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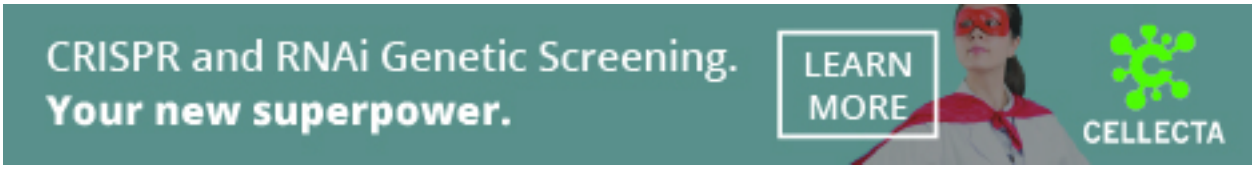

GENOME METHODS

Single Nucleotide Primer Extension: Quantitative Range, Variability, and Multiplex Analysis

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The quantitative measurement of transcription products from homologous alleles at a diploid locus has broad application for the study of mammalian gene expression. Single nucleotide primer extension (SNuPE) analysis is a simple and sensitive method for allelic transcript discrimination requiring only 1 bp difference between alleles. In this study the effective range, experimental variation, and the influences of poly(dT)-primed and gene-specific reverse transcriptions are characterized. The ability to analyze several genes from a single reverse transcription reaction is assessed as well. For the genes examined, the maximum range of detection is reached when the minor transcript represents 1/250 of the major allele. Relatively little error is seen within or between assays and linearity of response is maintained over an approximately thousandfold range.

Analysis of differential expression of maternal and paternal gene copies requires assays able to distinguish allelic transcription from each homolog. Several methods exist to quantitate the level of mRNA transcripts from cells or tissues. The most sensitive are quantitative PCR methods that utilize an internal standard compared to a sequence of interest (Gaudette et al. 1993). These methods depend on the analysis occurring in the logarithmic amplification phase of PCR, the conditions for which may vary between the standard and the gene of interest. Many components of the PCR, such as magnesium ion concentration and primer concentration, can substantially affect the data that are obtained from quantitative PCR.

Another method distinguishes homologous alleles amplified by PCR that differ at restriction sites. Digestion of radioactively labeled PCR amplified products distinguishes the two alleles (Kay et al. 1993). This technique limits analysis to polymorphisms that alter restriction sites and may have limited use for comparisons of organisms that do not maintain high degrees of polymorphism within the population such as inbred strains of mice.

RNase protection assays in conjunction with an internal standard have been used to quantitatively discriminate between different alleles (Kinloch et al. 1993). However, the method suffers from restriction to transcripts of relatively high abundance. Newer methods that couple PCR with RNase protection may alleviate the weak sensitivity of the RNase protection assay.

A third type of quantitative RNA analysis relies on the specificity of incorporation by DNA polymerase in a single nucleotide primer extension (SNuPE) reaction, as outlined in Figure 1. Reverse-transcribed RNA serves as a template for PCR amplification of a sequence of interest containing a single-base difference between two alleles. Each PCR-generated template is analyzed for the presence, absence, or relative amounts of each allele by annealing a primer that is 1 base 5′ to the polymorphism and extending by 1 radioactively labeled base. Only when the correct radionucleotide is available in the reaction will incorporation occur at the 3′ end of the primer. Extension products are electrophoresed on polyacrylamide gels and measured by autoradiographic methods. It is important to note that each sample is generated and processed identically until the primer extension step. Therefore, for an individual sample, the only point where experimental variation should have an impact on the result is at the final primer extension step itself. In studies to determine the relative abundance of two alleles of the same gene, each allele

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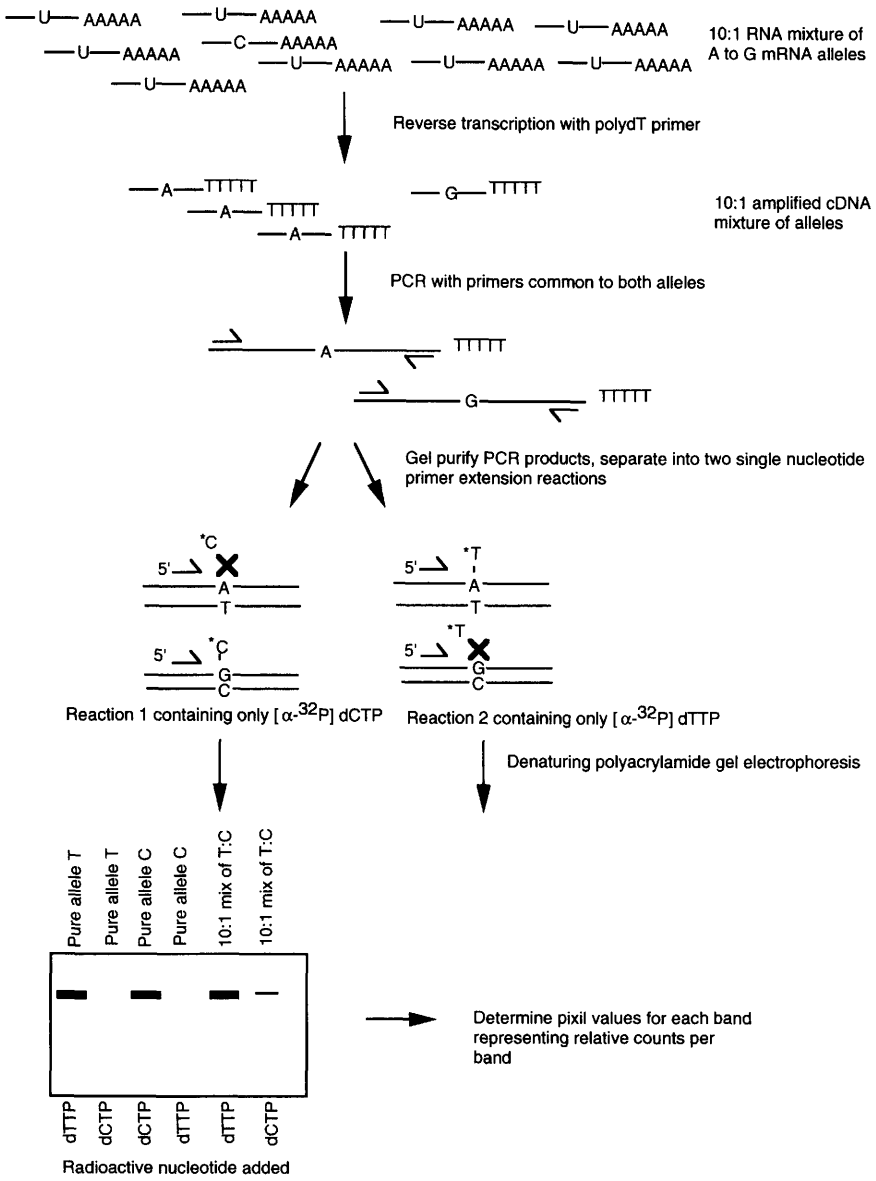

Figure 1 Schematic of a SNuPE assay for an mRNA containing one allele bearing a T and the other a C. RNA is reverse transcribed using a poly(dT) primer followed by PCR amplification with a primer pair recognizing both alleles. SNuPE follows with a primer common to both alleles terminating 1 base 5′ to the polymorphism. Extension reactions are carried out separately, one reaction adding dCTP as the only radioactive nucleotide and one reaction adding dTTP as the only radioactive nucleotide. Fragments are resolved by denaturing polyacrylamide gel electrophoresis, and the gels scanned using a phosphorescent screen and a phosphorescence detection system.

serves as an internal control for the other, obviating the requirement for an exogenous internal standard.

