybrid and backcross offspring were produced by inter-mating the F_1 hybrids and by crossing A/J males with F_1 females (backcross to strain A/J) and B6 males with F_1 females (backcross to strain B6).

When the mice were 41 d old they were individually weighed and given a single intraperitoneal injection of urethane (1 mg/g) made up as a 10% w/v solution in 0·9% saline. After approximately 6 months the mice were killed, and the lungs were expanded by intratracheal administration of formal saline. The number of tumours on the surface of the lungs was counted. A total of 162 F_2 hybrid, 22 $F_1 \times$ A/J and 24 $F_1 \times$ C57BL/6 mice completed the study.

(iii) *Genotyping*

DNA was prepared by proteolytic digestion and phenol extraction of samples of tail or liver tissue following digestion with proteinase K, or by incubating the tissues in TE buffer for 30 min at 55 °C and then boiling them for 8 min. The amount of DNA in solution was estimated using UV absorbance at 260 nm.

Most of the markers were microsatellites, but amplification of the *Kras2* polymorphic region used primers constructed according to the partial sequence of the *Kras2* gene described by You *et al.* (1992). The upper primer located at the 3′ end of the first exon had the sequence CTTGTGTGAGACATGTTC. The lower primer is located at the 5′ end of the first exon, and has the sequence CAGAGCTAGTCTGGTCTAC. These primers amplify a 609 base pair fragment in A/J and a 646 base pair fragment in C57BL/6 mice. The markers will be referred to collectively as 'STS' (sequence-tagged-site) markers.

Microsatellite PCR primers were obtained from the HGMP Resource Centre, Clinical Research Centre, Harrow, or were synthesized at the IRC for Mechanisms in Human Toxicity, University of Leicester. The primer sequences used are those reported by Love *et al.* (1990) and Hearne *et al.* (1991) (Nds series and named loci). The Mit series primers are those described by Dietrich *et al.* (1992), or were obtained from the Whithead Institute/MIT Genome Center. All markers were expressed codominantly.

The 'low' and 'high' mice (see below) were screened for polymorphic microsatellites at the following loci (chromosome): *Csfgm*, *D1Nds2* (1), *D2Mit30* (2), *Tshb*, *D3Nds1* (3), *D4Mit11*, *D4Mit15* (4), *D5Mit11* (5), *Kras2* (6), *Int-2*, *D7Nds1*, *D7Nds7* (7), *D8Mit4*, *D8Mit14* (8), *D9Mit11*, *D9Mit18* (9), *D10Mit10*, *D10Nds1* (10), *Hox2*, *D11Nds8* (11), *Odc*, *D12Mit5*, *IghV* (12), *D13Mit3* (13), *Hr* (14), *D15Mit5* (15), *D16Nds1* (16), *Qb1*, *Tnfb* (17), *D18Mit14*, *D18Mit20*, *D18Mit24* (18), *D19Mit9*, *D19Mit16*, *D19Mit19*, *D19Mit42* (19).

PCR amplification reactions were performed in volumes of 10 μl containing 44·5 mM Tris–HCl, pH 8·8, 11 mM ammonium sulphate, 4·5 mM $MgCl_2$, 6·9 mM 2-mercaptoethanol, 4·5 μm EDTA, 1 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 113 μg/ml BSA (Pharmacia), 50 ng primer, 100 ng of genomic DNA and 1 unit of Ampli Taq DNA polymerase (Applied Biosystems). Amplifications were carried out in a Perkin Elmer Cetus DNA thermal cycler 480. Two amplification programmes

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(standard and touchdown) were used depending on the primer specificity. The standard programme involved 32 cycles of 1·3 min at 96 °C, 1 min at 55 °C, 1 min at 70 °C, followed by a 1 min cycle at 67 °C and a 10 min cycle at 70 °C. The touchdown programme (Don *et al.* 1991) used two cycles of 2 min at 94 °C, 1·5 min at X °C and 2·5 min at 72 °C, where X was the annealing temperature. This was decreased 2 °C every two cycles from 72 °C down to 50 °C, at which temperature 21 cycles were carried out, followed by 7 min at 72 °C. All PCR products were visualized under UV light following electrophoresis with Tris-borate EDTA (TBE) buffer pH 8·0 in a 4% NuSieve or MetaPhor (FMC) agarose gel incorporating 0·5 μg/ml ethidium bromide.

