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Allele-specific quantification of *Drosophila* engrailed and invected transcripts

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ABSTRACT **Changes in levels of transcription can be difficult to gauge in animals with lethal mutations. For example, mutations in a regulatory region of an essential gene can have secondary consequences that complicate attempts to quantify the transcripts produced by the mutant gene. We describe a method that circumvents this problem by revealing the relative amount of transcript produced from each allele in a heterozygote. With this method, recessive lethal mutations can be analyzed in animals that are phenotypically wild type. We used this technique to analyze mutations in the regulatory region of the *Drosophila* engrailed gene and found that truncations reduce transcription to levels that depend both upon the tissue and upon the location of the chromosomal break. We also found that these mutations affect expression of the linked invected gene, suggesting that engrailed and invected share a complex set of regulatory elements that operate over at least 85 kb. We suggest that this technique will have general utility for the quantitation of allele-specific transcripts, even when amounts of tissue are limiting.**

Genes that are present in two copies in diploid organisms are assumed to contribute equally to RNA production. However, exceptions can arise in the course of normal development [e.g., allelic exclusion during the maturation of the immune (1) and olfactory (2) systems and parental imprinting (3)] or when mutations alter promoter utilization or RNA stability. Although there has been increasing interest in documenting such variations, the available methods for accurate and sensitive quantitation do not detect subtle changes. Northern blotting, RNase protection, and quantitative PCR can measure total transcript levels, but the crude comparisons they provide depend upon normalizing data to unrelated reference transcript abundance (4). However, reference transcripts can vary in different tissues or with changing growth conditions. Furthermore, since Northern blotting and RNase protection cannot, in general, distinguish transcripts contributed by each gene in a heterozygote, use of these methods has been limited mostly to studies of homozygous mutants. As a consequence, lethal mutations have been largely precluded from analysis.

Interest in measuring the relative abundance of transcripts in heterozygous human tissues and cells has increased as more mutant genes have been identified and isolated. An example is the myotonin–protein kinase gene, in which expansions of a repetitive trinucleotide sequence are associated with myotonic muscular dystrophy (5–7). Efforts to determine whether the level of myotonin–protein kinase gene transcripts is altered in heterozygotes carrying such an autosomal dominant mutation have been frustrated by the inadequacy of the available methods for analysis. Indeed, the use of quantitative reverse transcription–PCR to compare expression levels by two groups led to contradictory conclusions (8–11).

In this report, we describe a new method that yields a relative measure of transcript accumulation from heterozygous genes without standardization to a reference transcript. The rationale of the method is as follows. In a cell that has two different copies of an actively transcribed gene, the RNA pool should contain transcripts from each copy in proportion to their relative rate of synthesis and half-life. If the transcripts from the different copies can be distinguished by their size or sequence, then resolving such differences provides a measure of their relative abundance. Our method, termed allele-specific amplification (ASA), uses simple sequence polymorphisms to monitor the relative amounts of transcript produced by each of two alleles in a heterozygote. mRNA isolated from small amounts of tissue and used as a template for reverse transcription provided the template for PCR amplification of sequences chosen to include a relevant polymorphism and to span an intron. Sequence analysis of the two classes of DNA product yielded a quantitative measure of relative transcript abundance (Fig. 1).

