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Pentobarbital-mediated Regulation of Alternative Polyadenylation in *Drosophila* Glutathione *S*-Transferase D21 mRNAs*

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Two nearly identical, *gst*D21(L) and *gst*D21(S) mRNAs, whose polyadenylation sites differ by 19 nucleotides, are transcribed from the intronless glutathione *S*-transferase D21 gene in *Drosophila*. Both mRNAs are intrinsically very labile, but exposure to pentobarbital renders them stabilized beyond what can be attributed to transcriptional activation. We have reconstituted this PB-mediated mRNA stabilization in a transgene (D21L) that contains the full-length *gst*D21(L) sequence. We have also constructed a similar transgene (D21L-UTR), which matches D21L but excluded the native 3′-UTR. D21L-UTR produces a relatively stable RNA, whose stability is unaffected by pentobarbital. Following pentobarbital treatment of wild-type flies, the levels of *gst*D21(L) and *gst*D21(S) mRNAs hold at a relatively constant ratio (L/S) of 1.4 ± 0.2. In transgenic flies, heat shock induction of D21L mRNA changed the L/S ratio to 0.6 ± 0.1, and it was further reduced to 0.3 ± 0.1 as D21L mRNA accumulated in the presence of PB. The ratio returned nearly normal (1.1 ± 0.1) as the D21L mRNA decayed over 12 h after terminating induction. In constrast, when D21L-UTR was present, the ratio remained constant (1.7 ± 0.2) even under various induction conditions and during recovery. Thus, the 3′-UTR, which was the critical difference between these two transgenes, must have some role in determining the L/S ratio. Induced D21L mRNA alone is not sufficient to cause reversible changes in the ratio. Such changes require the presence of pentobarbital. Therefore, pentobarbital may regulate this L/S ratio by affecting the choice of polyadenylation sites for the *gst*D21 mRNAs through sensing the concentrations of the native 3′-UTR sequences.

The poly(A) tail found at the 3′-end of nearly every eukaryotic mRNA affects virtually all aspects of mRNA metabolism (for reviews, see Refs. 1 and 2). The process of polyadenylation is a complex and highly regulated process that involves well-characterized *cis*- and *trans*-acting factors (3–5). Although gene regulation of newly synthesized RNAs at the level of polyadenylation is not known to occur frequently, the number of reported examples of multiple polyadenylation sites in the non-coding 3′-exon is increasing (6–9). The efficiency of polyadenylation at various sites and/or the stability of mRNA polyadenylated at various sites have been shown to change with the metabolic state of a cell (6) or with stages of cell differentiation (8). The molecular basis of these observations, however, is currently not well understood. Furthermore, the potential effect of drugs and environmental chemicals on alternative polyadenylation has not yet been explored in detail.

The following communication details a more in-depth continuation of our previous investigations into pentobarbital (PB)[1]-mediated mRNA stability. Our results have directed us to focus attention on this region of the *gst*D21 mRNA molecule, the 3′-UTR as a possible critical site on mRNA stability, and alternative polyadenylation.

The glutathione *S*-transferases (GSTs, EC 2.5.1.18) are important Phase II detoxification enzymes (10), and all are products of a gene superfamily (11). The 10-member *Drosophila* *gst*D gene family (12–14) includes *gst*D1 and *gst*D21 genes, which we have been using as reporters for investigating drug-induced changes in mRNA stability (15–16). GST D1, the product of *gst*D1, is a DDT dehydrochlorinase, which converts DDT to DDE; its homolog *gst*D21 encodes a glutathione conjugate-binding protein with very low enzyme activities (17).

We have determined that PB, a general anesthetic, induces the expression of *Drosophila* *gst*D genes. This drug has been observed to induce *gst*D21 mRNA >10-fold, but most of this increase was thought to have come from changes in mRNA stability (15). In this communication, we report the mapping of two *gst*D21 mRNAs (L and S), which differ in polyadenylation sites. Both forms are equally inducible by PB, with the ratio (L/S) holding at relatively constant proportions in the wild type flies. We have reconstituted the PB-mediated *gst*D21 mRNA stabilization in a heterologous context and designed experiments that focus on the importance of the native 3′-untranslated region (3′-UTR) in determining *gst*D21 mRNA stability. We then used the induction and decay of this labile transgenic mRNA sequence to probe the effect of pentobarbital on alternative polyadenylation of the native *gst*D21 mRNAs. Our results show that a drug such as pentobarbital may regulate alternative polyadenylation. Moreover, this PB regulation appears to involve an important role for the level of the 3′-UTR sequence as a critical element recognized by the polyadenylation machinery.

EXPERIMENTAL PROCEDURES

Materials—Bacteriological media, laboratory chemicals, radioactive nucleotides, oligonucleotides, and enzyme reagents were products of

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[1] The abbreviations used are: PB, pentobarbital; AS, antisense; GST, glutathione *S*-transferase (EC 2.5.1.18); *gst*, glutathione *S*-transferase gene; HS, heat shock; HSPB, combined heat shock and PB treatment; nt, nucleotides; RPA, RNase protection assay; UTR, untranslated region (5′ or 3′) of a mRNA.

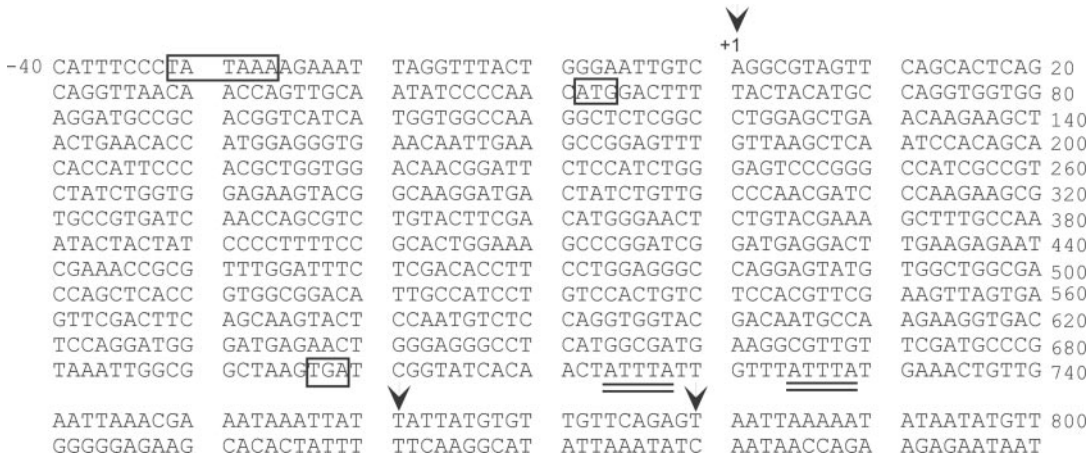

FIG. 1. **Complete sequences of the *Drosophila* gstD21 mRNAs.** The mRNA begins at +1 and ends at 780 for *gst*D21(L) or at 761 for *gst*D21(S) before the poly(A) tail (*arrows*). The putative TATA box sequence is from −27 to −32 (*boxed*). The initiation and termination codons are marked by *shaded boxes*. Two AUUUA motifs in the 3′-UTR are *underlined*. The poly(A) signal for *gst*D21(L) is most likely the sequence AAUAAA (751–756). The complete genomic sequence of *gst*D21 can be found under GenBank™ accession number M97702.