Singer-Sam et al. (1992a) were the first to use SNuPE for quantitation of transcripts from heterozygous alleles. The technique was shown to be extremely sensitive, amenable to processing large numbers of samples, and to require as little as 1 ng of input RNA. The SNuPE assay has been successfully extended to (1) the analysis of female mouse embryos for expression of paternal copies of the X-linked genes *Pgk-1* and hypoxanthine phosphoribosyl transferase (*Hprt*), (2) the quantitation of *Xist* expression levels in eight-cell stage female mouse embryos, and (3) the quantitation of the number of cells bearing mutations of the p53 gene in cells surrounding actinic keratoses (Singer-Sam et al. 1992b; Buzin et al. 1994; Ziegler et al. 1994).

The present study extends the analysis of the parameters affecting the SNuPE assay and estimates the range and quantitative response of the assay for three genes. The X-linked genes *Hprt*, *Mottled* (*Mo*, the mouse homolog of the Menkes gene), and ornithine transcarbamylase (*Otc*) are each tested for the detection limits, effective range, and reproducibility of their respective assays (Konecki et al. 1982; Veres et al. 1987; Levinson et al. 1994). The *Hprt* SNuPE assay is tested to compare the effects of poly(dT) versus gene-specific reverse transcription. Additionally, the results of the *Hprt* and *Otc* SNuPE assays are compared to RNase protection assays to show clearly the enhanced resolution of the former as compared to another commonly used technique. The findings demonstrate that the SNuPE assay remains linear over a large dilution range of allele ratios. Under several experimental conditions, the SNuPE assay shows little variation over its useful range and the results are highly concordant for different genes analyzed from the same reverse transcriptions.

RESULTS

Determination of Interspecific Polymorphisms

To distinguish different alleles by SNuPE, single-base polymorphisms between C57BL/6J(B6) and *Mus spretus* (spretus) were identified for each gene tested. Laboratory mouse strains and *M. spretus* are interbreeding species separated in evolution by ~3 million years and are known to maintain numerous sequence polymorphisms relative to each other (Silver 1994). Primers were chosen from published cDNA sequence to amplify and sequence the 3′ untranslated regions of *Hprt, Otc,* and *Mo* from both species. PCR primers for the SNuPE assay amplification were chosen to span intron/exon boundaries. In all cases, PCR primers were designed based on an annealing temperature of 55°C, lack of repeated sequence, and minimum primer length (Table 1). SNuPE primers were designed immediately 5′ of a polymorphism based on the highest possible annealing temperature, lack of repeated sequence, and minimum primer length.

Characterization of Sensitivity Range for SNuPE Assays

A series of preliminary experiments was performed to determine the optimal conditions for primer extension using the *Mo* SNuPE primer. Various parameters, including Mg^{2+} concentration, primer concentration, and gel-purified versus nonpurified SNuPE primer were tested. The best results were obtained with 1 mM Mg^{2+} and 0.1 μM gel-purified primer. Subsequent tests of the *Hprt* and *Otc* SNuPEs also performed well under these conditions. The effective range of the SNuPE assay was determined by diluting spretus RNA into B6 RNA at several dilutions. Total liver RNA from inbred B6 and spretus liver was quantitated by 260 nm UV absorption and diluted such that each sample contained 1 μg of RNA. Standard dilutions were made from working stocks of RNA diluted and requantitated by UV absorption each time a new series of dilutions was made. RNAs were diluted in three separate series. One series represented dilutions of B6/spretus RNAs in ratios of 1:1, 10:1, 50:1, 100:1, 250:1, 500:1, 750:1, and 1000:1 termed series A. A second group was diluted in similar ratios except the ratios represented spretus/B6 (series B). These extended ranges of ratios were used primarily to determine the sensitivity of the assays.

The third series represented B6/spretus dilutions of 1:10, 1:6, 1:4, 1:2, 1:1, 2:1, 4:1, 6:1, and 10:1 (series C). This narrow-range series was used to determine the linear response and sample-to-sample variation across the most robust dilution range. In addition to representing individual dilution range experiments, series A, B, and C RNA mixtures were prepared and reverse transcribed separately, thereby reducing the possibility of sample cross-contamination. In all cases, pure B6 RNA and spretus RNA alone served as controls for measuring misincorporation of the incorrect radioactive nucleotide.

Representative images of SNuPE assays performed on each dilution series are shown in Figure 2, A–C. In some experiments there was detectable misincorporation of the inappropriate radioactive nucleotide for each SNuPE reaction when a pure B6 or spretus template was used (Fig.

Table 1. PCR and SNuPE Primers for *Hprt, Mo,* and *Otc*

PCR primers	Primer sequence	Positions in published sequence	PCR product size	Polymorphism	Reference
OtcL	5'GTGGACAATCATGGCTGTCAT3'	997 to 1017	412 bp		Veres et al., 1987
OtcR	5'GTGTTTAATGTTTAGTGGAAGC3'	1409 to 1389			
HprtL	5'TACAGGCCAGACTTTGTTGGA3'	519 to 540	644 bp		Konecki et al., 1982
HprtR	5'GGGAAAATACAGCCAACACTG3'	1143 to 1163			
MoL	5'CAAGGAAAACAGTCAAGAGG3'	3968 to 3988	744 bp		Levinson et al., 1994
MoR	5'TCACTGTTCTCCCCTCTATATC3'	4680 to 4701			
SNuPE primers				B6 vs.spretus	
OtcSNuPE	5'CTCTCTTCAATTTACAAC3'	1306 to 1323		C to T	
HprtSNuPE	5'GCATGAACCTTCTATGAA3'	764 to 781		T to C	
MoSNuPE	5'CTAGTGAACCTGACAAGCA3'	4361 to 4379		C to T	

The *Otc, Hprt,* and *Mo* PCR primer pairs each span an intron and do not amplify genomic DNA. Annealing temperatures for all PCR primers are 55°C. All SNuPE primers terminate 1 base 5′ to the indicated polymorphism, and annealing temperatures are 42°C for *Otc,* 50°C for *Hprt,* and 56°C for *Mo.* SNuPE primers were gel purified to eliminate shorter products that could also anneal and extend. Primers were designed and annealing temperatures determined using Primer 3.0 (MIT).