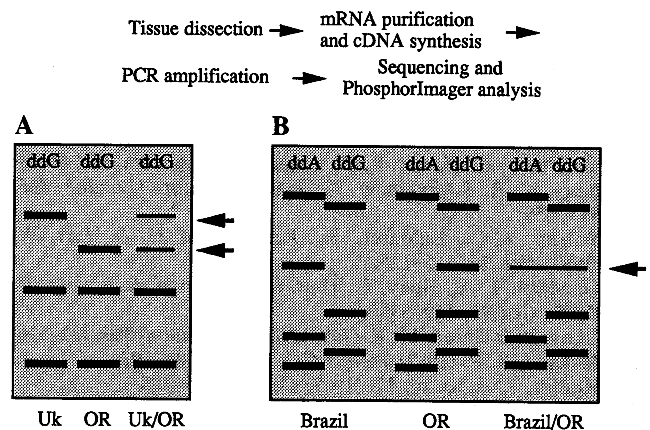

FIG. 1. Procedure for allele-specific quantification of transcripts. Tissue is dissected and mRNA is purified for first-strand cDNA synthesis and PCR amplification of engrailed or invected exons. The coamplification of cDNA derived from the mutant (M) and wild-type (WT) alleles removes the need to carefully calibrate PCR amplifications with reference transcripts. PCR primers span the polymorphism and an intron to allow the discrimination of transcripts and to avoid genomic DNA amplification. Polymorphisms revealed by sequencing of the PCR product and representing each transcript are quantified following gel electrophoresis by PhosphorImager analysis. (*A*) Hypothetical representation of gel bands obtained from a single sequencing reaction with the chain terminator dideoxyguanosine triphosphate (ddG) of engrailed cDNA derived from Ukraine (Uk) or Oregon-R (OR) homozygotes or heterozygotes (Uk/OR). The insertion polymorphism in the Ukraine cDNA produces a shift as indicated by the arrows. (*B*) Hypothetical representation of the invected cDNA sequence from Brazil or Oregon-R homozygotes or the heterozygote (Brazil/OR) containing a single base change polymorphism (arrow). Two sequencing lanes (ddG) and (ddA) for each cDNA preparation illustrate the polymorphism.

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Abbreviations: ASA, allele-specific amplification; M, mutant; WT, wild type; ddN, dideoxynucleoside triphosphate.

MATERIALS AND METHODS

Genomic DNA Amplification. Individual flies were homogenized in 100 μl of 6 M NaI (Bio 101) with a 1.5-ml Teflon pestle, and DNA was purified by binding to glass particles (Geneclean, Bio 101). DNA was eluted from the glass into 20 μl of water, 1 μl of which was used as a template for PCR amplification with exon-specific oligonucleotides.

***Drosophila* Crosses, mRNA Purification, and cDNA Synthesis.** Heterozygote male flies with the engrailed (*en*) breakpoint/Black cells (*Bc*) genotype were crossed to Ukrainian WT females and breakpoint/Ukraine larvae were identified by the absence of the Bc phenotype. Approximately 1–3 μg of tissue was dissected from late third instar larvae and lysed on poly(U) paper (2 mm^2; Hybond-mAP, Amersham) in the presence of 4 M guanidinium isothiocyanate/10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.5 M LiCl. mRNA is stable on this paper for at least 2 days at 4°C. After it had been washed two times with 200 μl of 0.5 M LiCl and two times with 70% EtOH, the poly(U) paper was briefly air dried, added to 10 μl of water, and heated for 10 min at 70°C. cDNA was prepared using Superscript II according to the manufacturer's (BRL) instructions. Reverse-transcription reactions were performed without the addition of oligo(dT) as the poly(U) bound to the Hybond-mAP paper served as an effective primer. Incubations were for 30 min at 42°C, 30 min at 45°C, and 10 min at 75°C.

PCR Amplification. Amplified products from engrailed and invected cDNAs were prepared as follows. Due to the lower efficiency with which the engrailed cDNA was amplified, a second amplification step (semi-nested PCR) was performed. engrailed cDNA (1 μl) was amplified in a 10-μl PCR reaction mixture containing final concentrations of 15 mM Tris·HCl at pH 8.8, 2.5 mM $MgCl_2$, 60 mM KCl, 0.4 mM dNTPs, 0.4 mM primer oligonucledotides [5′-ATCGTCCCAGCTCAGGAC-3′ and 5′-GTTGCTCGTTAACATTTCGC-3′ (nt 1151–1528 and 2332–2351, respectively)], and 0.4 unit of *Taq* DNA polymerase. Conditions for PCR were as follows: 30 cycles of 30 sec at 94°C, 30 sec at 65°C, and 60 sec at 72°C. A 1-μl aliquot of the primary amplification product was added to a 25-μl PCR reaction mixture with the primers 5′-TTGGCCCGCCTCAAGCGG-3′ and 5′-GTTGCTCGTTAACATTTCGC-3′ (nt 1609–1626 and 2332–2351, respectively). Both forward PCR primers were designed to span introns to avoid genomic DNA amplification.

invected cDNA was amplified in a 25-μl reaction mixture containing final concentrations of 20 mM Tris·HCl at pH 8.3, 1 mM $MgCl_2$, 50 mM KCl, 0.4 mM dNTPs, and 0.4 mM of primer oligonucleotides [5′-CAACTGCCTCCCTCCACTGG-3′ and 5′-TGGACGTCGCCGGCTTCTTGG-3′ (nt 772–791 and 1613–1633, respectively)]. PCR cycles were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. All invected PCR products spanned intron 2 and were sequenced with ddG and ddA to reveal polymorphisms and to allow quantitative analysis of the transcripts.