commercial suppliers as previously described (16). The plasmid pSP64(A) and a cDNA cloning kit were products of Promega (Madison, WI). RPA III kits were purchased from Ambion (Austin, TX). The *Drosophila* transformation vector pCaSpeR-*hs-act* was obtained from C. S. Thummel (18). T7 RNA polymerase was a generous gift from Bi-Cheng Wang (University of Georgia, Athens, GA).

Drosophila Strains, Transgenic Lines, and PB Treatment—Adult flies were maintained by the standard cornmeal agar medium in half-pint milk bottles at 21–25 °C as previously described (15–16). The laboratory stock Oregon-R (OR) was obtained from Tao-shih Hsieh of Duke University (Durham, NC). The Δ2–3 line {*P*[ry$^+$Δ2–3](99B)} (19) expressing the transposase and *yw* strain were obtained from Susan Abmayr and David Gilmour, respectively, both of this department. To clone full-length *gst*D21(L) (780 nucleotides, Ref. 13) into pCaSpeR-*hs-act*, we introduced EcoRI and BamHI restriction sites at the ends of the fragment by PCR amplification of the full-length *gst*D21(L) cDNA in pSP64(A), using the primers D21-FL-5′-RI (GGAATTCAGGCGTAGTTCAGCAC) and D21-FL-3′-Bam (CGGGATCCACTCTGAACAACACATAATA). This transgene is referred to as D21L (three lines: 7Y, 12OR, and 23R). Transgene D21L-UTR (D21L minus UTR, lines 4R, 2OR and 5Y), which contains 32 nucleotides of the 5′-UTR and the D21 coding sequence [nucleotides 20–699 of the *gst*D21(L) mRNA sequence in Fig. 1], was amplified with the primer set H17–5′ (5′-GGGAATTCGCAGGTTAACAACCAGTTGC-3′) and H17–3′ (5′-CGGGAATTCGGATCCTCATCACTTAGCCGCCAA-3′). All plasmid constructs were sequenced, and then microinjected into embryos to develop transgenic lines (19–20). In contrast to the chimeric D21 mRNAs, we refer to the two forms of endogenous *gst*D21 mRNAs as *gst*D21(L) and *gst*D21(S) mRNA, for "long" and "short," whose polyadenylation sites differ by 19 nucleotides (see Fig. 1).

Before PB treatment, adult flies (2–3-day-old) were distributed into clean milk bottles in approximately equal numbers for 5 h starvation at room temperature. A blotting paper strip (3 × 10 cm) saturated with a 5% sucrose solution (control flies) or 200 mg/ml PB in 5% sucrose (PB-treated flies) was placed in each bottle for two hours at room temperature. Heat shock of the flies was carried out in clean milk bottles containing 5% sucrose-saturated paper strips normally at 35 °C (or 37 °C where specified) for 1 h in a hybridization oven (Robbins Scientific Company, Sunnyvale, CA). It takes ~15 min for an empty milk bottle with a foam plug to warm to 35 °C. Once a bottle is removed from the 35 °C oven, the temperature inside the bottle drops to below 30 °C in 7.5 min. For the combined induction of transgene and endogenous *gst*D21 mRNAs, the flies were treated with PB at room temperature for 1 h and then at 35 °C (or 37 °C where specified) for the second hour. The flies ingested more PB at 35 °C (or 37 °C) than at room temperature. To determine the time course of decay of transgenic and endogenous *gst*D21 mRNAs, flies were allowed to recover at room temperature in milk bottles containing a paper strip soaked in 5% sucrose for varying durations following treatment: 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h. The flies were snap-frozen in liquid nitrogen and stored at −70 °C until use. The relative intensity of chimeric D21 mRNAs at each point of recovery was determined by a PhosphorImager (Molecular Dynamics, CA) and normalized against the RP49 signals. The value at zero time of recovery was set at 100%. The log [%RNA] at each time point of recovery was plotted against time of recovery to get a semi-log plot. Each point of the data set is the average from 3–4 independent experiments. The half-life of different D21 mRNAs was calculated using the trendlines of the Microsoft Excel program as described in Table I. Only data points from 2–10 h of recovery were used in the calculation of the half-life.

Mapping of gstD21 mRNAs—Two complementary experiments were carried out to map the start site of *gst*D21 mRNA transcription: S1 nuclease and primer extension (21). We used the E3.8 subclone of λGTDm101 genomic clone (13) for S1 mapping. The oligonucleotide D21-PE-5′ (5′-GACCGTGCGGCATCCTCCACCACC-3′) was used for primer extension and for generating sequencing ladders in S1 nuclease mapping. 3′ RACE was employed to map the 3′-end of *gst*D21 mRNA, which was PCR-amplified using a gene-specific primer (5′-GGAATTCATGGCGATGAAGGCGTTGTTCG, designated 5-RACE-D21, EcoRI site underlined) and an oligonucleotide with convenient restriction sites (*e.g.* HindIII, underlined) at its 5′-end [5′ CGGATATCGAATTCTCGAGAAGCTT$(T)_{17}$]. The PCR fragment was cloned into EcoRI-HindIII-digested pBluescript KS$^+$ vector. Because of the mobility differences of several cloned inserts in agarose gel electrophoresis, nine clones were sequenced to confirm the presence of two different 3′-ends.

Isolation and Analysis of RNA—Total RNAs from adult flies were isolated according to published procedures (22). The RNA purity was assessed by the A_{260}/A_{280} (≥1.8). Riboprobes were prepared from the antisense cDNA clones in pSP64(A) vector, pSP64(A).D21.AS, pSP64(A).D21.Act.AS, pSP64(A).49.AS by *in vitro* transcription of linearized plasmid DNA with T7 or SP6 RNA polymerase in the presence of [α-^{32}P]UTP according to Promega's instructions. Isolation of riboprobes from gels and RNase protection assays were performed according to the manufacturer's instructions RPA III kit (Ambion). Using RNase protection assay (RPA) we tracked the steady state levels of endogenous *gst*D21 mRNAs and of each constructed chimeric mRNA, under control (C), PB (PB), heat shock (HS), and combined heat shock and PB (HSPB) treatments. We also analyzed endogenous *gst*D21 mRNA levels by RPA in non-transgenic flies (*yw*, the parent line of the transgenic flies). For RPA results, we determined the radioactivity in each protected RNA fragment on each gel by a PhosphorImager (Molecular Dynamics, CA) with the ImageQuant software package. The intensity of each protected band in RPA was normalized against the RP49 mRNA signals before comparison.