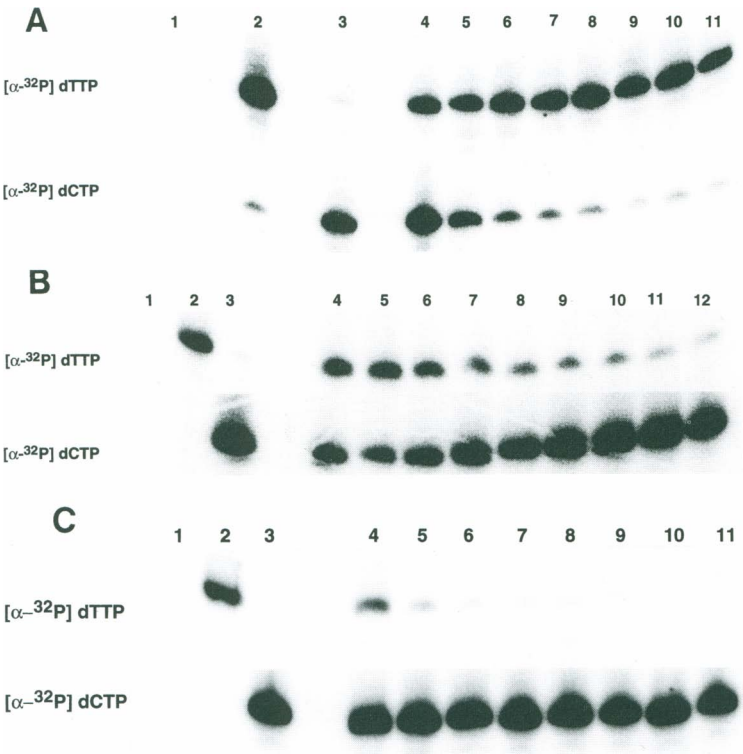

Figure 2 Representative images obtained from a phosphorescent event detector for *Hprt* SNuPE analysis. Aliquots (1 μg) of B6 and spretus liver RNAs were diluted to generate three series of dilutions incrementally, increasing or decreasing the relative amounts of B6 vs. spretus RNA. The B6 component represented between 1:1000 the total input RNA and 1:1 (series B), 0.1 and 10:1 (series C), and 1:1 and 1000:1 (series A). Each series was poly(dT) reverse transcribed, amplified for *Hprt*, and *Hprt* SNuPE analyzed. The B6 allele differs from the spretus allele by having a T instead of a C at position 782 of the cDNA. All ratios are expressed as B6/spretus allelic ratios. (*A*) SNuPE of 10 ng of gel-purified PCR products from series A. The *upper* gel represents the incorporation of dTTP, which will only extend in the presence of the B6 allele; the *bottom* gel represents incorporation of dCTP, which will only extend in the presence of the spretus allele. (Lane *1*) No template added; (lane *2*) B6 alone; (lane *3*) spretus template alone; (lane *4*) 1:1; (lane *5*) 10:1; (lane *6*) 50:1; (lane *7*) 100:1; (lane *8*) 250:1; (lane *9*) 500:1; (lane *10*) 750:1; (lane *11*) 1000:1. (*B*) Representative series C. (Lane *1*) No template; (lane *2*) B6 alone; (lane *3*) spretus alone; (lane *4*) 10:1; (lane *5*) 6:1; (lane *6*) 4:1; (lane *7*) 2:1; (lane *8*) 1:1; (lane *9*) 1:2; (lane *10*) 1:4; (lane *11*) 1:6; (lane *12*) 1:10. (*C*) Representative series B. (Lane *1*) No template; (lane *2*) B6 alone; (lane *3*) spretus alone; (lane *4*) 1:1; (lane *5*) 1:10; (lane *6*) 1:50; (lane *7*) 1:100; (lane *8*) 1:250; (lane *9*) 1:500; (lane *10*) 1:750; (lane *11*) 1:1000.

2A–C, cf. lanes 2 and 3). This represents the background contributed by *Taq* DNA polymerase error (see Methods for background adjustment calculations to compensate for this effect).

Because the misincorporation rate will determine the absolute range of each assay, the correct/incorrect nucleotide ratio was determined for pure B6 and spretus templates for all experiments. These values establish the boundaries beyond which the signal-to-noise ratio is too low for accurate data to be obtained. Following background adjustment and determination of the absolute range, the B6/spretus count ratios were graphed versus the input B6/spretus RNA ratios (Fig. 3).

The *Hprt* SNuPE assay remains linear across a range of 0.01–500. At a 500:1 dilution, the signal-to-noise ratio is still above the background incorporation determined during several repetitions of the assay. A somewhat lower sensitivity is seen at the other end of the range, as the 1:500 data point is not consistently above the incorrect nucleotide addition to spretus allele alone. Three complete repetitions of the experiment indicated that the interassay variation averaged 26% for the most robust portion of the curve (i.e., 0.01–100:1) but increased for more dilute samples. Most of the variation was contributed by one repetition of the assay for series B and A, for which all of the samples had slightly higher ratios. The greatest concordance was in the range from 0.1 to 100, with 20% variation at 100:1 and 5.8% at 6:1. The 10:1 data point varied relatively widely for all of the genes tested, indicating a possible dilution pipetting error or contamination that interfered with either the PCR amplification or the SNuPE extension. A best fit line was drawn from values between 0.01 and 100:1 representing the most robust portion of the curve. The slope of the line for *Hprt* was 0.8.

Total liver RNAs from three individual (B6 × spretus) F_1 hybrid female mice (BSp1, BSp2, and BSp3) were analyzed as test cases for determination of allelic expression ratios. RNA was reverse transcribed and analyzed for *Hprt* allelic expression using the same methods applied to the dilution series samples. Ratios obtained with animals BSp1, BSp2, and BSp3 were compared to the best fit line from the known dilution assays. The ratio values obtained from