DNA Sequencing. The engrailed PCR product was purified (Geneclean) following agarose gel electrophoresis and directly sequenced. Template DNA (200 ng) and primer [200 ng; 5′-TACTTTCGGCTATCCAGG-3′ (nt 2094–2111)] were mixed in a total volume of 4.5 μl of H_2O containing 10% (vol/vol) dimethyl sulfoxide. DNA was denatured at 94°C for 2 min and then quickly frozen on dry ice. Labeling reactions were performed with Sequenase according to the manufacturer's (United States Biochemical) instructions with the following modifications. Labeling reactions included 10% dimethyl sulfoxide and were terminated after 2 min with 5 μl of ddC containing 10% dimethyl sulfoxide. The invected PCR product from crosses of en^{49}, en^{63}, en^{59}, and en^{2} with the WT were sequenced with the primer 5′-TCCCAGACCCAATGCCGTGGC-3′ (nt 1088–1108). PCR products from crosses of en^{52}, en^{61}, and en^{67} with Brazil WT flies were sequenced with the primer 5′-ACCGGATGCTCCTGTGGCACC-3′ (nt 1486–1506).

Transcript Quantification. To quantify the relative abundance of transcripts, amplified products of M and WT mRNA preparations were fractionated and sequenced in standard sequencing gels. Gels were fixed and then exposed overnight to a Molecular Dynamics Phosphor screen. Data were obtained by scanning the exposed screen with a Molecular Dynamics PhosphorImager equipped with IMAGEQUANT Version 3.3 software.

The ddG-terminated sequence at position 2017 of engrailed was quantified as follows: The position of nucleotide 2017 derived from M and Ukraine (WT) mRNA is separated by six bases when sequenced from the 3′ side of each cDNA due to the presence of six additional nucleotides in the WT transcript. The relative abundance of these transcripts can be determined directly from the specific bands on a DNA-sequencing gel by calculating the average pixel value in the bands, subtracting background, and determining the area of the bands by integration. Expression of the M transcript was calculated as a percentage of the WT from the ratio of M and WT bands. Since the values for M and WT reflect the synthetic products from a single sequencing reaction after amplification of the same RNA preparation, controlling for variations in reaction conditions or loading efficiency was unnecessary.

For the analysis of invected transcripts, each assay involved two sequencing lanes, so it was necessary to compensate for variations in loading as follows. Corrections to differences in loading were made by normalizing the values of experimental bands in each lane to selected reference bands. For the crosses with en^{49}, en^{63}, en^{59}, and en^{2}, polymorphisms caused by C → T transitions at positions 826 and 834 gave rise to reciprocal changes in the amount of DNA in the ddG and ddA lanes at these positions. Reference bands were 821 and 823 for the ddG lane and 820 and 829 for the ddA lane. For the crosses with en^{52}, en^{61}, and en^{67}, polymorphisms caused by C → T transitions at positions 1341 and 1347 were also scored in both ddG and ddA sequencing reactions. Reference bands were nt 1348 and 1349 for the ddG lane and 1355 for the ddA lane. Normalized pixel values for the polymorphic bands are expressed as a percentage of WT.

RESULTS

To demonstrate the sensitivity and validity of ASA (see Fig. 1 and *Materials and Methods*), we studied the expression of engrailed and invected transcripts in several genetic contexts. Fig. 2 depicts these two linked genes, their opposing transcription units, and the positions of 11 mutations whose chromosomal rearrangements affect engrailed function to various extents. Polymorphic changes in the engrailed and invected sequences that could be used to distinguish between allelic transcripts were sought in several laboratory strains and in seven WT strains that had been isolated from geographically distant regions (e.g., Oregon-R, Canton-S, Brazil, Ukraine, Siberia, Australia, and Serbia). Sequencing engrailed and invected exons amplified from these strains revealed differences in the engrailed 3′-untranslated regions of the *CyO* chromosome [C → A (2236), +A (2262), −A (2265), and +T (2268)], the Ukraine chromosome [+TA (2015), +ATCCTA (2032), and C → T (2041)], and the en^{1} chromosome [+TA (2015) and C → A (2236)]. No sequence differences in the engrailed 3′ untranslated region were found in the other WT strains. No sequence differences were found in the 200-residue 3′ untranslated region of invected in these strains. However, comparisons of sequences of the invected exon 1 revealed differences in the Brazil chromosome [T → C (1161), C → T (1341), and C → T (1347)] and in the *CyO, Bc*, en^{49}, en^{63}, en^{59}, and en^{2} chromosomes [C → T (826 and 834)].