RESULTS

Mapping of gstD21 mRNAs—Our 5′-end mapping results reveal that the *gst*D21 mRNA start site, with an A at position 1, is 32 nucleotides downstream from a potential TATA sequence (Fig. 1). The 5′-UTR runs for 51 nucleotides, and is followed by a coding region of 215 codons. The *gst*D21 mRNAs take on two forms: a long form, *gst*D21(L) mRNA, which terminates at position 780, and a shorter form, *gst*D21(S) mRNA, which ends at position 761 (Fig. 1). The 3′-end cleavage site for

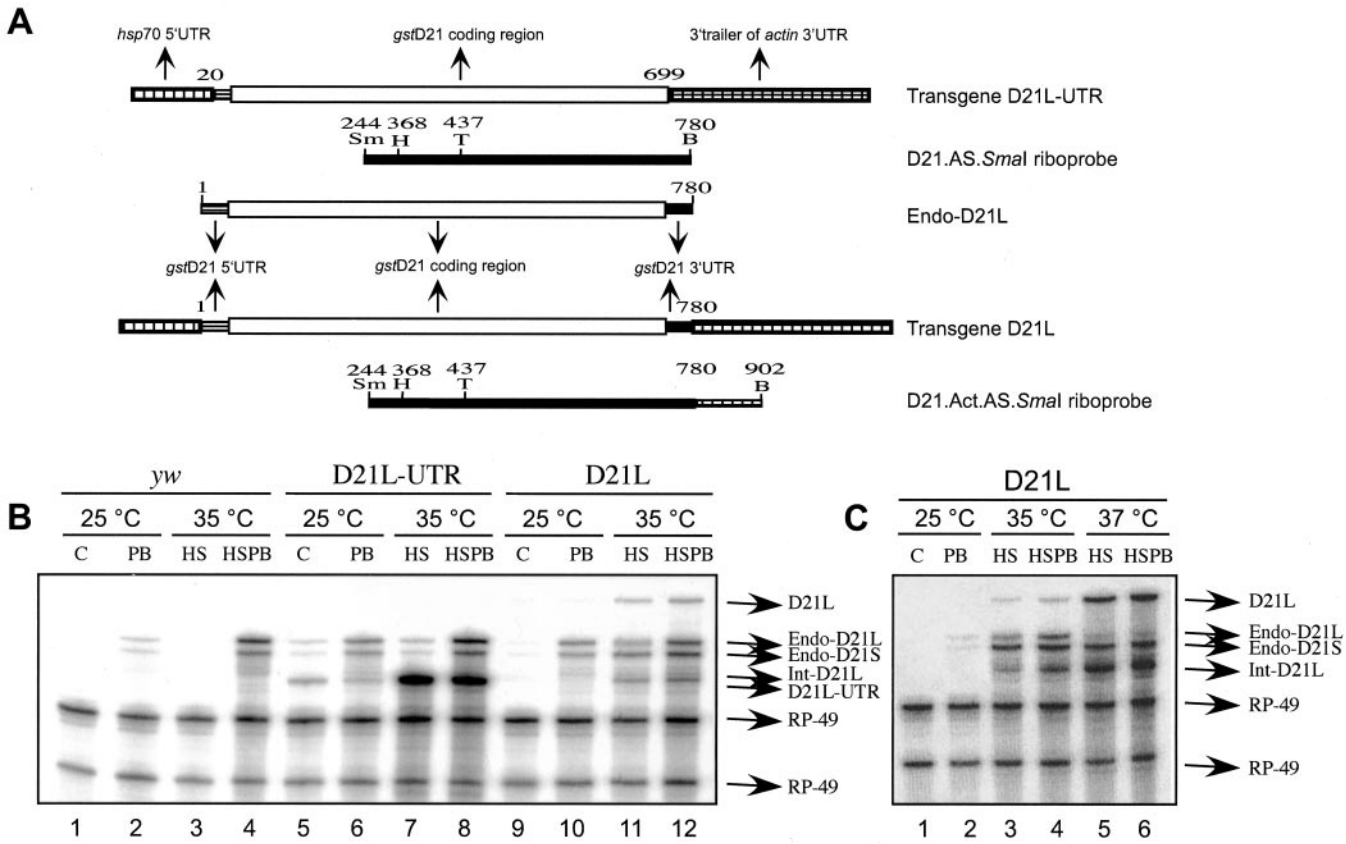

FIG. 2. **Effect of transgene expression on the ratio of *gst*D21(L) mRNA and *gst*D21(S) mRNA.** *Panel A*, diagrams of the transgenes D21L and D21L-UTR, the endogenous *gst*D21 mRNAs, and the riboprobe templates, SmaI-linearized D21-AS and SmaI-linearized D21.Act.AS. The numbers correspond to nucleotides in Fig. 1. The symbols are: *open bar, gst*D21 coding region, *horizontal bar*, *gst*D21 5′-UTR, *black bar, gst*D21 3′-UTR, *vertical hatch*, *hsp*70 5′-UTR, *black-bordered hatch*, *actin*5C 3′-trailer. The restriction sites are: *Sm*, SmaI; *H*, HindIII; *T*, TfiI; *B*, BamHI. *Panel B*, levels of the endogenous *gst*D21(L) and *gst*D21(S) mRNAs and chimeric D21 RNA from Transgene D21L or D21L-UTR in the total RNAs of different flies under control (*C*), PB treatment (*PB*), heat shock (*HS*), combined PB and heat shock treatment (*HSPB*). We determined the intensity of each band relative to the RP-49 RNA bands by a PhosphorImager. The D21.Act.SmaI probe protects 665 nt of the chimeric D21L mRNA (D21L), 536 nt of *gst*D21(L) mRNA (Endo-D21L), or 517 nt of *gst*D21(S) mRNA (Endo-D21S). The D21.AS.SmaI probe protects 455 nt of the chimeric D21 mRNA from transgene D21L-UTR (D21L-UTR), 536 nt of *gst*D21(L) mRNA (Endo-D21L), or 517 nt of *gst*D21(S) mRNA (Endo-D21S). The RP49 riboprobe (RP49.AS.SacI) protects two bands, 400 and 300 nt long. The *yw* strain is the non-transgenic control for D21L and D21L-UTR transgenic flies. Int-D21L is the major putative degradation intermediate of chimeric D21L mRNA. *Panel C*, effect of overinduction at a higher temperature of chimeric D21L mRNA on the endogenous D21L mRNA. Transgene D21L (line 23R) was maintained at 25, 35, and 37 °C in the presence and absence of PB treatment (C *versus* PB for 25 °C, non-heat shock temperature; HS *versus* HSPB for 35 °C and 37 °C, heat shock temperature). RPA conditions and patterns of protected bands are the same as those in *panel B*.