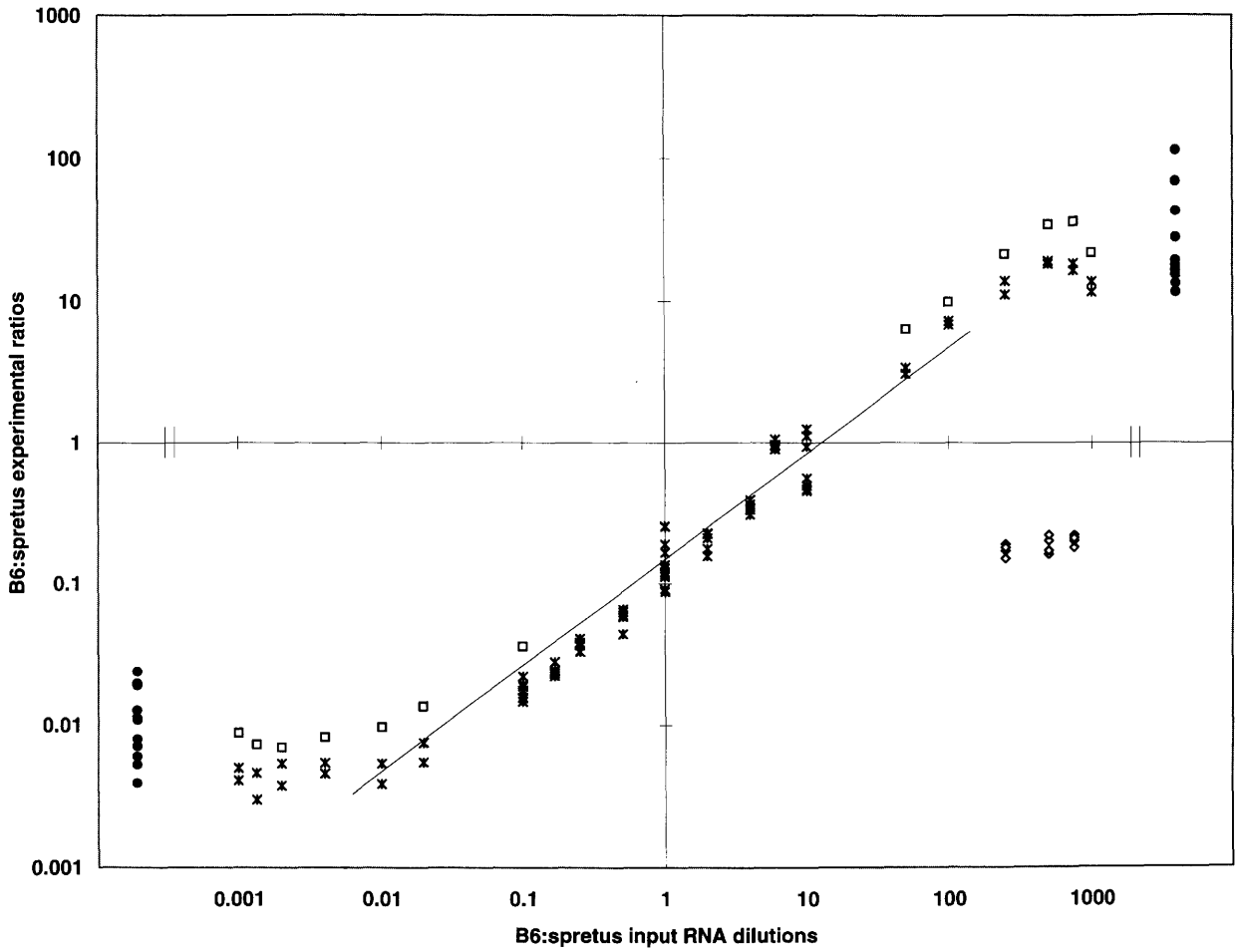

Figure 3 Graphic representation of the cumulative data for the three *Hprt* dilution series. (●) Values obtained using pure B6 or pure spretus RNA that define the absolute experimental range of the assay (double vertical lines on the *x*-axis denote that the absolute range values have no X coordinate). (□) Data points for a single experimental set where all values, including controls, were high with respect to the other repetitions. A best fit line for the data points from 0.01 to 100 is shown. Star symbols represent poly(dT) data points. (◇) The values for Bsp1, Bsp2, and Bsp3.

the standard curves gave B6/spretus allelic ratios of 1.7 ± 0.1, 1.6 ± 0.2, and 1.6 ± 0.1 for the three samples respectively. For each BSp mouse, sample assays were done in triplicate or more.

Poly(dT)-primed Reverse Transcription vs. Gene-specific RT–PCR

To test the effect of changes in reverse transcription conditions (i.e., primer, solutions, and transcriptases), series A dilutions were compared for poly(dT)-primed reverse transcription and gene-specific reverse transcription for *Hprt*. A separate series A set of dilutions was made, and reverse transcription was performed using a gene-specific primer, HprtR (Table 1) to prime cDNA synthesis. The subsequent amplification by PCR used the same primers as all previous experiments with *Hprt*. From a single set of PCR templates, triplicate SNuPE assays were done for the gene-specific RT–PCR. The linear response of the poly(dT) and gene-specific assays was evident in both cases (Fig. 4). However, the gene-specific RT–PCR had a somewhat broader range, as the signal-to-noise ratio remained higher for more dilute samples. This is similar to the findings of Singer-Sam et al. (1992a). Overall, the profiles of the assays were highly concordant. There was no large difference in standard error between (1) triplicate PCR with single SNuPE analysis of each amplification and (2) a single PCR and triplicate SNuPE. The gene-specific assay variation averaged 28%, with a minimum of 18% (at the 1000:1 dilution). The

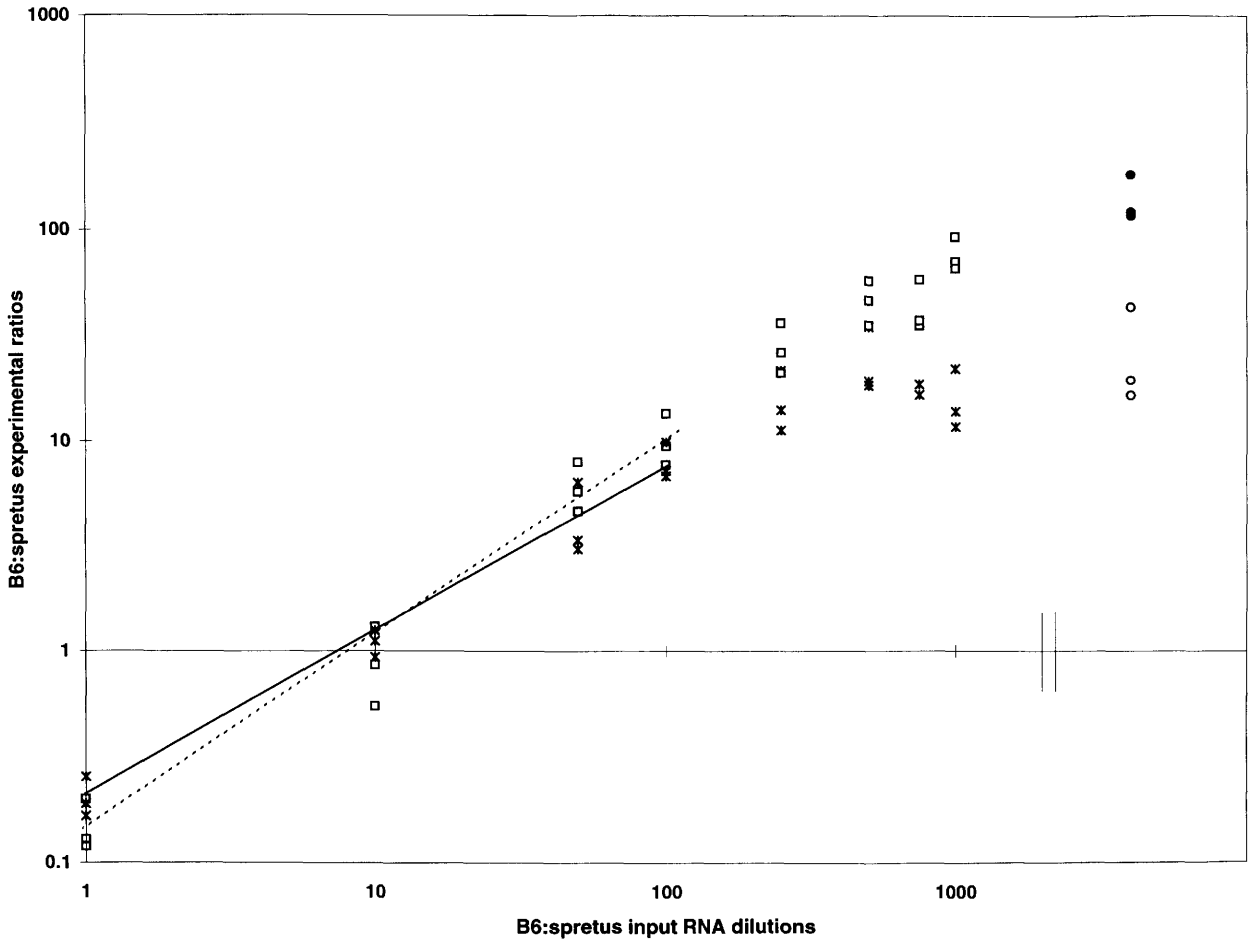

Figure 4 Comparison of poly(dT)-primed reverse transcriptions with AMV reverse transcriptase and HprtR-primed gene-specific reverse transcription with M-MLV. Poly(dT)-primed reverse transcriptions were PCR amplified for *Hprt* in triplicate and SNuPE analyzed. Gene-specific reverse transcriptions were SNuPE analyzed in triplicate. Star symbols represent poly(dT) data points. (□) Gene-specific reverse transcription data points. (○,●) Values obtained using pure B6 RNA that represent the absolute range for the poly(dT)-primed reverse transcription and gene-specific reverse transcription data sets, respectively. Best fit lines based on data points from 1 to 100 are shown.

slope of a best fit line for the most robust portion of the curve was 0.9.