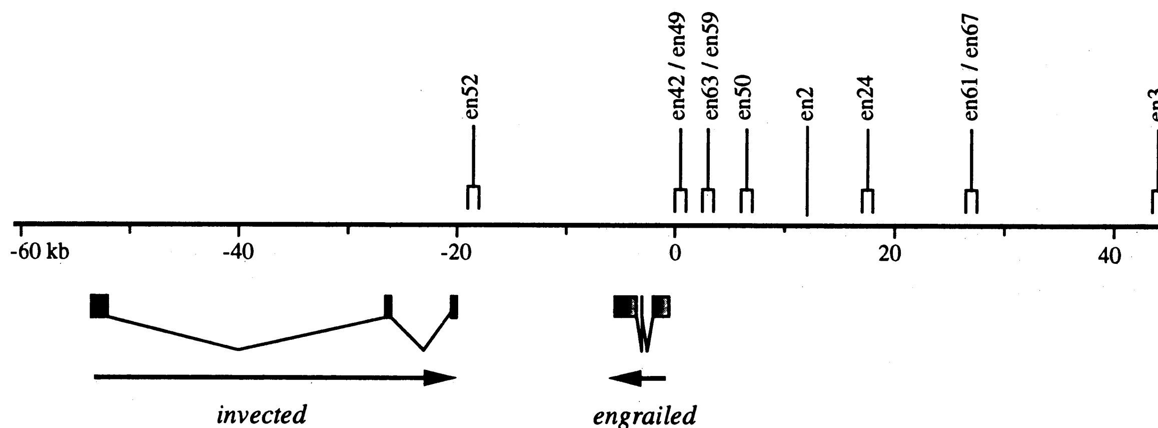

FIG. 2. Map of the engrailed/invected region. Genomic map (horizontal line) with the engrailed and invected transcription units indicated by arrows. Intron/exon organization of engrailed and invected is depicted, as are the approximate locations of 11 engrailed mutations (23). Scale is in kilobases with start site of engrailed transcription unit designated 0. Sequence polymorphisms in engrailed and invected transcripts: Ukraine DNA has six additional base pairs (ATCCTA) in the 3'-untranslated region between nt 2032 and 2033 of the engrailed transcript. All M chromosomes had the same sequence as Canton-S in this region. Two single base pair polymorphisms (C → T) in the first exon of invected from en^{49}, en^{63}, en^{59}, and en^{2} at positions 826 and 834 allowed the identification of transcripts derived from these chromosomes. Transcripts from the Brazil chromosome were identified by two single polymorphisms (C → T) at nt 1341 and 1347.

To gauge how well the proportion of input mRNA correlates with the sequenced product after ASA, separate preparations of RNA isolated from Oregon-R and Ukraine flies were copied into cDNA, amplified, and spectrophotometrically quantified. These DNA preparations were then mixed in various proportions, amplified, and sequenced. The ratio of DNA in the ddG bands at position 2017, which distinguishes Oregon-R- and Ukraine-derived transcripts, revealed an excellent correlation between the ratio of input cDNA and the ratio of amplified product (Fig. 3). Furthermore, genomic DNA isolated from Oregon-R/Ukraine heterozygotes yielded equivalent amounts of sequenced products after PCR amplification (not shown). Additional controls exploited the polymorphic differences between the 3' untranslated regions of the *CyO*, Oregon-R, and Ukraine engrailed transcripts to demonstrate that the proportion of amplified product is equivalent in each type of heterozygote fly (for Oregon-R/Ukraine, see Fig. 4; others not shown). We conclude that the WT engrailed genes from Oregon-R, *CyO*, and Ukraine flies produced equivalent amounts of transcript and that the ASA method accurately assesses the relative abundance of initial template.

To demonstrate the utility of ASA for quantifying relative levels of transcripts, various mutations in the regulatory region of engrailed were analyzed. Although the engrailed transcription unit is relatively small (approximately 3.7 kb), it is contained within a large regulatory region that has been defined by mutations that truncate the gene as far as 15 kb downstream or 45 kb upstream of the transcription unit (12). These mutants are recessive and, with the exception of en^{61}, en^{67}, en^{1}, and en^{3}, are all embryonic lethal when hemizygous or homozygous. Since the developmental program of engrailed expression includes spatially restricted domains in all developmental stages, it is of interest to know how the various gene truncations alter spatially and temporally regulated expression. The early developmental lethality associated with these mutants, however, has precluded their analysis at later stages. ASA provides a convenient way to study these lethal mutations, because M transcripts can be distinguished in tissue obtained from phenotypically normal, viable heterozygotes.