the *gst*D21(L) mRNA has the consensus recognition sequences of the mammalian polyadenylation system (4, 23–24). The ubiquitous poly(A) addition signal, AAUAAA (nt 751–756) is located 30 nucleotides upstream of the cleavage site. Next, a GU-rich sequence, proposed to enhance the efficiency of 3′-end processing, is located 16 nucleotides downstream from the 3′-cleavage site (24–25). The *gst*D21(S) mRNA may have its own poly(A) addition signal in an AUUAAA motif (nt 742–747), and followed by a GU-rich sequence (UGUGUUGUU, nt 766–774) 14 nucleotides downstream.

Reconstitution of PB-mediated gstD21 mRNA Stabilization in a Heterologous Context—In order to investigate PB-mediated stabilization of *gst*D21 mRNA, we established two chimeric constructs in the same heterologous UTR context of the pCaSper-*hs-act* vector for *Drosophila* transformation. The D21L transgene expresses a chimeric D21 mRNA composed of the 5′-UTR of the *hsp70* mRNA, the full-length *gst*D21(L) sequence (1–780, Fig. 1), and the 3′-UTR of *actin5C* from the pCaSpeR-*hs-act* vector (18). The D21L-UTR transgene retains the same UTR context as the chimeric D21L mRNA but expresses only a partial D21 sequence (20–699 of Fig. 1). This latter construct thus leaves out the native 3′-UTR, but includes 32 nucleotides of the 5′-UTR, in order to preserve, as much as possible, the context of *gst*D21 mRNA translation. And by this design, we hoped to minimize translation as a contributor to *gst*D21 mRNA turnover.

Upon induction by HS, each of these transgenic lines produced chimeric mRNA composed of the *hsp*70 5′-UTR, the cloned fragment (Fig. 2*A*) and the 3′-trailer of the *actin*5C 3′-UTR (18). We know that HS treatment does not affect transcription from the endogenous *gst*D21 promoter. In contrast, PB treatment induces its mRNA exclusively. Hence, any increase in the level of a transgenic mRNA under combined PB and HS (HSPB) treatment, beyond what results from HS alone, must be the result of mRNA stabilization by PB treatment. Results are shown in Fig. 2, *B* and *C*.

Under control conditions, endogenous *gst*D21 mRNAs were almost undetectable (15), and heat shock affected neither the induction nor the stability of transcripts (*lanes 1 versus 3*, Fig. 2*B*). We observed that PB induced both *gst*D21 mRNAs (*lanes 1 versus 2*, Endo-D21L and Endo-D21S, Fig. 2*B*) ~10-fold in control flies (*yw*), with induction even more pronounced at 35 °C (heat shock). We attributed this difference to the increased ingestion of PB solution by the flies at higher temperatures (*lanes PB versus HSPB* under *yw* (*2 versus 4*), D21L-UTR (*6 versus 8*), and D21L (*10 versus 12*)). Two faint but clearly visible bands to which we will tentatively refer as putative decay intermediates of endogenous *gst*D21 mRNAs ap-

pear below the protection product of *gst*D21(S) mRNA. Their intensities increase along with rising levels of endogenous *gst*D21 mRNA (*lanes PB versus HSPB* under *yw* (*2 versus 4*), D21L-UTR (*6 versus 8*), and D21L (*10 versus 12*) in Fig. 2*B*), suggesting a precursor-product relationship. The chimeric D21 mRNA is detectable in the D21L-UTR lines (Fig. 2*B*, *lanes 5* and *6*) from basal level of transcription of the *hsp*70 promoter, which is known to be leaky at room temperature. The enduring presence of this chimeric D21L-UTR mRNA suggests that under control conditions, this transcript, which lacks the native 3′-UTR of *gst*D21, is more stable than endogenous *gst*D21 mRNA. We also noticed that the D21L-UTR lines expressed detectable basal levels of endogenous *gst*D21 mRNA under control conditions. These levels, while produced independent of heat shock (*lanes 5 versus 7*, Fig. 2*B*), were noticeably elevated by PB induction (*lanes 6 versus 8*, Fig. 2*B*).

Chimeric mRNA from the transgene D21L is induced to a lesser extent by heat shock than that of D21L-UTR (*lane HS*, *7 versus 11*; Fig. 2*B*). This behavior accords with our premise that chimeric D21L mRNA, which contains the full-length *gst*D21L sequence, is unstable, and may explain why the same leaky *hsp*70 promoter produced no detectable chimeric D21L mRNA in transgenic flies at room temperature (*lanes 9* and *10* under D21L, 25 °C). The enhanced presence of endogenous *gst*D21 mRNAs in D21L flies untreated by PB, then, suggests a contribution of heat shock-induced chimeric D21L mRNA (*lanes 11 versus 9*, Fig. 2*B* (*5* or *3 versus 1*), Fig. 2*C*). We are proposing that these populations of endogenous *gst*D21 mRNAs are able to resist decay because of RNA stabilization involving chimeric D21L mRNA, which contains the full-length *gst*D21(L) sequence in a heterologous context. In other words, as far as RNA stability is concerned, *gst*D21(L) sequence in the chimeric D21L mRNA is counted as the pool of endogenous *gst*D21(L) mRNA but D21L-UTR RNA, which lacks the 3′-UTR and 19 nucleotides of the 5′-UTR, is not.

PB induction of endogenous *gst*D21 mRNAs in the D21L transgenic flies led to a 1.2–1.5-fold increase in levels of heat shock-induced chimeric D21L mRNA (lanes HSPB *versus* HS; Fig. 2B (*12 versus 11*) and 2C (*4 versus 3* and *6 versus 5*)). The unexpected general increase in mRNAs, both chimeric and endogenous, suggests that PB stabilization equally affects both endogenous D21 mRNAs and the chimeric D21L RNA. Moreover, we observed that the intensity of the bands representing putative decay intermediates (Int-D21L and the ones from the endogenous *gst*D21 mRNAs) increases in parallel to expression levels of both chimeric D21L mRNA and endogenous *gst*D21 mRNAs [lanes PB, HS, HSPB under D21L, Fig. 2*B* (*lanes 10–12*) and 2*C* (*lanes 2–6*)). Considering that the critical difference between the two chimeric transcripts of D21 transgenes D21L and D21L-UTR is the presence or absence of the native 3′-UTR, these results point to this native 3′-UTR as a critical determinant in PB-mediated *gst*D21 mRNA stabilization.