(iv) *Screening of 'high' and 'low' mice*

Twenty-eight F_2 hybrid mice with no tumours ('low') and 38 mice with 15 or more tumours ('high') were identified and typed at each locus, a strategy recommended by Lander & Botstein (1989). Where there was a possible departure from the expected 1:2:1 ratio (a significance level of $P < 0{\cdot}1$ was used), the remaining F_2 hybrids and the backcross mice were typed at that locus. Some samples of DNA failed to amplify with some primers, hence the number of mice typed varied slightly with each locus.

(v) *Statistical analysis*

Departure from the expected 1:2:1 ratio for each STS marker in the 'high' and 'low' mice was tested using the chi-squared test.

A square-root transformation of the lung tumour counts was used to normalize the data for statistical analysis, though untransformed data are also presented. Inspection of the results showed clearly that *Kras2* was a major locus governing susceptibility. Accordingly, for the loci where all mice were genotyped, a general linear model analysis of variance involving the *Kras2* locus and each of the other loci in turn was carried out. This estimates significance levels, possible interactions between *Kras2* and other loci, least squares means and standard deviations of tumour counts and square root tumour counts, eliminating variation caused by the *Kras2* locus. Multiple linear regression was used to quantify the contribution of the four genetic loci and sex to lung tumour susceptibility. The contribution of each factor to the total variability was estimated as the coefficient of multiple correlation (R^2) associated with each factor. Multiple regression was also used to estimate the effects of substitution of a B6 allele for an A/J allele at each locus.

3. Results

(i) *Re-analysis of AXB and BXA recombinant inbred strain data*

There was no obvious discontinuity in tumour counts among 37 AXB and BXA recombinant inbred strains which would suggest the segregation of a major susceptibility locus. However, one-way analysis of variance of the square-root transformed tumour counts in the AXB and BXA recombinant inbred strains according to the *Kras2* and *H2* genotypes showed a highly significant effect of *Kras2* ($F_{1,35} = 18{\cdot}07$, $P < 0{\cdot}01$, $R^2 = 0{\cdot}39$), with mean root counts of $3{\cdot}64 \pm 1{\cdot}12$ (13·3 tumours, $n = 19$) in mice homozygous for the strain A/J allele and $1{\cdot}94 \pm 1{\cdot}06$ (3·8 tumours, $n = 18$) in mice with the B6 allele, as found by Malkinson *et al.* (1985) using different statistical methods. There was also a highly significant effect due to the *H2* locus ($F_{1,32} = 6{\cdot}85$, $P < 0{\cdot}01$, $R^2 = 0{\cdot}18$), with mean root tumour numbers being $3{\cdot}27 \pm 1{\cdot}36$ (10·7 tumours per mouse, $n = 16$) for mice carrying the strain A/J allele and $2{\cdot}13 \pm 1{\cdot}18$ (4·5 tumours per mouse, $n = 18$) for mice carrying the B6 allele. While both *Kras2* and *H2* influenced tumour counts, the effect of *Kras2* was substantially stronger, accounting for nearly 40% of the between-strain variation compared with only 18% for the *H2* complex. As individual tumour counts were not available, the within-strain variation was not quantified.

(ii) *Distribution of tumour counts in F_2 hybrid and backcross mice*

There was some evidence of bimodality in the F_2 animals, largely due to excess of mice without any tumours, but no evidence of bimodality in either of the backcrosses, as would be expected with segregation of a single major locus. However, too few backcross mice were studied to give a reliable indication of bimodality.

(iii) *Screening of 'high' and 'low' tumour count mice*

Table 1 shows the genotypes of mice with the 'low' and 'high' tumour count for those loci where there was some evidence ($P < 0{\cdot}1$) for departure from the expected 1:2:1 Mendelian ratio, which might imply genetic linkage, and for loci on the same chromosome. As a result of this screen, all mice were typed for *Kras2*, *Tnfb*, *D9Mit11* and *D19Mit16*.