A representative set of engrailed mutants (en^{52}, en^{42}, en^{49}, en^{63}, en^{50}, en^{1}, en^{2}, en^{24}, en^{61}, en^{67}, and en^{3}) was selected for analysis. These mutants lack the 6-bp insertion present in the Ukraine WT engrailed gene, so ASA was used to compare the relative proportion of engrailed RNA produced by the M genes in heterozygous animals. engrailed mRNA was isolated from tissues of third instar larva in which engrailed is expressed, including wing imaginal discs and brains. In tissue from M/Ukraine (WT) heterozygous larvae, the proportion of product derived from the M chromosome varied considerably in a manner that was dependent upon both the particular mutant and the tissue type (Fig. 4). In wing discs, mutants with lethal breakpoints in the 20 kb immediately upstream of the engrailed transcription start (en^{42}, en^{49}, en^{63},

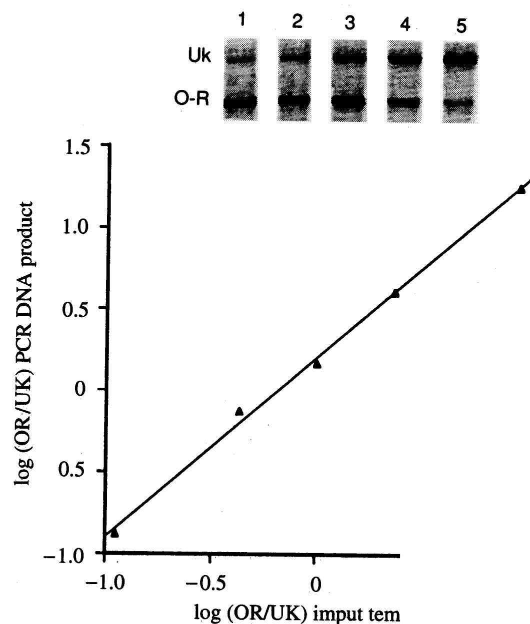

FIG. 3. Quantitative ASA of different ratios of Ukraine and Oregon-R engrailed cDNAs. cDNA prepared from homozygous Ukraine and Oregon-R larval tissue was amplified, quantified by absorbance at 260 nm, mixed in various proportions, reamplified, and sequenced. (*Inset*) The region of a sequencing gel that includes ddG 2017 and reveals the 6-bp polymorphic difference between these two strains (see legend to Fig. 2). Lane 1, 10% Ukraine:90% Oregon-R; lane 2, 30% Ukraine:70% Oregon-R; lane 3, 50% Ukraine:50% Oregon-R; lane 4, 70% Ukraine:30% Oregon-R; and lane 5, 90% Ukraine:10% Oregon-R. Graphical representation of this data shows the linear relationship between the ratio of input cDNA (OR/UK) to the ratio of OR/UK product.