Otc and *Mo* SNuPE Assays

To assess consistency of the assay between different genes from the same reverse transcriptions, SNuPE assays were performed for two additional X-linked genes: *Otc* and *Mo* (Fig. 5). The poly(dT)-primed reverse transcription series used to generate the *Hprt* data were also used for *Otc* and *Mo* analysis. Series B and C were PCR amplified once for *Otc*, with one subsequent SNuPE analysis of each sample. In processing, however, the 0.001 sample became contaminated with amplified product and was excluded from analysis. Series A was PCR amplified once and SNuPE analyzed in triplicate.

The profile of the assay is essentially identical to the *Hprt* analysis with a slope of a best fit line equal to 0.8. The range, variation, and overall profile of the assays were very similar to those established for *Hprt*. For series A of *Otc*, variation ranged from a minimum of 19% at the 1:1 dilution to 21% at the 750:1 dilution.

Mo was assayed with cDNA-specific primers Mo L and Mo R. Variation was 11% at a 2:1 dilution and 18% at a 6:1 dilution. Again the 10:1 sample varied the most of all samples. The 6:1 dilution became contaminated with amplified product and was excluded from further analysis. The absolute range and linear response in series B

was not as extensive and may represent a specific limitation of analysis for this gene. The slope of the best fit line was 0.8.

Hybrid liver RNAs from animals BSp1, BSp2, and BSp3 were also analyzed in triplicate for *Otc* and *Mo* from the same reverse transcriptions used to generate the *Hprt* SNuPE data. B6/spretus ratios were 2.0, 1.1, and 1.0 for Bsp1, Bsp2, and Bsp3, respectively, for the *Mo* assay. For *Otc*, the ratios were 0.6 ± 0.2, 0.5 ± 0.3, and 0.5 ± 0.2 for BSp1, BSp2, and BSp3, respectively.

RNase Protection

As a direct comparison, RNA dilutions similar to series A and C were made to test the effective range of standard RNase protection assays. Antisense riboprobes representing base pairs 469–754 of the *Hprt* cDNA and 187–442 of the *Otc* cDNA were used to distinguish B6 from spretus alleles. Note that these probes recognize different polymorphisms from the ones detected by SNuPE for the same genes. Protected fragments were resolved by polyacrylamide gel electrophoresis (Fig. 6A,B). For *Hprt*, at a 50:1 dilution, the spretus signal is no longer conspicuous (Fig. 6C). Both *Hprt* and *Otc* were linear in the effective range between 0.1 and 10, although *Otc* demonstrated a less consistent response beyond the 4:1 dilution. The respective slopes of the best fit lines were 0.7 for *Hprt* and 0.4 for *Otc*. To assess differences in the quality of the sample RNAs, Northern blot hybridization analysis was performed on a parallel dilution series prepared identically to those used for the RNase protection experiment. RNA hybridization signal across all of the diluted samples was equivalent when probed with the *Otc* and *Hprt* PCR products; however, a slightly higher degree of hybridization was seen in the pure B6 sample. The B6 and spretus samples

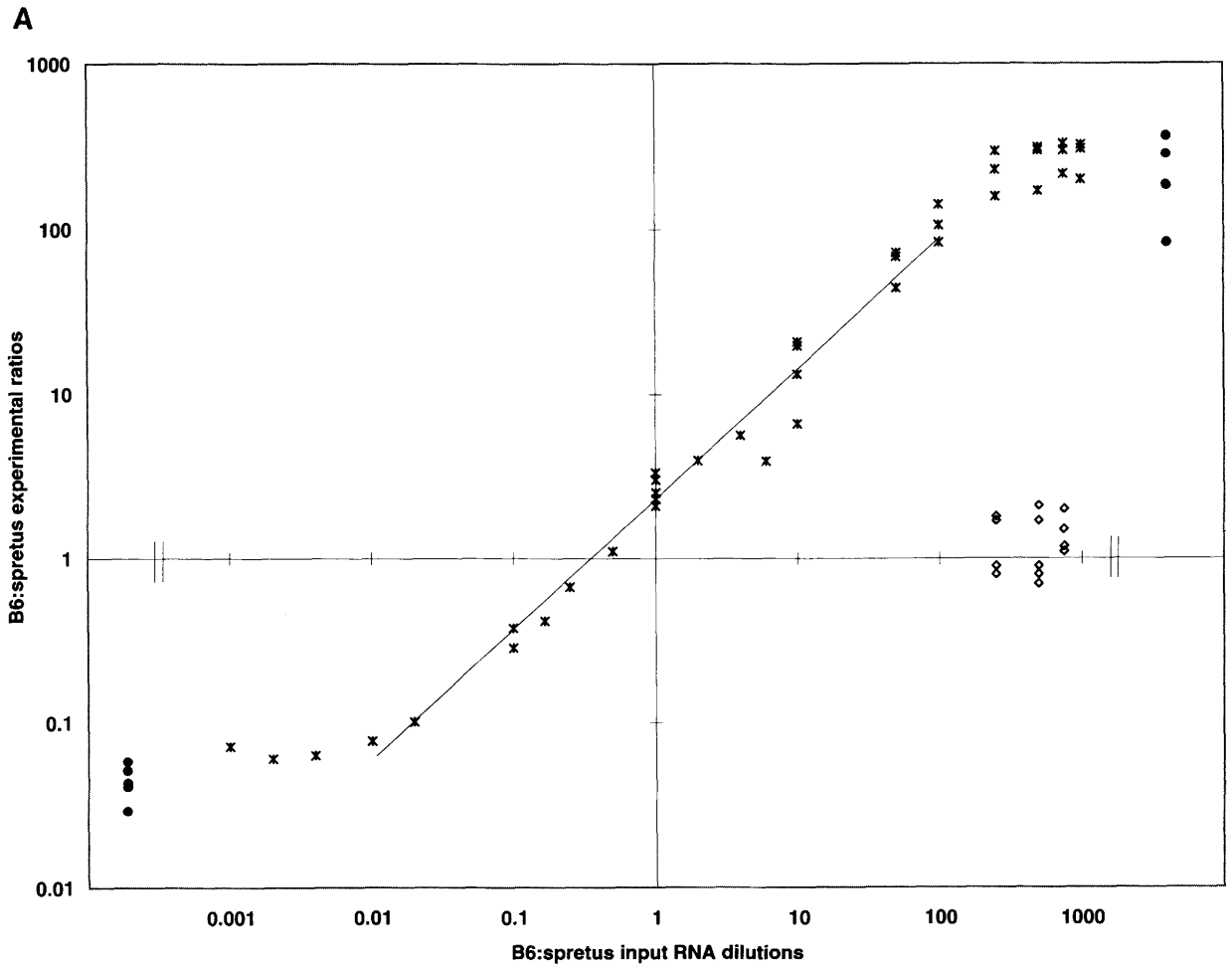

Figure 5 (*See facing page for* B *and legend.*)

showed minimal degradation and appeared to be equivalent by ethidium bromide staining (data not shown).