(iv) *Tumour numbers in F_2 hybrid and backcross mice*

No significant deviation from the expected 1:2:1 genetic ratios in the full sample of mice at the *Tnfb* (in the H2 complex), *Kras2* and *D9Mit11* loci and the expected 1:1 ratio for the sex of the mice was

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Table 1. *Screening results with 'low' and 'high' tumour mice*

Probe/locus	Chromosome	Location	Tumour number	Genotype at marker locus AA	AB	BB	Chi-square and P[1]
Kras[2]	6	70	Low	0	1	26	73·2***
			High	22	16	0	26·4***
D9Mit11[2]	9	42	Low	8	10	9	1·9
			High	14	19	4	5·4*
D9Mit18	9	65	Low	9	12	6	1·0
			High	7	20	11	0·9
Qb-1	17	19	Low	3	18	6	3·7
			High	16	18	1	12·9**
Tnfb[2]	17	19	Low	3	17	6	3·2
			High	17	20	1	13·6**
D19Mit42	19	2	Low	6	11	11	3·1
			High	13	21	4	4·7*
D19Mit16[2]	19	14	Low	6	12	9	1·0
			High	13	19	3	6·0**
D19Mit19	19	25	Low	6	12	9	1·0
			High	11	15	9	0·9
D19Mit9	19	34	Low	7	9	10	3·2
			High	10	16	10	0·4

[1] Deviation from expected 1:2:1 ratio, * $P < 0·1$, ** $P < 0·05$, *** $P < 0·01$.
[2] All mice were typed at these loci.

Table 2. *Mean*[1] *tumour numbers (square root scale) in F_2 and backcross mice by STS genotype*

Factor	Type[2]	Backcross to A Mean	S.E.[4]	N	F_2 hybrids[3] Mean	S.E.	N	Backcross to C57BL/6 Mean	S.E.	N
Sex	M	5·23	0·29	9	2·93	0·10	94	1·61	0·19	12
	F	4·17	0·23	13	2·48	0·12	68	1·54	0·25	12
Tnfb	AA	4·58	0·37	8	3·14	0·15	45			
	AB	4·64	0·28	14	2·69	0·11	88	1·44	0·21	11
	BB				2·19	0·20	29	1·67	0·19	12
Kras2	AA	5·22	0·24	12	4·29	0·17	35			
	AB	4·19	0·28	10	3·21	0·17	78	2·61	0·16	17
	BB				0·63	0·13	49	0·55	0·27	7
D19Mit16	AA	5.00	0·32	10	3·05	0·18	31			
	AB	4·31	0·27	12	2·81	0·11	78	1·71	0·19	12
	BB				2·48	0·14	53	1·22	0·25	12
D9Mit11	AA	4·82	0·29	12	3·17	0·15	40			
	AB	4·44	0·34	10	2·97	0·12	78	1·35	0·34	11
	BB				2·16	0·16	34	1·47	0·18	13

[1] Means are for the joint least squares analysis of Kras with every other factor.
[2] A is the strain A/J allele, B is the C57BL/6 allele.
[3] Differences among F_2 genotypes within a factor (sex, *Tnfb* etc.) were statistically significant at $P < 0·000$ for *Kras2*, $P = 0·008$ for sex, $P < 0·000$ for *Tnfb*, $P < 0·000$ for *D9Mit11* and $P < 0·049$ for *D19Mit16*. Differences among backcross genotypes for these factors were not significant ($P > 0·05$) except at the *Kras2* locus ($P < 0·01$).
[4] S.E. = Standard error of the mean.

observed. However, there was a significant ($P < 0·05$) deficiency of mice of the AA genotype, and an excess of mice with the BB genotype at the *D19Mit16* locus. Whether this is a real effect or is due to sampling variation is not clear.

Least squares means of square roots of the tumour counts for sex and the other four loci using a general linear model analysis of variance with *Kras2* as one factor are given in Table 2. In the F_2 hybrids, the effects of these loci on tumour counts were significant (to three decimal places) at $P < 0·000$ for *Kras2*, $P = 0·008$ for sex, $P < 0·000$ for *Tnfb*, $P < 0·000$ for *D9Mit11* and $P < 0·049$ for *D19Mit16* (see Table 3). The reality of the effect associated with *D19Mit16* would be debatable on the basis of these data, but this linkage has been independently confirmed by