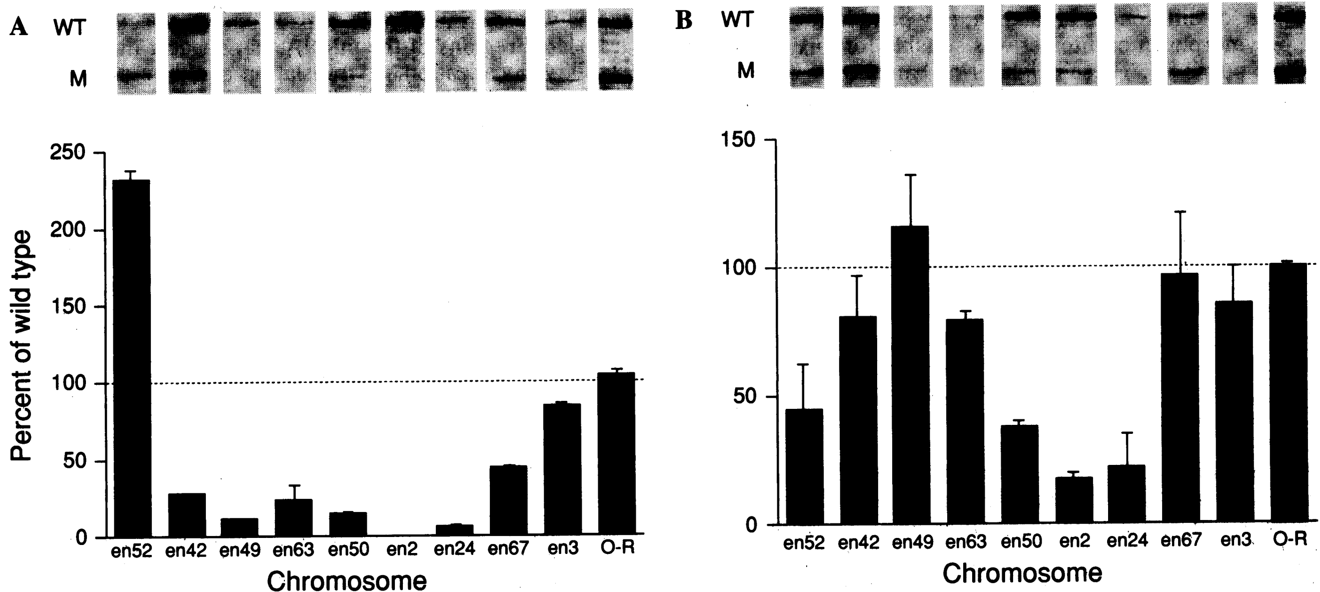

FIG. 4. Quantitative analysis of engrailed expression in heterozygous larval tissues. The relevant portion of each sequencing gel is pictured above a graphic representation of the data. The lanes are arranged from left to right with the proximal to distal order of the positions of the mutant breakpoints. Relative levels of expression are shown as a percentage of WT (100%). (*A*) Wing imaginal discs (approximately five discs per sample). (*B*) Larval brain (approximately five brains per sample). Each bar represents the average of at least two independent preparations. The values obtained from multiple preparations were highly reproducible. Expression of engrailed from the WT Oregon-R (O-R) chromosome is shown and was equal to that of Ukraine (100% ± 6%). The horizontal broken lines on each graph represent WT levels of engrailed expression.

en^{50}, en^{2}, and en^{24}) reduced expression from the M chromosome to less than 30% of WT. In contrast, the nonlethal breakpoint mutation, en^{3}, which is located 45 kb upstream, reduced expression in wing disc expression by only about 10%. Curiously, en^{52}, which has a lethal breakpoint 15 kb downstream of the engrailed transcription unit, had 2-fold greater expression from the M chromosome. Values obtained from multiple preparations were highly reproducible.

Preparations from larval brain (Fig. 4*B*) yielded quantitatively different results. The alleles with breakpoints in the region immediately upstream of the engrailed transcription start site (en^{42}, en^{49}, and en^{63}) had only marginal effects in the brain. In contrast, the more distal breakpoint mutations en^{50}, en^{2}, and en^{24} had increasingly severe effects on expression levels. This inverse relationship between length of the 5′ regulatory region and the level of expression suggests that the sequence elements regulating transcription in the larval brain are both numerous and varied in their function.

Located approximately 20 kb downstream of the engrailed transcription unit is a gene, invected, whose developmental program of expression is virtually identical to that of engrailed (Fig. 2*A*). invected encodes a protein that is closely related to the engrailed protein (13). We analyzed invected transcripts in heterozygous engrailed mutants to determine whether gene truncations in the engrailed regulatory region also affect invected expression. RNA isolated from heterozygous larval wing imaginal discs was used for ASA by taking advantage of polymorphisms in the first exon of invected to distinguish WT from M chromosome transcripts (Fig. 2*C*). Quantitative sequence analysis revealed that the engrailed mutants substantially alter invected expression (Fig. 5). All the mutants reduced expression, to 50% of WT levels in en^{52} and en^{67} heterozygotes, and to less than 15% of WT in en^{63} and en^{59} heterozygotes.

DISCUSSION

We have shown that ASA can be used to accurately quantify the relative abundance of particular transcripts derived from multiple gene copies in a diploid nucleus. Application of ASA to the changing number of engrailed and invected transcripts that were produced from mutant chromosomes illustrates its utility and sensitivity. Unlike other quantitative PCR methods, ASA requires neither careful calibration of each PCR amplification reaction nor quantitative restriction enzyme digestion (14, 15). Rather, in this method, WT transcripts serve as an internal reference for the expression of the M gene. Since ASA amplifies transcripts from both the WT and M chromosomes together, using the same set of primers, no calibration to a standard is necessary. This simplifies and improves the reproducibility and accuracy of the analysis. In practice, we found it necessary to calibrate sequencing

