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Evidence for a Stabilizer Element in the Untranslated Regions of *Drosophila* Glutathione *S*-Transferase D1 mRNA*

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The neighboring genes *gst*D1 and *gst*D21 share 70% sequence identity. *gst*D1 encodes a 1,1,1-trichloro-2,2-bis-(*P*-chlorophenyl)ethane dehydrochlorinase; *gst*D21, a ligandin. Both of their mRNAs are inducible by pentobarbital but otherwise behave very differently. Intact *gst*D21 mRNA is intrinsically labile, but becomes stabilized when separated from its native untranslated region (UTR). In contrast, whereas *gst*D1 mRNA is very stable in its entirety, without its native UTRs it becomes even more labile than that of *gst*D21. Decay patterns from four chimeric D1-D21 mRNAs, designed to reveal the individual importance of each molecular region to stability, strongly indicate the presence of destabilizing elements in the coding region of *gst*D1 mRNA. Thus, the UTRs of this molecule must contain a dominant stabilizer element that overrides the destabilizing influence of the coding region and confers overall stability to the entire molecule. The suspected presence of such a stabilizer element in *gst*D1 mRNA extends a concept from mRNA metabolism in yeast and cultured mammalian cells to include a multicellular organism, *Drosophila melanogaster*. The complementary presence of destabilizing and stabilizer elements on the same mRNA reveals a regulatory mechanism by which an abundant mRNA can be further induced by a chemical stimulus, or otherwise be returned to normal levels during recovery.

An effective way to regulate gene expression involves controlling mRNA stability (1–3). The major measure of mRNA stability is its half-life (1, 4), which determines the time required for a mRNA to reach a new steady state following a change in transcription rate (*e.g.* by inducers such as pentobarbital).

We have been using mRNAs of the *Drosophila* glutathione *S*-transferase (*gst*)[1] genes D1 and D21 as reporters to investigate pentobarbital-mediated changes in mRNA stability. The early paradigm for RNA metabolism associates mRNA decay rates largely with the strength of the destabilizing sequences of the molecule (1–3, 5). But the recent discovery of a handful of active stabilizer elements (STE) in certain mammalian and yeast mRNAs (6–9) has called for a revision to this model.

Although *gst*D21 mRNA is labile, the coding region of the gene gains stability when separated from its native UTRs. Just the opposite is true for mRNA of the D21 homologue, *gst*D1. This mRNA is very stable, but the coding region of the molecule alone, without native UTRs, is even more labile than intrinsically unstable *gst*D21 mRNA. To further investigate the nature and the cause of this instability, we assembled chimeric D1-D21 mRNAs containing various segments of the D1 coding sequence. We observed that these chimeras were also unstable in the same context of heterologous UTRs as the D1 coding sequence. We repeatedly detected putative decay intermediates from the D21 portion, but seldom the D1 segment of these chimeric mRNAs. Such patterns are strong evidence for the presence of cryptic destabilizing *cis*-acting elements in the coding region of *gst*D1 mRNA. Our observations also suggest that the stability of already abundant *gst*D1 mRNA is maintained by a stabilizer element in its UTRs, which overrides any destabilizing elements in the coding region. We speculate that this combination of stabilizer and destabilizing elements helps to regulate *gst*D1 mRNA levels in response to pentobarbital induction and to generally maintain mRNA stability. As we compare the characteristics of labile *gst*D21 mRNA with those of *gst*D1 mRNA, in which a completely different arrangement of *cis*-acting elements govern RNA metabolism, we note the potential for significant diversity in the regulation of different members of a single multigene family.

EXPERIMENTAL PROCEDURES

Materials—Bacteriological media were purchased from Invitrogen, and chemicals from ICN, Invitrogen, or Sigma. Oligonucleotides were products of either Integrated DNA Technologies, Inc. (Coralville, IA) or Invitrogen. Radioactive nucleotides ([α-^{32}P]UTP) were purchased from ICN (Irvine, CA). RPA III kits were purchased from Ambion (Austin, TX). Restriction enzymes were products of New England Biolabs (Beverly, MA) or American Allied Biochemicals (Aurora, CO). T4 polynucleotide kinase and T4 DNA ligase were products of New England Biolabs and Promega (Madison, WI), respectively. *Pfu* DNA polymerase was purchased from Stratagene (San Diego, CA). SP6 RNA polymerase and the plasmid vector pSP64(A) for *in vitro* transcription were purchased from Promega. Tobacco acid pyrophosphatase was a product of Epicentre Technologies (Madison, WI). T7 RNA polymerase was a generous gift from Bi-Cheng Wang (University of Georgia, Athens, GA). *Escherichia coli* DH5α competent cells and *Pfx* DNA polymerase were products of Invitrogen. The plasmid vector pCaSpeR-hs-act for *Drosophila* transformation was obtained from C. S. Thummel of the University of Utah (10). The Δ2–3 line {$P[ry^+\ \Delta 2\text{–}3](99B)$} (11) expressing transposase and the *yw* line were obtained from Susan Abmayr and David Gilmour, respectively, both of the Department of Biochemistry and Molecular Biology, The Pennsylvania State University. The *E. coli* expression plasmids for GST D1 (pGTDm1-KK) and GST D21 (pGTDm21-KK) were previously reported (12). The plasmids for C-

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[1] The abbreviations used are: *gst*, glutathione *S*-transferase gene; GST, glutathione *S*-transferase (EC 2.5.1.18); Int, putative decay intermediate; PB, pentobarbital; RPA, RNase protection assay; STE, stabilizer element on an mRNA; UTR, untranslated region (5′ or 3′) of an mRNA; nt, nucleotide(s); RT, reverse transcriptase.

A

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5'-UTR -
CAGTGCTACTTTTCATTTGATACAGTGTACATCGCGAGTTTCACAACAGAAGAAAAAAT(↓)T
AAA(ATG)

3'-UTR -
GCCTGATATTCACGTTTTTATACCCGTACATATGATGTAGTATTTATATTCACGTTCACAAACA
ACAATTCCAAATTCGCCTGTCTCCAAAGACAATAAATAAGTGTTCTTTTTTTTGAATGCACAAA
ATAC
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B

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D1      2    DFYYLPGSSPCRSVIMTAKAVGVELNKKLLNLQAGEHLKPEFLKINPQHTIPTLVDNGFA 61
             DFYY+PG   CR+VIM AKA+G+ELNKKLLN   GE LKPEF+K+NPQHTIPTLVDNGF+
D21     1    DFYYMPGGGGCRTVIMVAKALGLELNKKLLNTMEGEQLKPEFVKLNPQHTIPTLVDNGFS 60

                 *
D1      62   LWESRAIQVYLVEKYGKTDSLYPKCPKKRAVINQRLYFDMGTLYQSFANYYYPQVFAKAP 121
             +WESRAI VYLVEKYGK D L P  PKKRAVINQRLYFDMGTLY+SFA YYYP      P
D21     61   IWESRAIAVYLVEKYGKDDYLLPNDPKKRAVINQRLYFDMGTLYESFAKYYYPLFRTGKP 120