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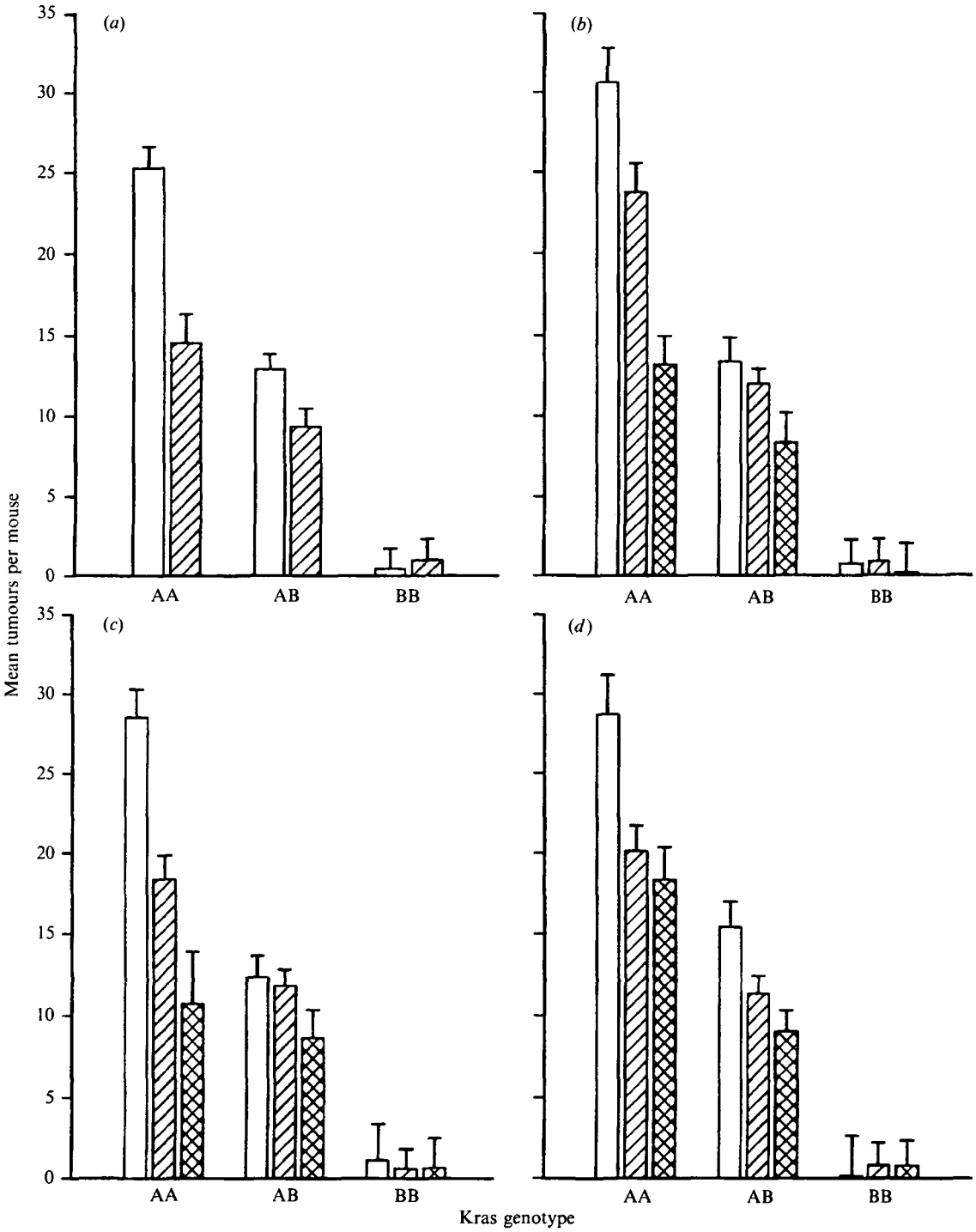

Fig. 1. Mean tumour counts according to (*a*) sex (□, male; ▨, female) and genotype at the *Kras2*, (*b*) *D9Mit11* (□, AA; ▨, AB; ⊠, BB), (*c*) *Tnfb* (□, AA; ▨, AB; ⊠, BB) and (*d*) *D19Mit16* (□, AA; ▨, AB; ⊠, BB) loci.