Effect of Pentobarbital and Transgene Expression on the Ratio of gstD21(L) to gstD21(S) mRNAs—Following PB induction, the L/S ratio stays at ~1.4 ± 0.2 in *yw* (Fig. 2*B*, *lanes 2* and *4*) and OR flies (data not shown). In D21L-UTR flies, this L/S ratio is slightly higher at 1.7 ± 0.2 (Fig. 2*B*, *lanes 6* and *8*). This ratio is reduced to 0.3 ± 0.1 and 0.6 ± 0.1, however, when the D21L transgene is expressed under HSPB and HS conditions, respectively (Fig. 2*B*, *lanes 11* and *12*). We pushed production of the chimeric D21L mRNA level further by administering a more severe HS (to 37 °C) and saw a dramatic increase in chimeric D21L mRNA (Fig. 2*C*, 35 °C *versus* 37 °C) accompanied by a corresponding decrease in the *gst*D21(L) mRNA such that the L/S ratio fell to < 0.1 (Fig. 2*C*, *lanes 3*, *4 versus 5*, *6*; Endo-D21L). But accounting for both chimeric and endogenous D21L sequences, the adjusted L/S ratio came closer to the normal value of 1.4 (Fig. 2*C*). In two separate lines of our D21L transgenic flies, the endogenous *gst*D21 mRNAs, which are labile under control conditions, now survive and accumulate to increased levels in the absence of PB (Fig. 2*B*, *lane 11*). The observed increase cannot be attributed to heat shock, which is known not to activate the native *gst*D21 promoter. Moreover, endogenous *gst*D21 mRNAs are intrinsically labile under both control and heat shock conditions (Fig. 2*B*, *lanes 1 versus 3* and *9 versus 11*). Thus, the appearance and survival of endogenous *gst*D21 mRNAs in the absence of PB strongly suggests that the chimeric D21L mRNA somehow stabilizes them. It is critical to note that D21L mRNA is the chimeric transcript that contains the full-length *gst*D21L mRNA sequence. No such effect was observed in the D21L-UTR transgenic lines whose chimeric D21 mRNA contains no native 3′-UTR (Fig. 2*B*, *lanes 5–8*).

Conservation of Relative Ratios of gstD21(L) to gstD21(S) mRNAs—It captured our interest that the L/S ratio is affected by the presence of D21L mRNA but not the D21L-UTR mRNA. The ratio was 1.4 ± 0.2 in PB-treated *yw* after PB treatment and during recovery from PB treatment (Fig. 3). To test whether the lowered L/S ratio observed in HSPB-treated D21L flies could be reversed back toward normal, we tracked this ratio over a 12-h time course after the heat-shock and PB treatment had ceased.

In the transgenic line D21L-UTR, over-expression of the chimeric D21L-UTR mRNA, which lacks the native 3′-UTR, did not significantly perturb the ratios (1.7 ± 0.2) under HSPB treatment (Fig. 3*A*). Nor did the ratio shift as the D21L-UTR mRNA decayed over a 12-h period. In the D21L transgenic lines (7Y or 23R), whose chimeric D21L mRNA contained the full-length *gst*D21(L) sequence, the L/S ratio remained at 0.6 ± 0.1 throughout the 12-h recovery period following heat shock (*i.e.* no additional induction from the native *gst*D21 promoter) (Fig. 3, *A* and *B*). Under HSPB treatment, on the other hand, which induced both the chimeric D21L mRNA and the endogenous *gst*D21 mRNAs, the ratio dropped to 0.3 ± 0.1 soon after treatment, but thereafter increased gradually toward 1.1 ± 0.1 over a 12-h recovery period (Fig. 3*C*). If our evidence holds, the difference in L/S ratios and their shift patterns between the two transgenic lines (*i.e.* D21L-UTR *versus* D21L) reflects the sequence disparity between transgenes: the D21L-UTR transgene lacks the native 3′-UTR and the first 19 nucleotides of 5′-UTR in contrast to the D21L transgene, which possesses both. Thus, we have reason to suspect that the *gst*D21(L) sequence, especially the native 3′-UTR in the chimeric D21L mRNA, is equally recognized by the cellular polyadenylation machinery as is native *gst*D21(L) mRNA. The combined account of chimeric D21L mRNA and endogenous *gst*D21(L) mRNA, however, balances out a near normal ratio of 1.4 over the *gst*D21(S) mRNA in the D21L transgenic lines (*e.g.* Fig. 2*C*). As the unstable chimeric D21L mRNA decayed over a period of 12 h after HS and PB treatment, the L/S ratio recovered toward 1.1, which was much closer to the value of 1.4 ± 0.2 observed in the *yw* control (Fig. 3, *C* and *D*). In D21L flies, the extent to which the L/S ratio recovers after HSPB treatment corresponds to the decay rate of the chimeric D21L mRNA. For example, the L/S ratio was < 0.1 when D21L mRNA was maximally induced; that is, at 37 °C and with PB treatment. But in the 12 h after the stimuli were removed, this ratio would recover toward 1.1. It exhibits a decay pattern similar to that in the parental line *yw* only when chimeric D21L mRNA is included in calculations of L/S ratio (Fig. 3, *C* and *D*). In contrast, the same ratio in D21L-UTR flies is unaffected by the inclusion or exclusion of the D21L-UTR mRNA, which contributes no 3′-UTR sequence. These results seem to link L/S ratio and the