                                                              ⇓
D1      122  ADPEAFKKIEAAFEFLNTFLEGQDYAAGDSLTVADIALVATVSTFEVAKFEISKYANVNR 181
                E  K+IE AF FL+TFLEGQ+Y AGD LTVADIA+++TVSTFEV++F+ SKY+NV+R
D21     121  GSDEDLKRIETAFGFLDTFLEGQEYVAGDQLTVADIAILSTVSTFEVSEFDFSKYSNVSR 180

D1      182  WYENAKKVTPGWEENWAGCLEFKKYFE 208
             WY+NAKKVTPGW+ENW G +  K  F+
D21     181  WYDNAKKVTPGWDENWEGLMAMKALFD 207
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C

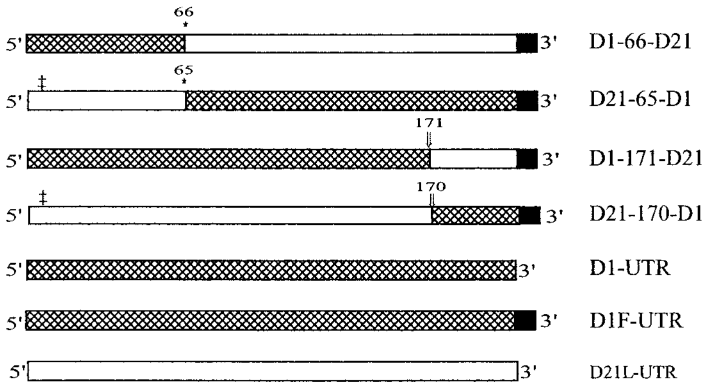

FIG. 1. **Complete *gst*D1 mRNA sequence and organization of chimeric D1-D21 genes.** *Panel A*, the 5′-UTR and 3′-UTR of *gst*D1 mRNA. An analysis of the genomic sequence (20) revealed the presence of a 627-nucleotide intron between the 4th and 5th nucleotides upstream of the ATG initiation codon. The gene orthologous to *gst*D1 in the housefly *Musca domestica* also contains an intron at this same position (21). *Panel B*, a comparison of GST D1 and GST D21 amino acid sequences (20, 22). Arg^{66} of GST D1 is marked by an *asterisk* (*). Phe^{171} of GST D1 and Phe^{170} of GST D21 are marked by a *vertical arrow* (⇓). The extra C-terminal sequence of D21, ARKLAAK (22), is not shown in *panel B*. *Panel C*, DNA fragments were cloned into pCaSpeR-hs-act for microinjection, a step toward establishing transgenic lines. All chimeric mRNAs contain the 5′-UTR of *hsp*70 (not shown), CCCCAAC of the 5′-UTR of *gst*D21 (not shown), the coding region of *gst*D1, *gst*D21, or chimeric D1-D21 with the FLAG octapeptide (*filled squares*) at the C terminus, and the 3′-UTR of *actin5C* (not shown). The *open rectangle* represents the D21 coding sequence (214 amino acids) and the *cross-hatched rectangles* represent the D1 coding sequence (208 amino acids) (22). ‡, G8S,G9S mutations in GST D21.

terminal FLAG derivatives of D1 (D1-F) and D21 (D21-F), as well as plasmid for the G8S,G9S mutant of D21-F, were unpublished laboratory stocks.

Transgenic Constructs and Nomenclature—Site-directed mutagenesis was carried out according to the QuikChange™ mutagenesis procedure (Stratagene). All clones were sequenced at the Penn State Nucleic Acid Facility prior to microinjection into embryos.

The primers D1-F-5′ (GGAATTCCCCAACATGGTTGACTTCTACTACC) and D1-F-3′ (CGGGATCCGTGAATATCAGGCTTACT) were used to PCR amplify the *gst*D1.F coding region. PCR amplification (12) of the D1-D21 and D21-D1 chimeras was set up using the appropriate pair permutations of primers from the set D1-F-5′, D1-F-3′, D21-F-5′ (GGAATTCCCCAACATGGACTTTTACTACATGCC), and D21-F-3′ (CGGGATCCTCGTGATACCGATCACTTG).

The coding region of *gst*D1, with a FLAG octapeptide at its C terminus (*gst*D1-F), was PCR-amplified from pGTDm1-FLAG-KK using primers D1-F-5′ and D1-F-3′ (KK refers to the pKK223-3 expression vector (Amersham Biosciences)). The *Bam*HI-*Eco*RI-digested fragment was cloned into pCaSpeR-hs-act (10) to obtain pBA1-CaSpeR. The transgene is called D1F-UTR (D1-FLAG − UTR). Primers D21-F-5′ and D21-F-3′ were used to introduce by PCR *Bam*HI and *Eco*RI sites at the ends of the D21-F-G8S,G9S coding region. The PCR products were then digested with *Bam*HI and *Eco*RI and ligated into *Bam*HI-*Eco*RI-digested pCaSpeR-hs-act to generate pBA3-CaSpeR.

To switch segments of GST D1 and GST D21 at the 66th and 65th residues (Fig. 1), primers D1-F-R66-*Sma*I-S (GTGGGAGTCCCGCGCCATCCAGGTG) and D1-F-R66-*Sma*I-AS (CACCTGGATGGCCCGGGTCTCCCAG) were used to introduce a *Sma*I site into pGTDm1-KK by site-directed mutagenesis without changing the amino acid sequence encoded by the template. The *Sma*I fragment of the resulting plasmid (pGTDm1-*Sma*I-KK) was cloned into calf intestinal alkaline phosphatase-treated, *Sma*I-digested pGTDm21-FLAG-G8S,G9S-KK to obtain pBA12-KK. The transgene, which contains the N-terminal 65 amino acids of D21 and C-terminal 150 amino acids of D1F, is called D21-65-