Devereux *et al.* (in press). As the association between tumour count and the two markers *D9Mit11* and *D19Mit16* only represents a genetic linkage, and the tumour susceptibility loci have not yet been accurately mapped, the influence of these loci may be underestimated. In all cases except that of *D9Mit11* there was also a highly significant ($P < 0{\cdot}01$) *Kras2* × Locus interaction. The minor loci and sex only had a detectable effect when there was at least one strain A allele at the *Kras2* locus. The mean tumour counts (untransformed data) for sex and the three 'minor' loci according to the *Kras2* genotype are shown in Fig. 1. Alleles at all four genetic loci behaved in an essentially additive manner on the transformed scale, with heterozygotes being intermediate in tumour number between the two homozygotes (Table 2). Among the backcross mice only the effects of the *Kras2* locus were statistically significant, but the power of these analyses was very low due to the small numbers of mice.

The contribution of each factor to the total phenotypic variation using multiple linear regression

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Table 3. *Estimates of the magnitude of the genetic effects associated with each factor, on a square root scale*

Factor	% of variation[1]	Mean difference[2]	Mean difference (standard deviations)[3]	Partial regression coefficient[4]	*P*-value from general linear model analysis of variance[5]
Kras2	59·76	3·66	4·11	1·96	< 0·000
Tnfb (H2)	7·66	0·95	1·07	0·44	< 0·000
D9Mit11	3·67	1·01	1·13	0·46	< 0·000
Sex	2·16	0·45	0·51	0·18	0·008
D19Mit16	1·82	0·57	0·64	0·33	0·049

[1] The percent of total variation (R^2) associated with each factor using multiple linear regression.
[2] Difference in tumour counts between mice of type AA and BB or between males and females.
[3] Difference in tumour counts between animals of type AA and BB or between males and females in units of the within-genotype standard deviation. The standard deviation (0·89 units) was estimated from the deviations from regression after fitting all five factors in the multiple regression equation.
[4] Estimated from the multiple regression analysis. Indicates approximately the effect of substitution of an allele (or a different sex) on tumour numbers.
[5] Obtained from the general linear model joint analysis of variance of each factor with *Kras2*, and expressed to three decimal places.

on the square root scale is given in Table 3. The *Kras2* locus accounted for 60% of the total variation, followed by *Tnfb (H2)*, *D9Mit11*, sex and *D19Mit16* which together accounted for a further 15% of the variation. Variation due to all four loci and sex accounted for 75·1% of the variation. The multiple regression equation was:

$$\text{Root Tumour count} = 6{\cdot}14 - (0{\cdot}18*\text{sex}) - (0{\cdot}44*\text{Tnfb}) - (1{\cdot}96*\text{Kras}) - (0{\cdot}33*\text{D19Mit16}) - (0{\cdot}46*\text{D9Mit11}),$$

where males are coded as 0 and females as 1, and 'Tnfb', 'Kras' etc. represent the number of strain B6 alleles present (i.e. 0, 1 or 2). The magnitude of each regression coefficient indicates the effect of substituting a strain A/J allele at that locus on the tumour score on the square root scale. For example, a male mouse homozygous for strain A/J alleles at all loci would have a predicted score of 6·14 tumour units (approximately 37·7 tumours/mouse), as 'sex', 'Tnfb', etc. would all equal zero. A female mouse homozygous for B6 alleles at all loci would have a predicted tumour score of −0·42 (zero tumours). The importance of the *Kras2* locus is shown by the relatively large size of the regression coefficient. As a result, a male mouse homozygous for strain A/J alleles at the three minor loci (*Tnfb*, *D9Mit11* and *D19Mit16*) but homozygous for strain B6 alleles at the *Kras2* locus would have a predicted tumour score of 2·22 tumours (approximately 4·9 tumours per mouse). In contrast, a male mouse which is homozygous for strain B6 alleles at the three minor loci, but has strain A/J alleles at the *Kras2* locus would be expected to develop a higher score of 3·68 (approx. 13·5 tumours/mouse). Thus, allelic substitution at the *Kras2* locus would more than counterbalance all three of the minor loci. The influence of each of the factors is further quantified in terms of the difference between mice of type AA and BB or between males and females in standard deviation units (Table 3).