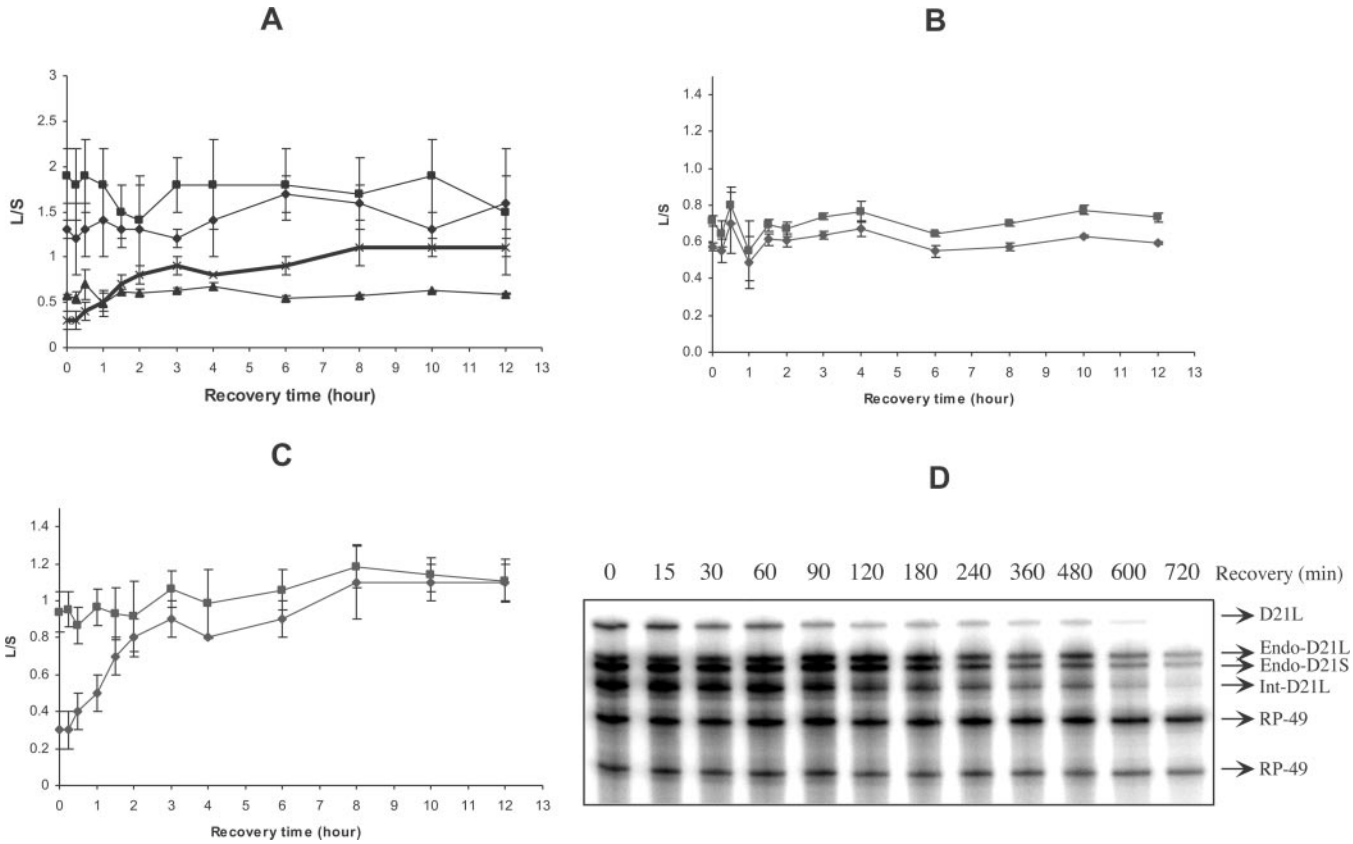

FIG. 3. **Effect of native 3′-UTR of gstD21(L) mRNA on the alternative polyadenylation of gstD21 mRNAs.** We isolated total RNAs for RPA from *yw,* D21L (line 23R), and D21L-UTR (line 4R) after HS, PB, or HSPB treatment at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 h of recovery. Riboprobes were the same as those in Fig. 2*A*. Following the RPA assays, the intensity of each band was normalized against RP-49 signals before calculation of the L/S ratio. *Panel A*, the L/S ratio is plotted against recovery time after induction of the endogenous *gst*D21 gene by PB treatment and/or the D21 transgene by heat shock. The chimeric D21L mRNA was not combined with endogenous *gst*D21(L) in calculating the ratio. *Yw*, PB-treated flies (PB, ♦); transgene D21L-UTR, combined heat shock and PB-treated (HSPB, ■); transgene D21L, heat shock-treated (HS, ▲) or combined heat shock and PB-treated (HSPB, X). In *panel B* (HS-treated D21L flies) and *panel C* (HSPB-treated D21L flies), the chimeric *gst*D21 mRNA was included (■) in or excluded (♦) from the calculation of L/S ratio in the total *gst*D21(L) mRNA population. Each data point is the average of 3–4 RPA analyses on two separate preparations of total RNAs. *Panel D* is one of the RPA gel patterns from which the graphs in *panel C* (HSPB-treated D21L flies) are derived. We determined the intensity of each band relative to the RP-49 RNA bands by a PhosphorImager. The D21.Act.SmaI probe (Fig. 2*A*) protects 665 nt of the chimeric D21L mRNA (D21L), 536 nt of *gst*D21(L) mRNA (Endo-D21L), or 517 nt of *gst*D21(S) mRNA (Endo-D21S). The D21.AS.SmaI probe protects 455 nt of the chimeric D21 mRNA from transgene D21L-UTR (D21L-UTR), 536 nt of *gst*D21(L) mRNA (Endo-D21L), or 517 nt of *gst*D21(S) mRNA (Endo-D21S). The RP49 riboprobe (RP49.AS.SacI) protects two bands, 400 nt and 300 nt long.

concentration of the D21 3′-UTR sequence, including even those in the middle of a chimeric mRNA. Without pentobarbital, the L/S ratio in D21L lines was 0.6 ± 0.1 after heat shock and remained so during recovery. Now we propose to implicate PB in the polyadenylation machinery that it senses the concentration of certain sequence element in the 3′-UTR or a sequence interacting with it. In such a role, PB would act at the level of alternative polyadenylation, restoring the L/S ratio toward normal following its activation of transcription of the *gst*D21 promoter (15).

There are two possible mechanisms by which the L/S ratio can remain relatively constant in the D21L transgenic lines. The first mechanism requires the selective degradation of the *gst*D21(S) mRNA alongside the decay of chimeric D21L mRNA. A normal L/S ratio would be maintained by selective stabilization of the *gst*D21(L) mRNA during the recovery period. Evidence against selective degradation of the *gst*D21(S) mRNA is suggested by the apparent half-lives of the endogenous D21(S) and (L) mRNAs, which are nearly identical in D21L flies under both HS and HSPB conditions: 1.7 *versus* 1.7 and 7.2 *versus* 10 h, respectively (Table I). Thus, the recovery of the L/S ratio from 0.3 to 1.1 (1.1/0.3 = 3.7-fold) as D21L mRNA decays after HSPB treatment could not be due entirely to a change in mRNA stability.