D1. The opposite swap, resulting in pBA10-KK, was made by inserting the *Sma*I fragment of pGTDm21-FLAG-KK into calf intestinal alkaline phosphatase-treated, *Sma*I-digested pGTDm1-FLAG-KK. The transgene, which contains the N-terminal 66 amino acids of D1 and C-terminal 157 amino acids of D21F, is called D1–66-D21. The exchange at the 171st (D1) and 170th (D21) amino acid position was initiated by the introduction of an *Xho*I site into both pGTDm1-FLAG-KK (D1F-F171L-*Xho*I-S (GCAGGTGGCCAAACTCGAGATCAGCAAGTAC) and D1F-F171L-*Xho*I-AS (GTACTTGCTGATCTCGAGTTTGGCCACCTC-G)) and pGTDm21-FLAG-G8S,G9S-KK (D21-F170L/D171E-*Xho*I-S (GTTCGAAGTTAGTGATCTCGAGTTCAGCAAGTACTCC) and D21-F170L/D171E-*Xho*I-AS (GGAGTACTTGCTGAACTCGAGCTCACTAA-CTTCGAAC)), which yield pBA1-*Xho*I-KK and pBA2-*Xho*I-KK, respectively. The *Xho*I-*Sma*I fragment of pBA2-*Xho*I-KK was cloned into *Xho*I-*Sma*I-digested pBA1-*Xho*I-KK to generate pBA13-KK. This led to the transgene D1–171-D21, which contains 171 amino acids of D1 followed by 52 amino acids of D21-F at the C terminus. Because the *gst*D21 coding region has a second *Sma*I site that interferes with cloning, the *Xho*I-*Pst*I fragment of pBA1-*Xho*I-KK was cloned into pBA2-*Xho*I-KK, resulting in pBA15-KK. The corresponding transgene is called D21-170-D1, which contains 170 amino acids of D21 followed by 45 amino acids from the C-terminal of D1-F. Prior to cloning into pCaSpeR-hs-act DNA, *Bam*HI and *Eco*RI restriction sites were introduced by PCR at the desired ends of the constructs described thus far, using the appropriate pair of starting primers from the set D1-F-5′, D1-F-3′, D21-F-5′, and D21-F-3′. A FLAG-less version of pBA1-CaSpeR (*i.e.* pBA22-CaSpeR) was constructed using the same strategy, but changing the 3′-end PCR primer to D1-3′ (CGGGATCCGTGAATAT-CAGGCTTATTC) from D1-F-3′. This transgene is called D1-UTR. Construction of transgene D21L-UTR will be described elsewhere.

All clones in pKK223-3 and pCaSpeR-hs-act vectors were sequenced at the Penn State Nucleic Acid Facility. Results also showed that the four chimeric proteins (D1-66-D21, D21--65-D1, D1-171-D21, and D21-170-D1) were successfully expressed from the pKK223-3-based expression constructs in *E. coli*.[2] Plasmid DNA was prepared for microinjection using a Concert™ rapid plasmid DNA isolation kit (Invitrogen). Microinjection of embryos was subsequently performed, as previously described (13, 14). The newly enclosed G_0 flies were crossed singly to *yw* to remove any transposase background. Yellow- to red-eyed G_1 progeny with longer body bristles (Sb^-) were re-crossed with *yw*. Stable lines were established through sibling crossings of colored-eye G_2 virgin flies. Three separate lines were maintained for each transgene.

Pentobarbital and Heat Shock Treatments—Adult flies (2–3 days old) were distributed into clean milk bottles in approximately equal numbers for 5 h starvation at room temperature (21–23 °C) (15). Control flies received a blotting paper strip (3 × 10 cm) saturated with a solution of 5% sucrose; PB-treated flies received a strip soaked in 5% sucrose plus 200 mg/ml PB. The strips were placed in the fly bottles for 2 h at room temperature. Heat shock was administered by incubating flies at 35 °C for 1 h in clean bottles containing 5% sucrose paper strips in a Robbins Scientific Co. (Sunnyvale, CA) hybridization oven (model 2000). (An empty milk bottle with a foam plug requires ~15 min to reach 35 °C from room temperature and takes ~6 min to drop to 31 °C after removal of the bottle from the 35 °C oven.) In addition, heat shock treatments of varying duration were carried out at 35 °C for 5–40 min (instead of 1 h) to detect labile transgenic mRNAs. The flies were subsequently snap-frozen in liquid nitrogen and stored at −70 °C until use.

RNA Isolation and RPA Analysis—RNA was isolated from pulverized flies according to a protocol from Ullrich *et al.* (16). The templates used to prepare our radiolabeled riboprobes, pSP64(A).D21AS, pSP64(A).D1AS, and pSP64(A).RP-49AS, were constructed by RT-PCR amplification. Each plasmid DNA was linearized with an appropriate restriction enzyme then transcribed *in vitro* using [α-^{32}P]UTP. RPA analyses of 40 μg of total RNA samples were conducted according to procedures specified by Ambion, manufacturer of the RPAIII kits. In our figures, we call the protection product of endogenous *gst*D1 mRNA "endo-D1." For transgenes, protection products of expected sizes are labeled as "transgene"; those smaller than the expected sizes are called "decay intermediates" (Int).

Mapping the 5′ End of gstD1 mRNA—The 5′ end sequence of the *gst*D1 mRNA was determined by primer extension using the primer 5′-AGCGGCAGGGGGAGGAGCCGGGCA-3′ and by circular RT-PCR (17, 18). Decapping, DNase I treatment, and circularization of RNA were carried out according to a procedure by Couttet *et al.* (18). 5 μg of circularized RNAs was used for reverse transcription using a *gst*D1-specific primer 5′-GCGGATCCTTGGCGGTCATGATCACGGAGC-3′. The resulting cDNA reaction mixture was boiled for 5 min and then digested with a mixture of RNase A and RNase T1. The treated cDNA was recovered by phenol extraction and ethanol precipitation. One percent of the recovered cDNA was taken for PCR amplification, using 1 unit of *Pfx* DNA polymerase and the primer pair, 5′-GCGGATCCTT-GGCGGTCATGATCACGGAGC-3′ and 5′-GCGAGCTCTCCCGGAT-GGGAGGAGAACTGGGC-3′, as instructed by Invitrogen. The PCR product was digested, gel-purified, and then cloned into *Bam*HI-*Sac*I-digested pSP64(A). Two clones were randomly selected for sequencing to determine the 5′ and 3′ end sequences of the *gst*D1 mRNA. Finally, Southern blot analysis was carried out with 5′-end-labeled oligo(dT)$_{18}$ to determine the length of the poly(A) of each mRNA (19).

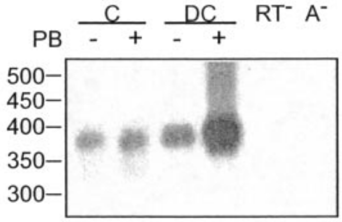

FIG. 2. **Detection of uncapped *gst*D1 mRNA by circular RT-PCR and determination of poly(A) length.** Southern blot analysis of circular RT-PCR products from *gst*D1 mRNAs with 5′-end-labeled oligo(dT)$_{18}$. Proportionally equal amounts of materials were used in each step of the experiment. *C*, RNAs ligated without decapping; *DC*, RNA ligated after decapping by tobacco acid pyrophosphatase. The two negative controls are without reverse transcriptase (*RT*$^-$) or *in vitro* transcribed poly(A)$^-$ *gst*D1 mRNA (*A*$^-$). The DNA size markers are indicated to the *left* of the *panel*. There is no difference in the sizes of cRT-PCR products between control and PB-treated RNAs. The sum of the 5′ and 3′ ends of *gst*D1 mRNA in the PCR product is 330 nucleotides without any poly(A). The hybridization signals have an electrophoretic mobility of ~370 nucleotides. Therefore, the poly(A) tail length of *gst*D1 mRNA is estimated to be ~40.