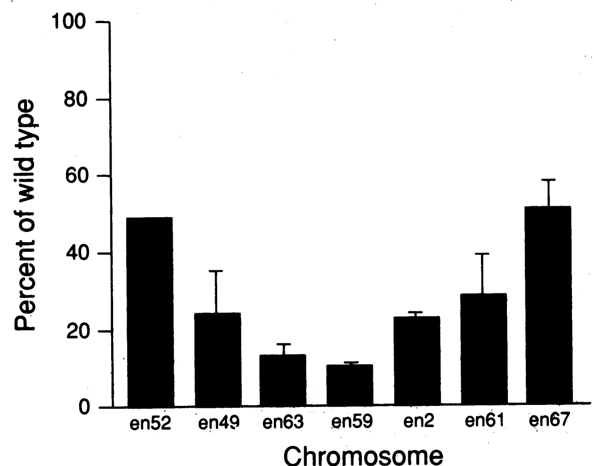

FIG. 5. Quantitative analysis of invected expression in heterozygous wing imaginal discs. Graphic representation of ASA from heterozygous engrailed Ms reveals that invected expression depends upon the integrity of the regulatory region surrounding the engrailed gene. The first exon of invected was amplified by PCR with primers that flank sequence polymorphisms. The lanes are arranged from left to right with the proximal to distal order of the positions of the mutant breakpoints. Relative levels of expression are shown as a percentage of WT (100%). Expression of invected from the WT Oregon-R chromosome was equal to that from Brazil (data not shown).

samples for differences in loading; any band in the region of the polymorphism of interest was suitable for this and comparable purposes. These neighboring bands were composed of the reaction products from both the WT and M transcripts.

Clearly it is essential that the polymorphic differences that facilitate the separate quantitation of the WT and M transcripts not affect the efficiency of reverse transcriptions, amplification, or utilization by the sequencing reaction. In the examples used in this study, simple sequence polymorphisms were chosen so that the capacity of PCR to amplify the surrounding sequences should not be affected. This contrasts with the protocol of Fu *et al.* (8) in which reverse transcription through amplified repeat regions may have introduced artifactual variations. We found identification of suitable polymorphisms to be straightforward and the *Drosophila* genome to be rich in the type of sequence diversity suitable for ASA.

Our application of ASA to monitor levels of engrailed and invected transcripts revealed several unanticipated consequences of engrailed mutations. Although en^{42}, en^{49}, and en^{63} truncate the engrailed regulatory region within 1 kb of the start site for transcription, engrailed transcripts were produced in both wing discs and larval brain. The high levels of expression in the brain in these mutants (80–115% of WT levels) suggests that any enhancer elements activating expression in the brain are downstream of the start site. In contrast, mutations with more distal breakpoints (en^{50}, en^{2}, and en^{24}) reduce expression in the brain substantially (18–38% of WT levels), suggesting, perhaps, that regulatory sequences with inhibitory effects dominate in these rearrangements. These suggestions are consistent with the results of previous studies of the engrailed regulatory region that assayed *P* element transformants of fusion constructs. Although incomplete, these studies located enhancers both upstream and downstream of the transcription start site (16) and obtained evidence for redundancy as well as for functional heterogeneity of these elements (C.-N. Chen and T.B.K., unpublished data).

Perhaps most surprising is the 50–90% reduction of invected expression in wing imaginal disc in the engrailed mutants. We find it difficult to explain how a rearrangement breakpoint, for instance, en^{61}, that is 85 kb downstream of the invected transcription start site reduced invected transcripts to less than 30% of WT levels. Nevertheless, our results are consistent with earlier studies by Gustavson (17), who concluded that a common set of regulatory elements are responsible for the similar patterns of engrailed and invected expression. Such a model might explain why engrailed breakpoint mutations outside of the engrailed transcribed region have more severe phenotypes than point mutations that are presumed nulls (premature termination codons). We suggest that invected protein has a function similar to engrailed but is redundant in its presence. Mutants with premature termination codons in engrailed would therefore retain some "engrailed-like" function in the form of the invected protein. Indeed, our unpublished observations suggest that invected protein is an "active" repressor in tissue culture cells, similar to that described for the engrailed protein (18). At a minimum, such a model accounts for the indistinguishable patterns of expression of these two genes.

We have shown that simple sequence polymorphisms can be used to quantify relative transcript abundance. We suggest that ASA should be generally applicable to studies of recessive genes in a heterozygous and phenotypically normal state, as well as lethal alleles with dominant phenotypes. Examples might include the Huntington-disease gene or genes where trinucleotide expansions can occur in the untranslated regions of the mRNA that suggest that transcript stability, localization, or translation could be affected (19). ASA might also benefit the analysis of the mechanisms involved in sex-specific dosage compensation, transcriptional effects in trisomics, parental imprinting, transvection, and the apparent reduction of mRNA stability by nonsense codons (20–22).