TABLE I

Half life of endogenous and chimeric D21 mRNAs in transgenic flies

The half-life (in hours) with S.D. were calculated from the slopes (Slope = $-k/2.303$ where k is the first order decay rate constant) of semi-log plots of mRNA decay patterns (*i.e.* log [%RNA] *vs* time, not shown). The half lives ($t_{½}$) are then calculated by the relationship of $t_{½} = 0.693/k$. We used data points between 2 and 10 hours of the decay patterns for the calculation of half-lives to minimize contributions of continued induction by residual PB inside the flies after termination of induction. Each data point was the average of 3–4 experiments.

Strain condition	$t_{½}$ h			
	D21L-UTR		D21L	
	HS	HSPB	HS	HSPB
Transgene	5.6 ± 0.2	5.5 ± 0.3	2.3 ± 0.1	9.5 ± 0.5
*gst*D21 (S)	ND[a]	5.0 ± 0.2	1.7 ± 0.1	7.2 ± 0.4
*gst*D21 (L)	ND	4.6 ± 0.2	1.7 ± 0.1	10.0 ± 0.3
Int-D21L			2.1 ± 0.1	2.3 ± 0.2

[a] ND represents "not determined" due to very low levels of expression.

The alternative mechanism, which we prefer, would require a selective polyadenylation favoring production of *gst*D21(L) mRNA during recovery from HSPB treatment. To assess whether the L/S ratio is restored by selective polyadenylation at the L form, we analyzed the decay pattern of PB-induced endogenous *gst*D21 mRNAs over a 12-h period in both *yw*

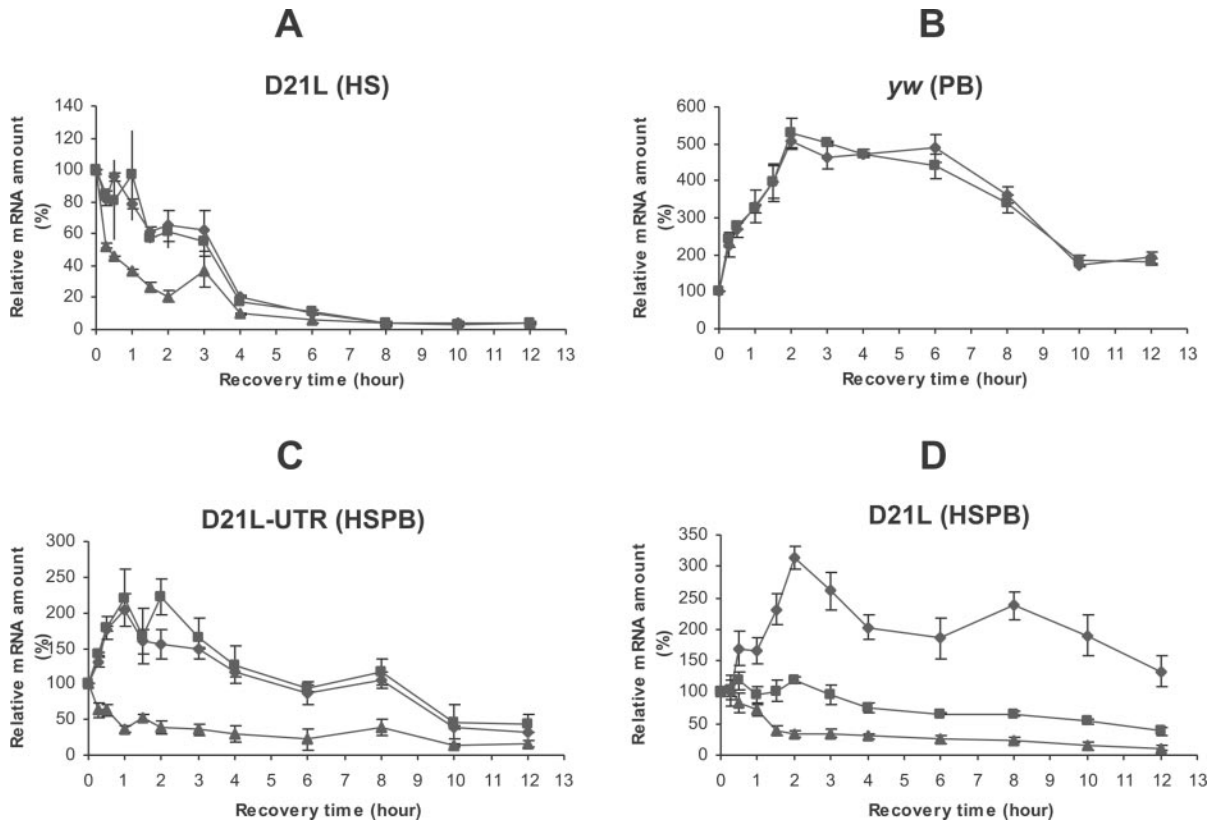

FIG. 4. **D21L and D21L-UTR transgene expression affect alternative polyadenylation of gstD21 mRNAs.** Total RNAs from yw, D21L (line 23R), and D21L-UTR (line 4R) were isolated after PB treatment (*yw*), heat shock (D21L), or combined heat shock and PB treatment (D21L and D21L-UTR) after 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 h of recovery. The riboprobes used for RPA were the same as those in Fig. 2. The intensity of each protected D21 RNA species was normalized against the RP49 signals. *Panels* are: *A*, D21L flies treated with heat shock HS; *B*, *yw* flies treated with PB; *C*, D21L-UTR flies treated with HS and PB; *D*, D21L flies treated with HS and PB. The symbols are: ◆, endogenous *gst*D21(L) mRNA, ■, endogenous *gst*D21(S) mRNA; ▲, chimeric D21L-UTR mRNA (*panel C*) or chimeric D21L (*panels A* and *D*) mRNA.

(parental) and transgenic lines of D21L and D21L-UTR. We found that the decay patterns of both endogenous *gst*D21 mRNAs are nearly identical to each other throughout the 12 h recovery period for D21L flies under heat shock (Fig. 4*A*), PB-treated *yw* flies (Fig. 4*B*), and in HSPB-treated chimeric D21L-UTR flies (Fig. 4*C*). In the HSPB-treated D21L transgenic flies (Fig. 4*D*), however, the decay patterns of the *gst*D21(L) mRNAs are influenced by the levels of the chimeric D21L mRNA present.

Following HSPB treatment, the induction pattern of the *gst*D21(L) mRNA in D21L flies is similar to those for other lines (Fig. 4, *A–C versus D*). But, the *gst*D21(S) mRNA expression is reduced in the presence of PB (Fig. 4*D*, 0–2 h). As the chimeric D21(L) mRNA decays, the L/S ratio is gradually restored to its normal level by PB-mediated preferential polyadenylation of the *gst*D21(L) mRNA while PB speeds up transcription from the *gst*D21 promoter (15).