RESULTS

Complete Sequence of the gstD1 mRNA—Based on sequencing results obtained for the cRT-PCR clones, the 5′-UTR of *gst*D1 mRNA spans 64 nucleotides, and primer extension yielded multiple bands, marking D1 mRNAs with 5′-UTR sequences of 67, 66, 64, 63, 61, and 60 nucleotides in length (data not shown). Two cRT-PCR clones were sequenced. One had a 5′-UTR of 63 nucleotides, the other, one of 64 nucleotides. The 3′-UTR of *gst*D1 mRNA is 132–135 nucleotides long, with variation because of uncertain cleavage over a stretch of As in the genomic sequence (20, 22). Total RNAs from control or PB-treated flies yielded cRT-PCR products of *gst*D1 mRNA. Moreover, they did so regardless of decapitation by tobacco acid pyrophosphatase, indicating the presence of uncapped D1 mRNA (Fig. 2). The presence of multiple primer extension products of varying size supports the notion that some *gst*D1 mRNAs are uncapped and missing a few nucleotides at the 5′ end.

In contrast, the same preparations of RNAs yielded no cRT-PCR products for *gst*D21 in the absence of tobacco acid pyrophosphatase when a D21 primer pair was used under the same set of experimental conditions (data not shown). This affirms that a small contingent of stable, uncapped *gst*D1 mRNA exists among a capped majority population whose molecules have an average of ~40 As at the 3′ ends under both control and PB treatment conditions. The uncapped and shorter than full-length *gst*D1 mRNA molecules with short poly(A) tails are probably decay intermediates stabilized by a stabilizer element (Ref. 9 and see "Discussion").

Identification of Cryptic Destabilizing Element(s) in the gstD1 mRNA—Despite a slow transcription rate, *gst*D1 mRNA is relatively abundant under control conditions. In contrast, *gst*D21 mRNA, which has a faster transcription rate than

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[2] B. Akgül, Y.-S. L. Tu, and C.-P. D. Tu, unpublished results.

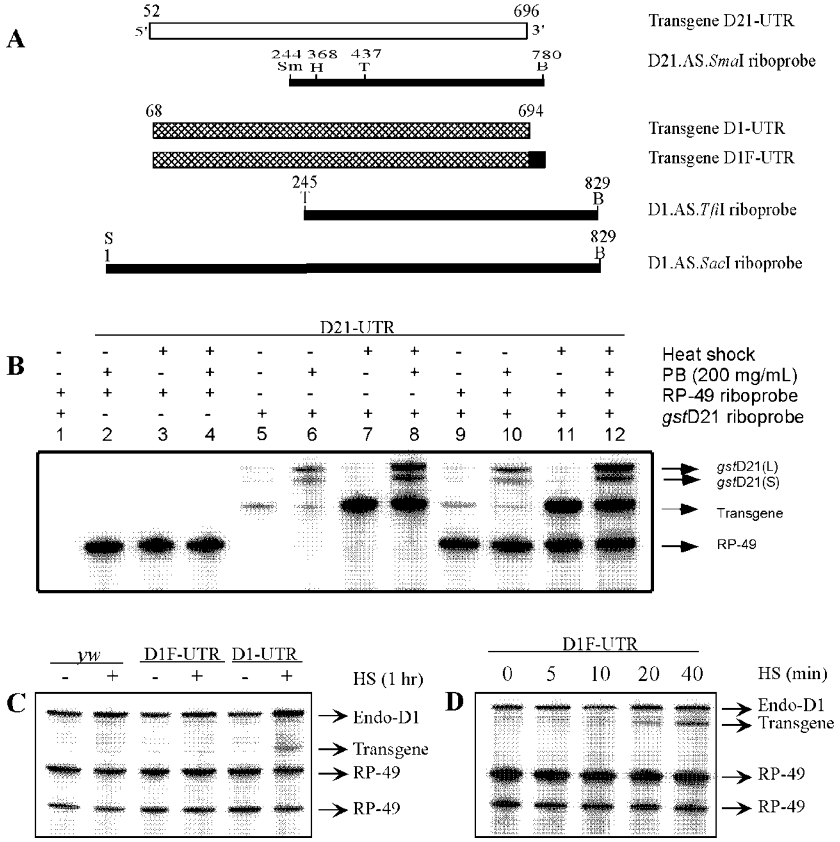

FIG. 3. **Instability of the coding sequence of *gst*D1 mRNA in a transgenic model.** Nucleotide position 1 is the beginning of the full-length *gst*D1 mRNA (1–829). *Panel A* illustrates the D21L-UTR transgene (D21 coding region, *open rectangle*), and the two transgenes of D1 (*hatched rectangle*) and D1-F (*hatched rectangle* with a *filled square*) coding sequences and the antisense riboprobes used in the RPA analyses. *Panel B*, RPA analyses of total RNAs from the transgenic line D21L-UTR under control (*lanes 5* and *9*), PB-treated (*lanes 2*, *6*, and *10*), heat-shocked (*lanes 3*, *7*, and *11*), combined heat shock and PB treatment (*lanes 4*, *8*, and *12*) conditions with RP-49 probes alone (*lanes 2–4*), D21.AS.*Sma*I riboprobes alone (*lanes 5–8*) and both riboprobes (*lanes 9–12*). *Lane 1* is a negative control containing yeast tRNAs only. The sizes of the protected fragments are 536 (*gstD21(L)*), 517 (*gstD21(S)*), 455 (chimeric D21L-UTR RNA from the *transgene*), and 400 (*RP-49*) nucleotides. *Panel C*, total RNAs from transgenic lines *D1F-UTR* (D1-F coding region) and *D1-UTR* (D1 coding region) were hybridized with D1.AS.*Tfi*I and RP-49.AS.*Sac*I riboprobes in RPA analyses. Heat shock (*HS*) was performed at 35 °C for 1 h or for the time indicated *above* the *panel*. Protected fragments are identified by *arrows* to the *right* of each panel. *Endo-D1*, 589-nucleotide protection product of endogenous *gst*D1 mRNA; *transgene*, 449-nucleotide protection product of chimeric D1 mRNAs; *RP-49*, 400 and 300 nucleotides for the two RP-49 bands. *yw* is the parental line of the transgenic lines. *Panel D*, total RNAs from D1F-UTR flies after a time course of heat shock were hybridized with D1.AS.*Sac*I and RP-49.AS.*Sac*I riboprobes in RPA analyses. The sizes of the protected fragments are 829 nucleotides for endogenous *gst*D1 mRNA (Endo-D1), 627 nucleotides for the transgene, and 400 and 300 nucleotides for the two RP-49 bands.