4. Discussion

The rapid development of microsatellite markers has made it practicable for the first time to attempt to dissect out and quantify the effects of individual loci controlling polygenic characters by looking for associations between marker loci and the character of interest in segregating populations. Urethane-induced lung adenomas in mice represent a good model system for testing these methods. Falconer & Bloom (1962) showed that tumour multiplicity was a quantitative polygenic character, but provided evidence of a major susceptibility locus segregating in crosses between susceptible strain A and resistant C57BL mice. Their major susceptibility locus was almost certainly *Kras2*. The surprising findings reported here are that these methods can identify as many as five factors controlling susceptibility, with individual loci accounting for as little as 2–3% of the phenotypic variation, though the reality of the effect associated with chromosome 19 would be open to doubt had it not been discovered independently by other workers.

Three of these factors had already been identified. Ryan *et al.* (1987) found that the *Kras2* locus exerted a major effect on lung tumour susceptibility in the AXB and BXA recombinant inbred strains. This can be regarded as the *Pas-1* locus. The availability of H2 congenic strains on a strain A/J inbred background made it possible to show that a gene or genes within the *H2* complex influenced tumour counts. Further analysis of the AXB and BXA recombinant inbred strains reported here supports the finding that the H2 complex contains a *Pas* gene. However, although the

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AXB and BXA recombinant inbred strains represent one of the largest sets of RI strains in the mouse, there was still no evidence from the strain distribution pattern for the segregation of a major gene.

With this microsatellite analysis, not only has it been possible to quantify the contribution of the *Kras2* and *H2* (marked in this study by the *Tnfb* microsatellite) complexes to overall tumour susceptibility in F_2 hybrids, but two additional linkages have been found. The one on chromosomes 19 (designated *Pas-3*) was independently discovered by Devereux *et al.* (in press) and was detected in this study even though it only accounted for 1·8% of the total variation. The one on chromosome 9 (designated *Pas-4*) has not been previously reported, but it accounted for 3·7% of the total variation.

The study has also shown that at each of the four *Pas* loci, the genes are acting additively, with no sign of dominance when transformed to an appropriate scale. The total of $3 \times 3 \times 3 \times 3 = 81$ susceptibility genotypes in F_2 hybrids and $2 \times 2 \times 2 \times 2 = 16$ genotypes in the recombinant inbred strains per sex explains why segregation of individual loci could not easily be seen in a large set of recombinant inbred strains, even though one of the loci (*Kras2*) was exerting a major effect.

The finding of a significant sex difference in tumour counts is surprising. It seems to have been widely accepted that there are no sex differences in susceptibility. Accordingly, some investigators have pooled their results without looking for a sex difference (e.g. Miyashita & Moriwaki, 1987; Ryan *et al.* 1987), and others have given a fixed dose per mouse regardless of body weight (e.g. Stoner & Shimkin, 1985). As females weigh less than males, the higher dose per gramme of body weight in this case could compensate for their lower susceptibility, resulting in approximately equal numbers of tumours per mouse, and the conclusion that there are no sex differences. Absence of sex differences when dose is given according to individual body weight should no longer be assumed.

Apart from the *Kras2* locus, none of the minor genes has yet been identified. If the markers used here turn out to be distant from the actual *Pas* gene, then the effect of the gene on tumour counts will have been underestimated. On the other hand, the fact that these four genes plus the sex of the mouse account for over 75% of the variation suggests that there are no more major genes to be identified. Much of the remaining variation must be due to non-genetic factors, as there is still substantial inter-individual variation within an isogenic strain. It is not known whether the same or other loci and alleles will segregate in crosses between other strains.

What is the nature of the three minor *Pas* genes identified here? Their effects are of similar magnitude. Individually, they can approximately halve the number of tumours when a *Kras2* susceptible allele is present, and collectively they can reduce tumour numbers by about two thirds. Although their effects on lung tumours are quite small, they may well be coding for xenobiotic metabolizing enzymes, growth factors, oncogenes, or DNA repair enzymes which have an influence on susceptibility to other types of tumours. For example, *D9Mit11* appears to map to the same chromosomal region as the mouse homologue of the DNA mismatch repair gene hMLH1 (Bronner *et al.* 1994) which is known to be expressed in many tissues including the lung (Papadopoulos *et al.* 1994), and is associated with colon cancer. It is plausible, though highly speculative, that genetic variation at this locus could influence lung adenoma susceptibility in mice. With the ever increasing number of mapped markers in the mouse, accurate localization of the minor *Pas* loci should provide a reasonable chance that these loci can soon be identified.

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