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1. Robey, E. & Fowlkes, B. J. (1994) *Annu. Rev. Immunol.* **12**, 675–705.
2. Chess, A., Simon, I., Cedar, H. & Axel, R. (1994) *Cell* **78**, 823–834.
3. Holliday, R. (1990) *Development (Cambridge, U.K.)* Suppl., 125–129.
4. Foley, K. P., Leonard, M. W. & Engel, J. D. (1993) *Trends Genet.* **9**, 380–385.
5. Mahadevan, M., Tsilfidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barceló, J., O'Hoy, K., Leblond, S., Earle-Macdonald, J., de Jong, P. J., Wieringa, B. & Korneluk, R. G. (1992) *Science* **255**, 1253–1255.
6. Brook, J. D., McCurrach, M. E., Harley, H. G., Buckler, A. J., Church, D., Aburatani, H., Hunter, K., Stanton, V. P., Thirion, J.-P., Hudson, T., Sohn, R., Zemelman, B., Snell, R. G., Rundle, S. A., Crow, S., Davies, J., Shelboune, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P. S., Shaw, D. J. & Housman, D. E. (1992) *Cell* **68**, 799–808.
7. Fu, Y.-H., Pizzuti, A., Fenwick, R. G., Jr., King, J., Rajnarayan, S., Dunne, P. W., Dubel, J., Nasser, G. A., Ashizawa, T., de Jong, P., Wieringa, B., Korneluk, R., Perryman, M. B., Epstein, H. F. & Caskey, C. T. (1992) *Science* **255**, 1256–1258.
8. Fu, Y.-H., Friedman, D. L., Richards, S., Pearlman, J. A., Gibbs, R. A., Pizzuti, A., Ashizawa, T., Perryman, M. B., Scarlato, G., Fenwick, R. G., Jr., & Caskey, C. T. (1993) *Science* **260**, 235–238.
9. Sabouri, L. A., Mahadevan, M. S., Narang, M., Lee, D. S., Surh, L. C. & Korneluk, R. G. (1993) *Nat. Genet.* **4**, 233–238.
10. Roses, A. D. (1994) *Science* **264**, 587.
11. Fu, Y.-H., Pearlman, J. A., Pizzuti, A., Fenwick, R. G., Jr., Friedman, D. L., Perryman, M. B., Richards, S., Gibbs, R. A., Ashizawa, T., Scarlato, G. & Caskey, C. T. (1994) *Science* **264**, 587–588.
12. Drees, B., Ali, Z., Soeller, W., Coleman, K., Poole, S. & Kornberg, T. (1987) *EMBO J.* **6**, 2803–2809.
13. Coleman, K. G., Poole, S. J., Weir, M. P., Soeller, W. C. & Kornberg, T. B. (1987) *Genes Dev.* **1**, 19–28.
14. Gilliland, G., Perrin, S., Blanchard, K. & Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2725–2729.
15. Weksberg, R., Shen, D. R., Fei, Y. L., Song, Q. L. & Squire, J. (1993) *Nat. Genet.* **5**, 143–150.
16. Hama, C., Ali, Z. & Kornberg, T. B. (1990) *Genes Dev.* **4**, 1079–1093.
17. Gustavson, E. (1993) Ph.D. thesis (Univ. of California, San Francisco).
18. Jaynes, J. B. & O'Farrell, P. H. (1991) *EMBO J.* **10**, 1427–1433.
19. Nelson, D. L. & Warren, S. T. (1993) *Nat. Genet.* **4**, 107–108.
20. Schiavi, S. C., Wellington, C. L., Shyu, A. B., Chen, C. Y., Greenberg, M. E. & Belasco, J. G. (1994) *J. Biol. Chem.* **269**, 3441–3448.
21. Barker, G. F. & Beemon, K. (1994) *Mol. Cell. Biol.* **14**, 1986–1996.
22. Lim, S. K., Sigmund, C. D., Gross, K. W. & Maquat, L. E. (1992) *Mol. Cell. Biol.* **12**, 1149–1161.
23. Kuner, J., Nakanishi, M., Ali, Z., Drees, B., Gustavson, E., Theis, J., Kauvar, L., Kornberg, T. & O'Farrell, P. (1985) *Cell* **42**, 309–316.