*gst*D1, holds at barely detectable levels in the same environment. *gst*D1 mRNA must therefore be significantly more stable than *gst*D21 mRNA under control conditions (15). We observe that the coding sequence alone of *gst*D21 was stable as part of a chimeric RNA with the 5′-UTR of *hsp*70 and the 3′-UTR of *act5C* (*i.e.* transgene D21L-UTR, Fig. 3*B*). Thus, we attribute the instability of *gst*D21 mRNA to the influence of a *cis*-acting, destabilizing element in the UTRs. This association reflects the current paradigm that mRNA stability depends largely upon the strength of its destabilizing element (for reviews, see Refs. 1–3 and 5). We were surprised to observe, then, that contrary to the standing model, chimeric D1 mRNA, which contains only the D1 coding sequence, was actually very labile (Fig. 3*C*) in the same heterologous UTR contexts (*i.e.* D1-UTR) in which chimeric D21L-UTR mRNA was stable.

To test the effect of nonspecific 3′ extension on D1-UTR mRNA stability, we added the FLAG$^{\mathrm{TM}}$ sequence (GACTACAAGGACGACGATGACAAG) at the 3′ end of the D1 coding region to yield transgenic line D1F-UTR. We used RPA to compare the mRNA expression levels of three chimeric constructs: D1F-UTR (D1F-UTR = D1 coding sequence with FLAG$^{\mathrm{TM}}$ tag minus the native UTRs of *gst*D1 mRNA); D1-UTR, the same sequence minus the FLAG$^{\mathrm{TM}}$; and D21L-UTR, the D21L sequence minus the native UTRs. Whereas chimeric D21L-UTR mRNA expression was induced to a great extent by 1 h of heat shock at 35 °C (Fig. 3*B*), the same treatment reduced chimeric D1-UTR mRNA to barely detectable levels (Fig. 3*C*). Also, under the same conditions, control endogenous *gst*D1 mRNA levels were elevated 1.8 ± 0.4-fold by heat shock (Fig. 3*C*). Results of shortened heat shock treatments (of incubation lasting 5–40 min at 35 °C) showed that this chimeric D1F-UTR mRNA was inducible by heat shock but yielded very labile product (Fig. 3*D*). Chimeric D1F-UTR mRNA levels increased with the duration of heat shock for up to 40 min, but always remained much lower than those of endogenous *gst*D1 mRNA (Fig. 3, *C* and *D*). Thus, this particular nonspecific 3′ end extension to the D1 coding sequence did not sufficiently replace the function of the native 3′-UTR sequence.

The critical difference between the endogenous *gst*D1 mRNA and the transgenic D1 mRNAs, from D1-UTR and D1F-UTR, is in the presence or absence of native *gst*D1 UTRs. We had previously observed that endogenous *gst*D1 mRNA is stable under both control and PB treatment conditions (15). Given the current paradigm of mRNA stability, we did not anticipate the relative instability of the transgenic D1 mRNAs. These unexpected results strongly suggest that the coding region of *gst*D1 mRNA contains one or more cryptic destabi-

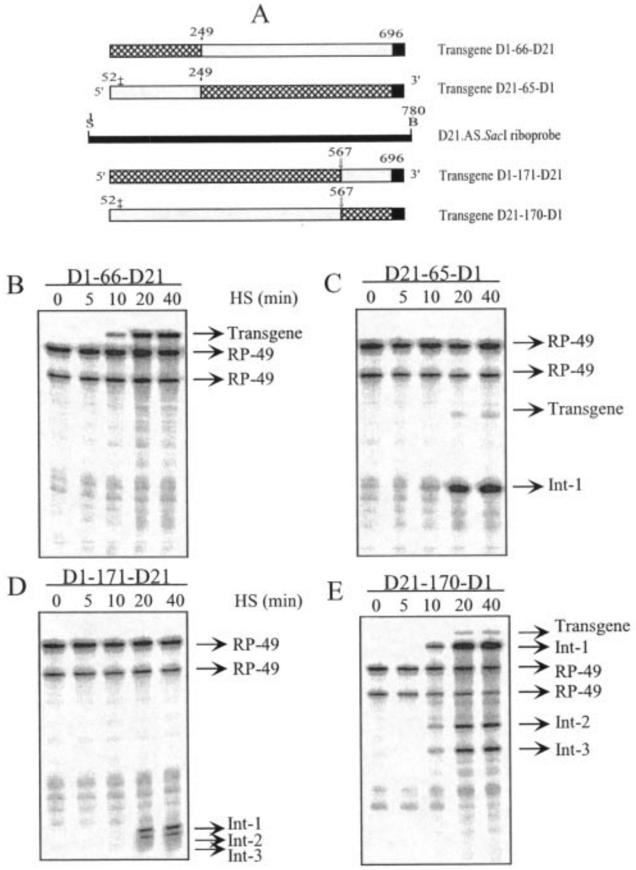

FIG. 4. **Evidence for destabilizing *cis*-acting element(s) in the *gst*D1 mRNA coding region.** *Panel A*, diagrams of chimeric D1-D21 mRNA sequences (*cross-hatched bars* for D1 and *open rectangles* for D21, see Fig. 1) and D21.AS cDNA linearized by *Sac*I used for riboprobe preparations. Riboprobe sequences overlapping with the *open rectangles* are protected in the RPA. The *nucleotide numbers* are coordinates for the D21 portion in each chimeric gene. The expected sizes of protected products are 450 nucleotides for D1-66-D21, 170 nucleotides for D21-65-D1, 130 nucleotides for D1-171-D21, and 490 nucleotides for D21-170-D1. *Panels B–E*, transgenic lines and the duration of heat shock at 35 °C are indicated *above* each panel (*B–E*). The full-length D21 riboprobe, D21.AS.*Sac*I, was hybridized with total RNAs from transgenic lines D1-66-D21 (*B*), D21-65-D1 (*C*), D1-171-D21 (*D*), and D21-170-D1 (*E*). The protection product of the expected size for each transgene is identified as transgene. Major decay intermediates (*Int*) are marked to the *right* of each *panel*.

lizing element(s), which exert their influence in the absence of the native UTRs.

Effect of the gstD1 mRNA Destabilizing Element(s) on gstD21 Coding Sequences—Recalling that D1 and D21 coding sequences share 70% sequence identity (Ref. 22 and Fig. 1), and given how the chimeric D21L-UTR mRNA, which contains the D21 coding region, is stable, we set out to localize these suspect destabilizing *cis*-acting elements in *gst*D1 mRNA and observe their destabilizing effect on an otherwise stable sequence. We constructed two pairs of chimeric D1-D21 genes, and established corresponding transgenic lines for each (D1-66-D21 and D1-171-D21 for D1-D21 chimeras; D21-65-D1 and D21-170-D1 for D21-D1 chimeras, see "Experimental Procedures" for nomenclature of chimeric genes). Each of the induced chimeric RNAs was analyzed by RPA with antisense D21 and D1 riboprobes. Results are shown in Figs. 4, *B–E*, and 5, *B–E*, respectively. The D21 probe detected an RNA band of ~450 nt (Fig. 4*B*, *D1–66-D21*) from the total RNAs of transgene D1–66-D21; the D1 probe, however, failed to detect anything (Fig. 5*B*).