DISCUSSION

The single-nucleotide primer extension is a very sensitive and reproducible method for determination of relative amounts of allelic transcripts in complex mRNA mixtures. The effective range of the *Hprt* assay spanned a B6/spretus ratio of 0.01 to 250:1 using total RNA reverse-transcribed samples. The variation between replicate assays was low but increased, as expected, at higher dilutions where the signal of the diluted allele approached the value of the misincorporation background. The SNuPE variation among triplicate PCR amplifications ranged from 5.8% for the 6:1 dilution to 20% at 100:1 in the most robust portion of the *Hprt* curve. Single PCR reactions assayed by SNuPE in triplicate demonstrated similar variation, indicating that both the amplification and extension steps contribute little variation to the final results. It should also be noted that series A, B, and C were each reversed transcribed separately. This difference contributed no apparent observable intra-assay variation even though the RNA used in generating the dilution series was only quantitated by spectrophotometry. Overestimation or underestimation of

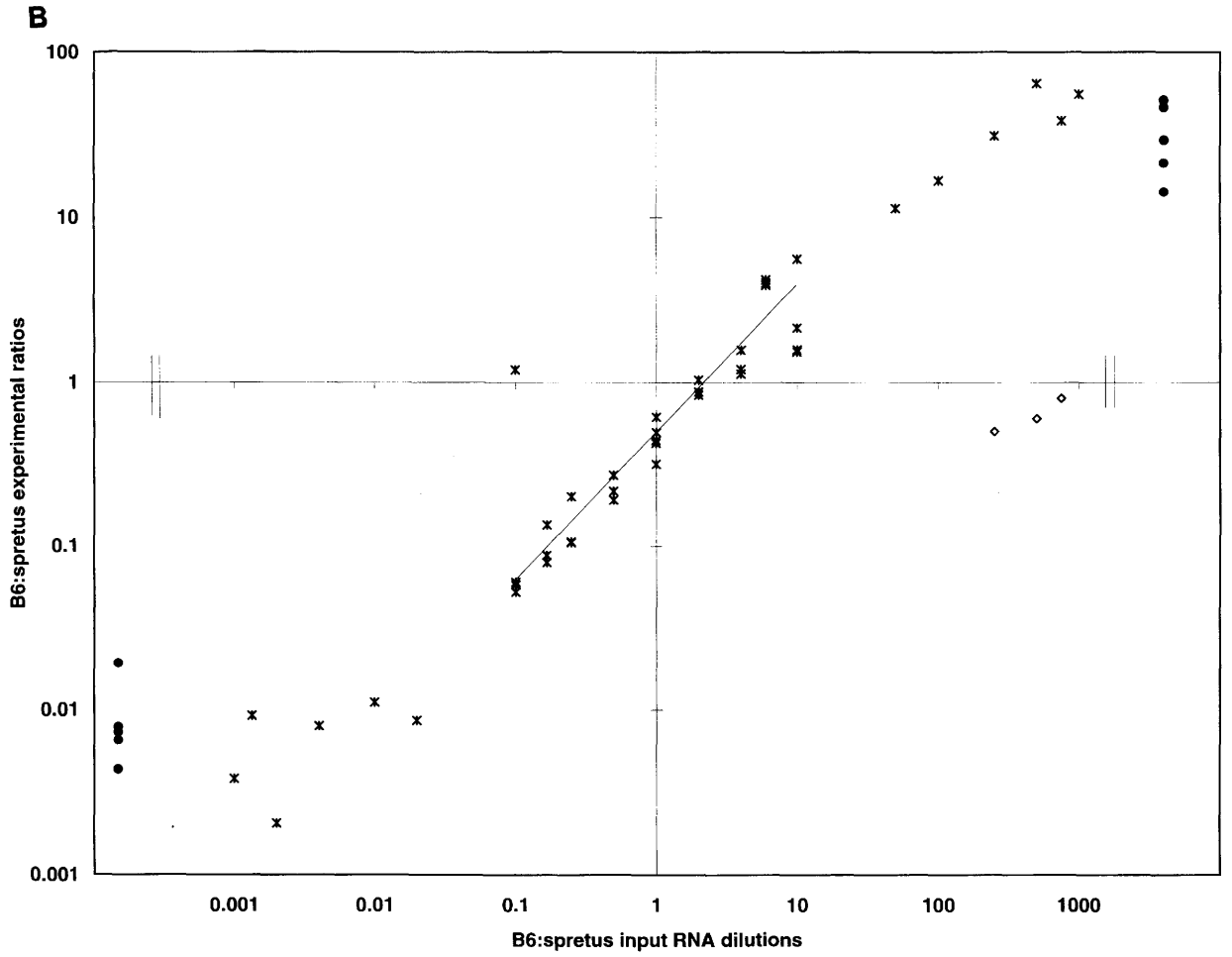

Figure 5 *Mo* and *Otc* quantitative ratio analysis. The poly(dT)-primed reverse transcriptions of series A, B, and C used in generating the *Hprt* data were PCR amplified and SNuPE analyzed for the *Otc* and *Mo* genes. (◇) Allelic ratios for hybrid animals Bsp1, Bsp2, and Bsp3 (*A,B*). (*A*) *Otc* data: Star symbols represent the dilution series data. (●) The absolute range determined in each experiment. Series A was SNuPE analyzed in triplicate. A best fit line is drawn through the 0.01 to 100:1 data points. (*B*) *Mo* data: Star symbols represent the dilution series data. (●) The absolute range determined in each experiment. Series C was done in triplicate. A best fit line is drawn through the 0.1 to 10 data points.