DISCUSSION

We mapped the transcription unit of *gst*D21 and identified two forms of mRNAs, *gst*D21(L) and *gst*D21(S), distinguished by their lengths. The mapping results confirmed that the *gst*D21 gene contains no intron (13). Thus, the investigation of its post-transcriptional regulation need not consider pre-mRNA splicing. We have shown previously that PB regulates *gst*D21 gene expression at the level of transcription and mRNA stability (15). In the presence of PB, there is a >10-fold increase in the steady state level of *gst*D21 mRNAs, a change that cannot be entirely attributed to a 2-fold increase in the transcription rate. We are currently exploring this PB-mediated stabilization in greater depth, with particular attention to the role of *cis*-acting element(s) on *gst*D21(L) mRNA in the process. We constructed two different transgenes to separate the 3′-UTR from *gst*D21(L) mRNA's coding region to determine its effect on mRNA stability. We have now reconstituted the PB-responsiveness of the *gst*D21(L) sequence in the context of heterologous UTRs of the chimeric D21L mRNA. This D21L mRNA is as labile as the endogenous *gst*D21 mRNAs after heat shock induction but can be stabilized by PB (under HSPB), just as are the endogenous *gst*D21 mRNAs. The *cis*-acting element responsible for the PB-responsiveness of full-length D21 mRNAs most likely resides in the 3′-UTR (and/or the last 32 nucleotides in the 5′-UTR). The D21L-UTR mRNA, which lacks both the native 3′-UTR and the first 19 nucleotides of the 5′-UTR, is itself stable and has no effect on the stability of *gst*D21 mRNAs (Fig. 2*B*, *lanes 5–8*). Finally, the half-life of chimeric D21L mRNA was lengthened by PB treatment (Table I) suggesting a form of positive PB-mediated stabilization of endogenous *gst*D21 mRNAs.

Our results on PB-mediated *gst*D21 mRNA stabilization are consistent with a model which involves a putative *trans*-acting factor(s) that bind to the native 3′-UTR in the absence of PB. This interaction destabilizes the *gst*D21 mRNA either alone or with help from other recruited factors (*e.g.* nucleases). In the presence of PB, then, the PB-mediated dissociation (or inactivation) of that *trans*-acting factor, along with its collaborating entourage of proteins from the *gst*D21 mRNA, brings about the stabilization of the *gst*D21 mRNA.

Three pieces of evidence strongly support this model. First, HS induction of the chimeric D21 mRNA in D21L flies has been shown to stabilize the endogenous *gst*D21 mRNAs (*lanes HS*

versus C under D21L in Fig. 2*B* (*11 versus 9*), 2*C* (*5*, *3 versus 1*)). This stabilization is presumably achieved by mutual competition for the same putative *trans*-acting factor(s) because both the D21L mRNA and the endogenous *gst*D21 mRNAs must be recognized by the same *trans*-acting factor(s). Since no PB was present to activate transcription from the *gst*D21 promoter, the appearance of *gst*D21 mRNAs must have come from the basal transcription and have been freed up from the *trans*-acting factor(s) by the heat shock-induced chimeric D21L mRNA. Accordingly, the chimeric D21L mRNA, which contains the full-length *gst*D21(L) sequence, must have been destabilized in its interaction with the same putative *trans*-acting factor(s) (*lanes* under *HS*; *lane 11* of Fig. 2*B* and *lanes 3*, *5* of 2*C*). Second, results in Table I showed a ~4-fold increase in the half-life of D21L mRNA by PB treatment. Third, the half-lives of the putative decay intermediate, Int-D21L, which lacked the native 3′-UTR and whose level increased with increasing levels of D21L mRNA, did not change by PB treatment (Table I).

PB-mediated *gst*D21 mRNA stabilization could not be explained by a simple titration mechanism that is prompted by an increase in levels of D21 mRNA. A PB-induced, 2-fold increase in transcription rate cannot fully account for a >10-fold increase in the steady-state *gst*D21 mRNA (Fig. 2*B*, *lanes 1 versus 2* and *3 versus 4*). Moreover, in D21L flies, the PB-mediated stabilization of endogenous *gst*D21 mRNAs is more pronounced than the effect of heat shock-induced chimeric D21L mRNA on these same molecules, which presumably works by the simple titration mechanisms. Therefore, we can confidently propose that some PB-responsive *trans*-acting factor(s) (or complex) must figure prominently in *gst*D21 mRNA stability.

We next present evidence for yet another level of regulation by PB: alternative polyadenylation. Alternative polyadenylation of mRNAs has been associated with temporal and spatial regulation of gene expression. Numerous genes possess multiple poly(A) sites in their 3′-UTRs (9). Multiple mRNAs resulting from alternative poly(A) sites retain the same open reading frame, but can influence translation efficiency and/or mRNA stability (1, 8). It has been proposed that the choice of poly(A) sites correlates with the strength of the core polyadenylation signals (3, 7, 26), the activity of certain basal factors (*e.g.* Cst-64), and interactions between the polyadenylation machinery and the splicing factors (5). The mechanism for regulating alternative polyadenylation is not well understood, however. Our results revealed that 3′-UTR (and sequences that interact with it) is a critical *cis*-acting element in alternative polyadenylation and that changes of 3′-UTR concentration due to mRNA turnover can influence alternative polyadenylation. We also showed that PB plays a critical role in the response to changing concentrations of the 3′-UTR sequence.

Both *gst*D21(L) and *gst*D21(S) mRNAs are inducible by PB (Fig. 2*B*). It is unexpected that the L/S ratio is influenced only by the expression of a chimeric D21L mRNA containing the native 3′-UTR but not by one without it (*i.e.* D21L-UTR) (Fig. 4). Thus, the D21 3′-UTR, either independently or in conjunction with certain sequence(s) in the rest of the mRNA, is a crucial determinant of the L/S ratio. We have just proposed that a *cis*-acting element(s) located in the native 3′-UTR of D21 mRNA and presumably with its repertoire of *trans*-acting factor(s) are responsible for PB-mediated stabilization of *gst*D21 mRNAs. It is not known whether the same factors are also involved in the signaling mechanism between RNA turnover in the cytoplasm and choice of alternative polyadenylation in the nucleus. The relatively constant ratio of *gst*D21(L) and *gst*D21(S) mRNAs under heat shock and chemical stresses (PB) implies biological relevance in regulating alternative polyadenylation in the *gst*D21 system. Thus, the PB signaling mechanism on alternative polyadenylation in the nucleus is an intriguing question.

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Pentobarbital-mediated Regulation of Alternative Polyadenylation in *Drosophila* Glutathione *S*-Transferase D21 mRNAs

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