These results suggest that the chimeric D1–66-D21 mRNA was unstable, particularly in the 201-nucleotide region of D1 (codons numbers 1–67). A time course analysis of heat shock induction (Fig. 4*B*, D1–66-D21) revealed that the chimeric mRNA was induced as soon as the inside of the experiment bottle reached 32 °C, between 5 and 10 min inside the 35 °C oven. This protected band from the D21 segment appeared exclusively for the transgene D1–66-D21 and was not observed in other transgenic or nontransgenic lines. The D1 portion, on the other hand, for which no band showed, probably degraded rapidly (Fig. 5*B*, *D1–66-D21*). Because the stable D21 component lies downstream from the D1 region in the chimeric mRNA (D1–66-D21), we know that degradation of the D1 sequence cannot be caused by 3′ → 5′ exonucleases from the poly(A) end (23–25).

In analyses of other chimeric mRNAs, the full-length D21 probe protected multiple fragments of the chimeric D21-170-D1 RNA (Figs. 4*E* and 5*E*). But this same probe protected only one fragment, and which was smaller than expected, in chimeric D21-65-D1 mRNA (Figs. 4*C* and 5*C*), and three very small fragments in D1-171-D21 mRNA (Figs. 4*D* and 5*D*). Recalling that endogenous *gst*D21 mRNAs are not induced by heat shock, the protected D21 subfragments must trace to the induced chimeric mRNAs.

The D1 riboprobe clearly protected endogenous *gst*D1 mRNA but as for the D1 portions of chimeric mRNAs yielded protection products at low to undetectable levels. No bands appeared for the D1-66-D21 and D21-170-D1 constructs (Fig. 5, *B* and *E*), and the D1 portions of chimeric mRNAs from D21-65-D1 and D1-171-D21 were detectable but only at very low levels (Fig. 5, *C* and *D*). Our results show these four D1-D21 (D21-D1) chimeric RNAs to be very labile, conceivably because of the presence of destabilizing D1 sequences. The instability of these chimeras was manifest very early into heat shock induction, with some decay intermediates appearing before induced chimeric mRNAs could be detected (Fig. 4, *D* and *E*). These decay intermediates were not generated during RNA isolation but, rather, increased along with the duration of heat shock. Meanwhile the reference RP-49 mRNAs remained intact throughout our time course analysis.

Results in Figs. 4 and 5 along with the demonstrated stability of the chimeric D21L-UTR mRNA (Fig. 3*B*), support the notion that the *gst*D1 coding sequence contains cryptic destabilizing elements. These *cis*-acting elements apparently exert their degradative influence in the absence of the native UTRs from the mRNA. A conceivable explanation, then, for how full-length *gst*D1 mRNA maintains its stability is that the UTRs contain a dominant STE(D1) that overrides any destabilizing influence from the coding region. This hypothesis, substantially supported by our findings, expands the current paradigm of mRNA stability regulation with this new detail of an additional stabilizer element.

Mapping Putative Decay Intermediates—We set out to identify a decay pattern for the D21 portion of the chimeric D21-D1 mRNAs by mapping the decay intermediates of each molecule from D21-65-D1, D1-171-D21, and D21-170-D1 flies with a nested set of D21 riboprobes. (We passed over the D1 portion because its intermediates were barely detectable.) RPA results are shown in Fig. 6. The decay intermediate from D21-65-D1 (Fig. 6*A*, *Int-Sa*) spanned ~100 nucleotides of the D21 sequence (numbers 81–181 of the 198 nucleotides from codons 1 to 66). The D1 portion of chimeric D21-65-D1 mRNA was barely detectable and only so very early into heat shock treatment (Fig. 5*C*). Three pieces of decay intermediates spanning ~50, 55, and 60 nucleotides were detected from D1-171-D21 by the D21 probe (Fig. 6*A*, *Int-1*, *Int-2*, and *Int-3*). As the sum of these lengths exceeds the entire D21 stretch (129 nt, numbers 567–