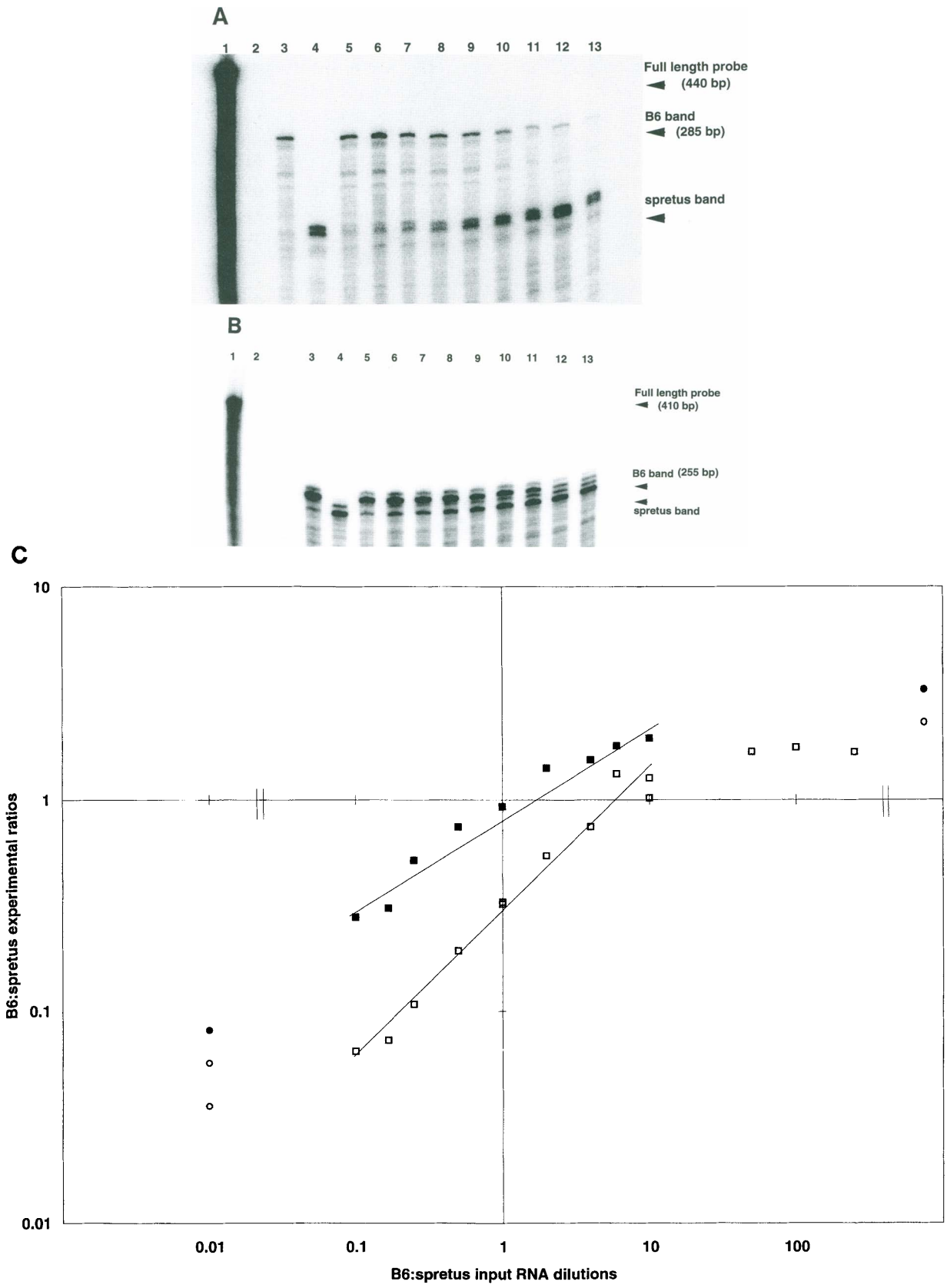

Figure 6 (*See facing page for legend.*)

one RNA species at the initial dilution would bias the range in favor of one allele. Because small dilution errors will have a maximum effect at the highest dilution, the useful range of the assays can be considered conservative.

One parameter that influences the effective range of the assay is the method employed for reverse transcription. The parameters that change between the two most common methods were the use of a gene-specific primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase versus poly(dT) oligonucleotides and AMV reverse transcriptase. Gene-specific reverse transcription of *Hprt* resulted in SNuPE values that remained linear to a dilution of 1000:1. The interassay SNuPE variation, however, was similar to results from poly(dT)-primed reverse transcription. The increased range provided by gene-specific analysis may be useful in situations where one gene is being analyzed for very low levels of allelic transcript variation. Alternatively, if several genes are to be tested, it is preferable to use poly(dT)-primed reverse transcription to minimize the variation in reagents and handling between genes.

As an interassay comparison, standard RNase protection assays for *Hprt* and *Otc* were compared to their respective SNuPE assays. For both *Hprt* and *Otc* SNuPE assays the range from 0.1 to 10 was consistent in reproducibility; however, unlike *Hprt*, the *Otc* dilutions from 4:1 to 10:1 began to deviate from linearity. The RNase protection effective range for *Hprt* did not exceed 50:1, representing a 10-fold decrease in range between SNuPE and RNase protection. As a practical point it is important to note that the RNase protection assays require 5–10 μg of total RNA for a single assay, whereas the SNuPE assay can begin with 1 μg of RNA for reverse transcription and generate 20–40 replicate assays.

Allelic bias was evident in a template-specific manner for each of the genes assayed. *Hprt, Otc*, and *Mo* exhibited a characteristic skewing of the line from a perfect *y*-axis intercept of zero. The *Otc* data favored the B6 allele by over twofold, *Hprt* favored the spretus allele by about ninefold, and *Mo* favored the spretus allele twofold. Because the genes were amplified from the same reverse transcriptions and skewing favored both B6 and spretus in a consistent fashion, it seems unlikely that simple handling error explains the deviations.

One explanation is that the level of transcription for each homologous allele of a gene may vary per unit of total RNA, leading to preferential detection of one allele versus the other. To address this possibility, additional experiments were performed. Both RNA and DNA were prepared from the combined fragments of female B6 and male spretus liver homogenized in the same tube. A SNuPE assay was performed on the genomic DNA derived from this mixed sample to determine the genome equivalents of each allele. For *Hprt*, comparison of the nuclear equivalents of DNA versus the RNA ratios indicated that the deviation from a perfect zero *y*-intercept is the result of a 9- to 10-fold higher transcription level of the spretus allele (data not shown). This is corroborated by the skew of the data in favor of the spretus *Hprt* allele of the RNase protection results, although it was somewhat less pronounced (Fig. 6C). The difference in expression of the two alleles did not entirely explain the skew at *Otc* in favor of B6, however. The RNase protection experiments, although less sensitive, had a *y*-intercept close to zero. The lack of concordance observed by these two methods indicates that the reverse transcription and/or the PCR reaction is biased in favor of the B6 allele for this assay. These alternatives have not been investigated further.

Quantitative and qualitative data can be ob-

Figure 6 RNase protection analysis for the *Hprt* and *Otc* genes. Spretus total liver RNA was diluted into B6 total liver RNA within a range of 0.1 to 10:1 for B6 and spretus, respectively. Ten micrograms of RNA was hybridized to a *Hprt* cDNA or *Otc* cDNA subclone, digested with an RNase A/T1 mixture, and the fragments were resolved on 5% denaturing polyacrylamide gels. Representative images are shown in *A* and all RNA ratios given as B6/spretus ratios. (*A*) *Hprt* RNase protection. (Lane *1*) Full-length probe; (lane *2*) Yeast tRNA, probe, and RNase A/T1; (lane *3*) 1:0; (lane *4*) 0:1; (lane *5*) 10:1; (lane *6*) 6:1; (lane *7*) 4:1; (lane *8*) 2:1; (lane *9*) 1:1; (lane *10*) 1:2; (lane *11*) 1:4; (lane *12*) 1:6; (lane *13*) 1:10. (*B*) A dilution series made in parallel was tested using an *Otc* probe. Dilution ratios are the same as in *A*. (*C*) Graphic representation of the *Otc* series C, *Hprt* series C, and *Hprt* series A B6/spretus ratios vs. the B6/spretus input RNA dilutions. (■) *Otc* data points; (□) *Hprt* data points; (●,○) Values obtained using pure B6 and pure spretus RNA that define the RNase protection assay absolute range for *Otc* and *Hprt*, respectively. Best fit lines are drawn through the 0.1 to 10 range.

tained regardless of the *y*-intercept skewing. However, it should be noted that the skewing effect was not equivalent in the F_1 hybrid animals and may reflect transcriptional differences in an interspecific hybrid genetic background. Taking the skew into consideration, the animals Bsp1, Bsp2, and Bsp3 give B6/spretus allelic ratio values of 0.17, 0.16, and 0.16 for *Hprt*, 1.0, 0.6, and 0.5 for *Mo*, and 0.2, 0.18, and 0.18 for *Otc*. The *Mo* ratios are substantially higher than *Hprt* or *Otc*, which may reflect the decreased linear response of the assay with respect to the other assays. For investigations of small qualitative changes in transcription from two homologous alleles, that is, loss of genomic imprinting, such minor deviations should have little impact (Feinberg 1993).

The SNuPE assay can be applied to the investigation of transcriptional differences between homologous loci in the same genome. The differential expression of homologous alleles has been observed in several mammalian genetic systems, including X chromosome inactivation, genomic imprinting, allelic exclusion, and RNA and DNA changes associated with cancer. The large effective range and low variability of SNuPE should make it possible to analyze early stages of these phenomenon in a sensitive manner. Additionally, small changes in allelic transcription in response to stimulus or time can be observed and monitored for multiple samples from multiple individuals. Thus, profiles of the transcriptional histories for specific alleles of genes will now be more accessible.

METHODS

RNA Preparation

Total Liver RNA was prepared using the RNAzol B protocol (Tel-Test, Inc.) from C57BL/6J(B6), *M. spretus/Ei* and (B6 × spretus) F_1 hybrid female mice (Jackson Laboratories, Bar Harbor, ME). RNA was stored in two aliquots: (1) stock aliquot in diethyl pyrocarbonate-treated ddH_2O was stored at −20°C, and (2) long-term storage as a suspension in 100% EtOH at −20°C.