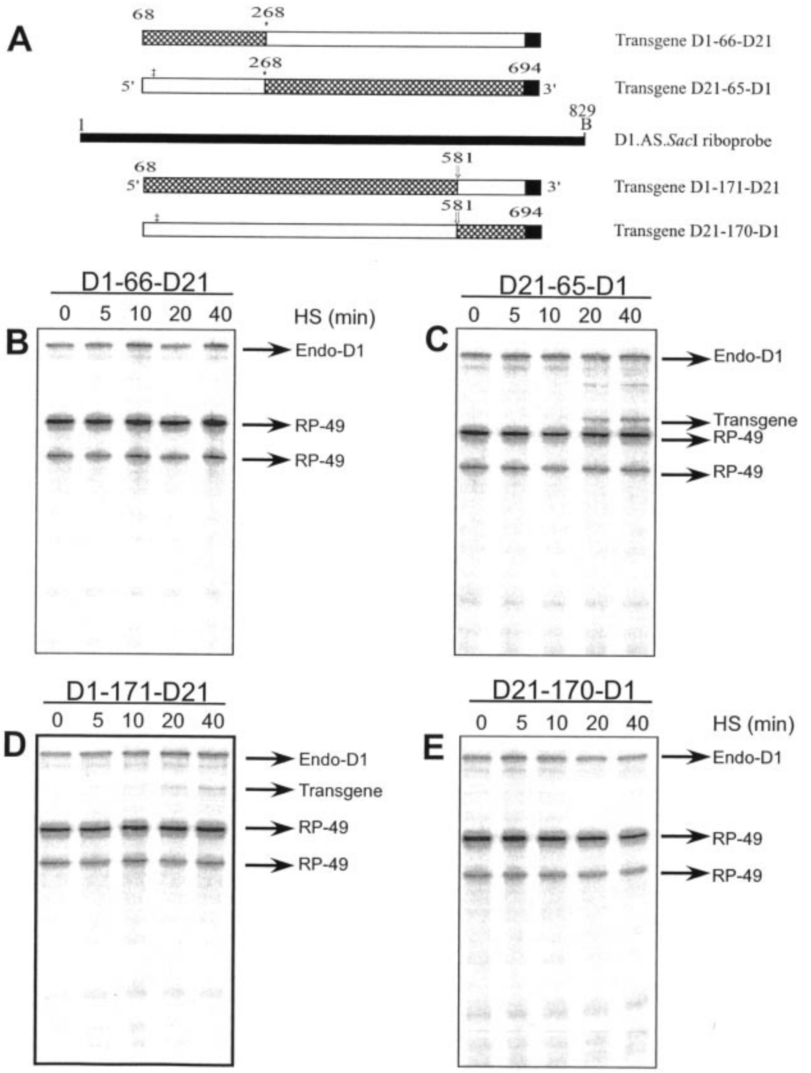

FIG. 5. **RPA analysis of chimeric D1-D21 mRNAs by full-length *gst*D1 riboprobe.** *Panel A*, diagrams of chimeric D1-D21 mRNAs in the context of the 5′-UTR of *hsp70* and the 3′-UTR of *actin 5C* (not shown). *Cross-hatched* and *open rectangles* represent D1 and D21 coding sequences, respectively. Regions of overlap with the *cross-hatched* segments are protected in the RPA. The coordinates of the *gst*D1 mRNA sequence are identified in the D1 portion of each chimeric gene. *Panels B-E,* total RNAs from transgenic lines D1-66-D21 (*B*), D21-65-D1 (*C*), D1-171-D21 (*D*), and D21-170-D1 (*E*) were hybridized with D1.AS.*Sac*I and RP49.AS.*Sac*I riboprobes. Heat shock (*HS*) was carried out at 35 °C for the times indicated *above* each panel. Each RPA product band is labeled to the *right* of each panel. *Endo-D1* represents protection product from endogenous *gst*D1 mRNA (824 nt) and, *transgene*, that from the various chimeric D1-D21 mRNA. The protected fragment from transgene D21–65-D1 is 418 nt and from transgene D1-171-D21 is 516 nt. The protected bands from RP-49 are 400 and 300 nt, respectively. No protection product was detected in the other two transgenic lines.

696 of the complete *gst*D21(L) mRNA) of the chimeric mRNA; they must partially overlap. Decay of the D21 part of the chimeric D21-170-D1 mRNA probably involves an endonucleolytic cleavage near the *Sma*I site (number 228 of the *gst*D21(L) sequence).[2] One major decay intermediate (*Int-1* in Fig. 4*E* and fragments marked by *arrows* in Fig. 6*B*) was mapped to the region of 81–547 of the D21 mRNA sequence, another intermediate (Int-2) to the region of 245–460, and a third (*Int-3* in Fig. 4*E* and *Int-3-Sa* in Fig. 6*B*) to the region of 81–245 (Fig. 6, *B* and *C*). The early appearance of stable decay intermediates from the induced transgene(s) indicates that the half-lives of the intact chimeric D21-D1 mRNAs are shorter than 20 min (Figs. 4 and 5). Given that the half-life of chimeric D21L-UTR mRNA in the same context of UTRs is much longer (Fig. 3*B*),[2] dramatically shorter half-lives for the D21-D1 chimeras strongly suggests, then, that the D1 coding sequence contains active destabilizing elements. The summary of mapping results (Fig. 6*C*) suggests that these destabilizing elements most likely are located in the first 67, and the last 37, codons of the D1 coding sequence. Chimeric RNAs containing these sequences are either undetectable (transgenes D1-66-D21 and D21-170-D1) or detectable only at very low levels (transgenes D21-65-D1 and D1-171-D21). These destabilizing elements are suppressed in endogenous *gst*D1 mRNA, which must contain the proposed dominant stabilizer element STE(D1) in its native UTRs.

DISCUSSION

mRNA stability is an important regulatory factor in gene expression. The relative stability of mRNA determines its lifespan and thus, its translatability, in the cytoplasm (1–5). Endogenous *gst*D1 mRNA is quite stable under both control and PB treatment conditions. A ~2-fold PB-induced increase in the transcription rate of *gst*D1 accordingly resulted in a ~2-fold increase in the steady-state level of *gst*D1 mRNA (15). How, then, does stable *gst*D1 mRNA return to normal levels after the PB inducer is removed from the flies? The presence of cryptic, *cis*-acting, destablizing elements in the coding region of *gst*D1 provides a possible avenue. Just how they exert their influence, however, remains to be elucidated.

In the absence of their native UTRs, D1 portions of the D1-D21 chimeric RNAs were shown not only to be degraded themselves, but also to destabilize segments of the D21 coding sequence that we know is stable in the absence of its native UTRs (Figs. 3 and 4). Mapped decay intermediates from the chimeric D1-D21 mRNAs display patterns that are consistent with the hypothesis that the destabilizing elements are, most likely, located in the N-terminal (codons 1–67) and C-terminal (codons 172–209) regions of the GST D1 coding sequence. It also indicates that endonucleolytic cleavage(s) are probably involved in the decay pathway(s).

The stability of endogenous *gst*D1 mRNA, therefore, must

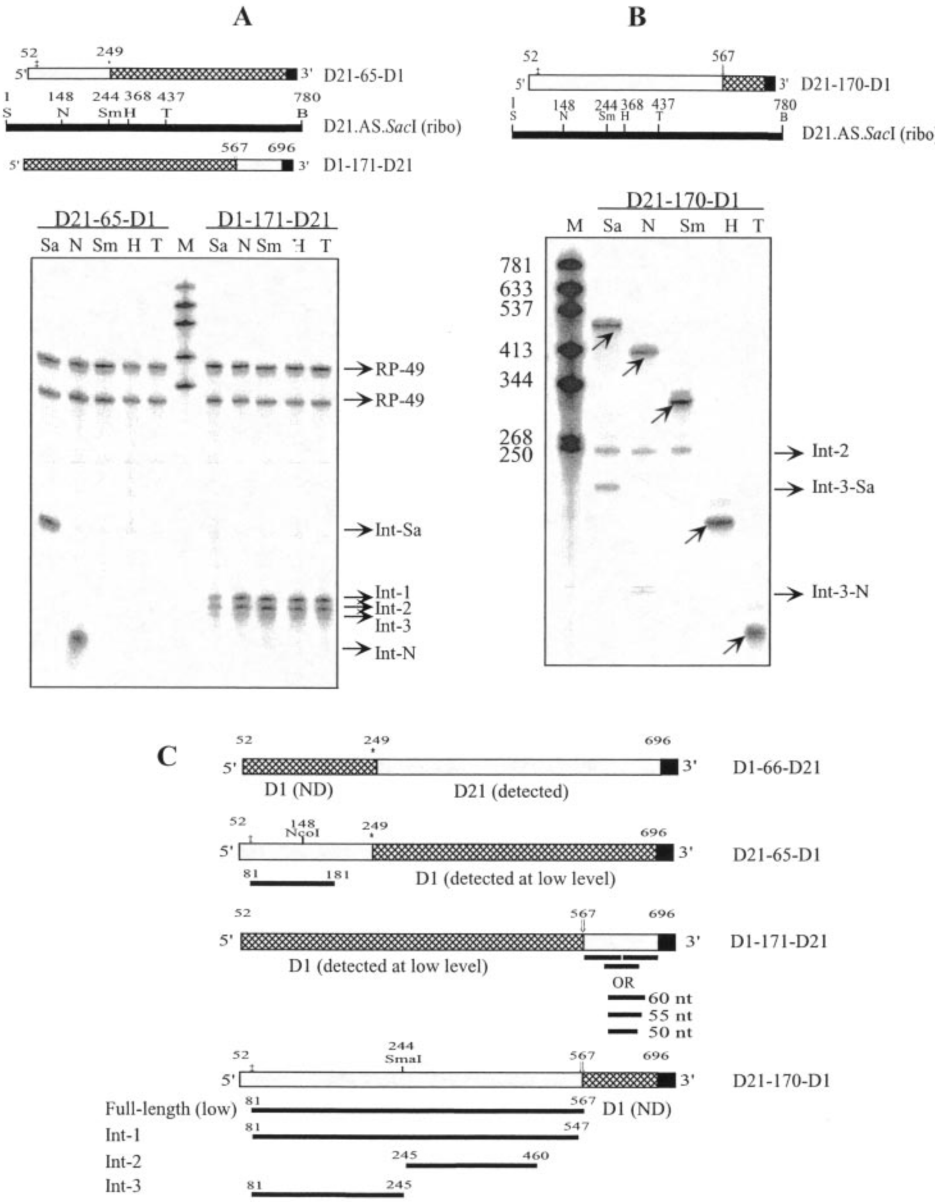

FIG. 6. **A summary of mapping the decay intermediates (*Int*) in transgenic lines.** A diagram of the transgene(s) is shown *above* each RPA pattern in both *panels A* and *B*. The restriction sites are indicated for D21.AS DNA used in riboprobe synthesis. Nucleotides of the complete *gst*D21(L) cDNA are numbered from 1 to 780, so are the D21 portions of each chimeric transgene. The same strategy was used to determine the sizes and the locations of the intermediates from total RNAs of D21-65-D1, D1-171-D21 (*Panel A*), and D21-170-D1 (*Panel B*). Total RNAs for the RPA assays were isolated from combined heat shock and PB-treated flies. Riboprobes were obtained from *Sac*I (*S*), *Nco*I (*N*), *Sma*I (*Sm*), *Hin*dIII (*H*), or *Tfi*I-digested D21.AS (*T*). The RP-49.*Sac*I riboprobe was included in the RPA analyses of D21-65-D1 and D1-171-D21 to demonstrate that the intermediates are not the result of poor RNA quality. Fragments protected in RPA are regions overlapping with the D21 portion (*open rectangles*) beginning from the G8S,G9S mutation. The expected sizes are 170 nucleotides for D21-65-D1, 130 nucleotides for D1-171-D21, and 490 nucleotides for D21-170-D1. The protected RP49 bands are 400 and 300 nucleotides. The sizes of the bands from Int-1 of D21-170-D1 mRNA (marked by *arrows*) are: 480 (*lane Sa*), 405 (*lane N*), 300 (*lane Sm*), 180 (*lane H*), and 110 (*lane T*) nucleotides. *Panel C*, a summary of RPA analyses of the four D1-D21 chimeric mRNAs by both the D1 and D21 riboprobes in Figs. 4 and 5. *Hatched rectangles* represent the D1 coding sequence and *open rectangles* represent the D21 coding sequence. *Solid squares* represent the FLAG epitope. Decay intermediates are indicated by *thick lines*. The G8S,G9S mutations in GST D21, Arg^{66} junction, and 170/171 junctions are indicated by ‡, *, and ⇓, respectively. *Numbers* indicate nucleotide positions of the full-length *gst*D21(L) sequence. *ND*, not detectable.

rely on a dominant STE that overrides these destabilizing elements. The fact that both chimeric D1 mRNAs from transgenes D1-UTR and D1F-UTR, which lack native UTRs, are both very labile is strong evidence that this putative STE(D1) resides in the UTRs of *gst*D1 mRNA. The presence of STE(D1) may also explain the occurrence of a small fraction of stable, decapped, and shorter than full-length *gst*D1 mRNAs in control and PB-treated RNA populations (see Fig. 2).

Studies in the yeast *Saccharomyces cerevisiae* have revealed that decapping triggered poly(A)-shortening leads to 5′ → 3′ exonucleolytic degradation (26, 27). This pathway of mRNA decay has also been detected in mammalian cells (18). If this same pathway also persists in *D. melanogaster* then the presence of stable decapped *gst*D1 mRNA would indeed be impossible without the function of a stabilizer element. The P-STE stabilizer, found in the coding region of the yeast *PGK1* mRNA has been shown to block deadenylation-dependent mRNA decay (9). The short poly(A) (~40 As) of the intact molecules and presence of decapped *gst*D1 mRNA in the natural population suggest that the putative STE(D1) functions similarly to the yeast P-STE by blocking 5′ → 3′ exonucleolytic degradation.

STEs are a known feature of yeast and mammalian mRNAs (6–9). Several yeast mRNAs that contain upstream open reading frames in the 5′-UTR are degraded through nonsense-mediated decay (3, 8, 28). But for certain genes, such as *GCN4* and *YAP1*, mRNAs are known to harbor a stabilizer element in the 5′-UTR just upstream of the main open reading frame. In *GCN4* mRNA this STE protects the molecule from rapid decay by interacting with the RNA-binding protein Pub1p, which is required in the nonsense-mediated decay pathway (8). Our findings provide solid evidence that such a STE(D1) works similarly on the mRNA of a multicellular eukaryotic organism. Moreover, this STE would be similarly located to the stabilizer element of the α-globin mRNA if it should fall in the 3′-UTR of *gst*D1 mRNA (6, 7). There is, however, no pyrimidine-rich segment in *gst*D1 UTRs as there is in the 3′-UTR of α-globin mRNA (7).

The *Drosophila gst*D1 and *gst*D21 genes are adjacently located but divergently transcribed (20). Although their coding sequences share 70% identity, their products perform very different enzymatic functions. GST D1 is a 1,1,1-trichloro-2,2-bis-(*P*-chlorophenyl)ethane dehydrochlorinase as well as a glutathione *S*-transferase. GST D21, on the other hand, does not exhibit normal GST activity (12) but may be an important ligand-binding protein (*i.e.* ligandin). The UTRs of both the *gst*D1 and *gst*D21 mRNAs appear to contain *cis*-acting regulatory element(s), but ones which function quite differently. The native UTRs of the *gst*D1 mRNA are essential to the stability of the molecule, whereas those of *gst*D21 mRNA contain one or more element(s) that render the molecule very unstable in the absence of PB.[2] The coding regions of *gst*D1 and *gst*D21 also exhibit contrasting behaviors with respect to mRNA stability. In the same context of the *hsp*70 5′-UTR and the *actin5C* 3′-UTR, we observe that, on the one hand, the D21 coding sequence remains very stable (Fig. 3*B*), but that, on the other, the D1 coding sequence becomes very labile. The stark differences in behavior between *gst*D1 mRNA and *gst*D21 mRNA with regard to stability show the potential for diversity in yet another aspect of expression regulation within a multigene family.

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Evidence for a Stabilizer Element in the Untranslated Regions of *Drosophila* Glutathione *S*-Transferase D1 mRNA

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