RNase Protection

The RNase protection assay was done according to the protocols provided by the Ambion RPAII kit (Ambion, Inc., Texas) The RNA probes used were prepared from a subclone of the C57BL/6J *Hprt* cDNA from position 469 to 754 cloned into pBluescript SK(−) and a subclone of *Otc* from 187 to 442 subcloned into the same vector. Antisense riboprobe synthesis was performed with ~300 ng of DNA in a buffer containing 0.04 M Tris-HCl (pH 8), 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, with 10 mM ATP, 10 mM CTP, 10 mM GTP, 10 mM NaCl, 20 units of RNasin (GIBCO–BRL), 15 units of T3 RNA Polymerase (Promega), and 50 μCi of [α-^{32}P]UTP (Amersham). The reactions were incubated at 39°C 1 hr followed by the addition of 20 units of RNase-free DNase I (GIBCO–BRL) and incubation for 15 min at 39°C. The probes were gel purified according to Ambion protocols. Determination of the probe radioactive incorporation (counts per minute) was by liquid scintillation counting. For each RNase protection, 1×10^5 cpm was added per reaction. A gel-fixing step in 5% methanol, 5% acetic acid, followed by drying under a vacuum at 80°C for 2–3 hr was included to improve the autoradiographic resolution of protected fragments.

Sequencing

PCR primers were designed using the Primer 3.0 program and published data from the two 3′-most exons and 3′-untranslated regions of *Otc, Hprt*, and *Mo* (Table 1). *Hprt, Otc*, and *Mo* primers span one or more introns (Table 1). PCR products were directly sequenced with the primers used to generate the PCR products. Primers were end labeled with [γ-^{33}P]dATP (Amersham) and sequenced according to the protocols of a double-stranded DNA cycle sequencing kit (GIBCO–BRL)

Reverse Transcription

Poly(dT)-primed reverse transcriptions for *Hprt, Otc*, and *Mo* were performed as described in Ausubel et al. (1991), except they were scaled down to 1 μg of input RNA. Twenty units of AMV reverse transcriptase (Molecular Genetic Resources) was diluted 10-fold in 10% glycerol, 10 mM potassium phosphate (pH 7.4), 0.2% Triton X-100, and 2 mM DTT and placed on ice for 30 min prior to addition to the reactions as recommended by the supplier.

Gene-specific reverse transcriptions for *Hprt* were performed using 1 μg of total liver RNA reverse transcribed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 2 U/μl of RNasin (GIBCO–BRL), 0.1 μM oligomer (HprtR), and 0.125 U/μl of M-MLV reverse transcriptase (BRL) in 20-μl reactions. Reactions were incubated at 42°C for 15 min, heat inactivated at 95°C for 5 min, and diluted to 100 μl with a master mix of (10 mM Tris-HCl (pH 8.3), 1 mM NH_4Cl, 1.5 mM $MgCl_2$, 100 mM KCl), 0.125 mM dNTPs, 10 ng/μl of HprtL and HprtR, and 0.75 units of *Taq* DNA polymerase (BRL), in preparation for PCR amplification.

PCR

PCR for each gene was performed with gene-specific primers spanning a known intron/exon boundary (see Table 1). All PCRs were done as 20μl reactions containing 10 mM Tris-HCl (pH 8.3), 1 mM NH_4Cl, 1.5 mM $MgCl_2$, and 100 mM KCl, 0.125 mM dNTPs, 10 ng/μl of each primer, and 0.75 units per reaction of *Taq* DNA polymerase (BRL). Cycling parameters were 94°C preheating step for 5 min followed by 94°C denaturing step for 1 min, 55°C annealing step for 2 min, and 72°C extension for 30 sec (or 1 min for *Mo*), with a 10-min 72°C final extension. *Hprt* and *Otc* were

amplified for 30 cycles in a 480 Thermal Cycler (Perkin Elmer). *Mo* was amplified for 40 cycles.

Purification of Templates

PCR products were gel purified as described by Zhen and Swank (1993). Briefly, PCR products were resolved on 1% agarose gels run in 0.04 M Tris-acetate, 0.001 M EDTA (1× TAE) buffer and stained ethidium bromide while visualizing with a UV light source. A trough was cut just in front of the band of interest and filled with 50–200 μl of 10% PEG in 1× TAE buffer. Electrophoresis was continued with continuous observation until the band had completely entered the trough. The contents of each trough were removed, phenol extracted, chloroform extracted, and precipitated in 0.1 vol of 7.5 M ammonium acetate and 2.5 vol of 100% EtOH. Samples were washed with 75% EtOH and dried under vacuum briefly. Quantitation of DNA yield was done by electrophoresis of a small aliquot on a 1% agarose gel in 1× TBE buffer with ethidium bromide staining and comparison to a known amount of size standard DNA.

Gel Purification of Primers Used in Primer Extensions

Primers used in the extension step were purified prior to use in SNuPE assays. Unlabeled primers were resolved on a 19% denaturing polyacrylamide gel with 250 μg of each primer formamide denatured prior to loading for each gel. The fluorescently stained band corresponding to the correct size species was cut from the gel and placed in a buffer containing 0.5 M ammonium acetate and 1 mM EDTA. The gel slices were incubated at 37°C overnight. Samples were centrifuged at 10,000 rpm for 10 min. Supernatants were loaded into a 5-ml syringe and filtered through a 0.45-μm filter. Samples were loaded onto a G-50 Sephadex column plugged at the bottom with glass wool and suspended in 50 mM triethanolamine (TEA). Six fractions were collected. Fractions containing DNA, as determined by spectrophotometry, were dried under vacuum and resuspended at a concentration of 1 μM.

Primer Extensions

Single nucleotide primer extensions were performed as in Singer-Sam et al. (1992a) except that 1 mM Mg^{2+}, 0.1 μM primer, and 0.05 μCi of DNA radionucleotide final concentration was used in each reaction. After primer extension, one-fifth volume of loading dye (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 mM EDTA) was added, and the entire sample electrophoresed in a 15% denaturing polyacrylamide gel. Gels were fixed in 10% methanol, 10% glacial acetic acid, and 10% glycerol for 35 min with agitation followed by transfer to a 10% glycerol solution and further soaking for 10 min. Gels were then placed between two pieces of cellophane presoaked in 10% glycerol. The gels and cellophane were sandwiched between two sheets of 3MM Whatman paper and dried for 3–5 hr under vacuum at 55°C.

Data Analysis

Dried gels from the RNase protection and SNuPE experiments were exposed to a Phosphorscreen (Molecular Dynamics) overnight for the RNase protection and 3–4 hr for SNuPE gels. The screen was then scanned into a 400E PhosphorImager (Molecular Dynamics), and values for signal intensities determined by the ImageQuant software version 3.3 (Molecular Dynamics). Each band on a scanned gel image was circumscribed with an equivalent size box. The volume, or number of radioactive events within the box, was then integrated and the values displayed as a table. The volume integration results were transferred to Microsoft Excel 5.0 for analysis. The ratio of misincorporated to correctly incorporated nucleotide was determined for pure B6 and spretus samples, respectively. This ratio was used to adjust the values obtained for dilutions series samples by the following formula: Corrected value $V = V_x (1 - V_y/V_w)$, where V_x is the raw count for incorporation of the correct base, V_y is the raw count of misincorporated signal in the control reaction, and V_w is the raw count for the correctly incorporated signal in the control reaction. The ratio of the B6/spretus signals was calculated using the corrected values and graphed.

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