C H A P T E R F I F T E E N

mRNA Decay Analysis in *Drosophila melanogaster*: Drug-Induced Changes in Glutathione *S*-Transferase D21 mRNA Stability

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Contents

Abstract

We have established an *in vivo* system to investigate mechanisms by which pentobarbital (PB), a psychoactive drug with a sedative effect, changes the rate of decay of *gst*D21 mRNA (encoding a *Drosophila* glutathione *S*-transferase). Here we describe methods for the use of *hsp*70 promoter-based transgenes and transgenic lines to determine mRNA half-lives by RNase protection assays in *Drosophila*. We are able to identify and map putative decay intermediates by cRT-PCR and DNA sequencing of the resulting clones. Our results indicate that the 3′-UTR of *gst*D21 mRNA is responsive to PB by regulating mRNA decay

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and that the *cis*-acting element(s) responsible for the PB-mediated stabilization resides in a 59 nucleotide sequence in the 3′-UTR of the *gst*D21 mRNA (Akgül and Tu, 2007).

1. INTRODUCTION

Xenobiotics and drugs affect gene expression at different levels, including mRNA stability (Akgül and Tu, 2005; Köhle and Bock, 2007; Nakata *et al.*, 2006). We have reported that the sedative drug pentobarbital (PB) can stabilize the mRNA of an intronless gene encoding glutathione S-transferase (gst) D21 in *Drosophila melanogaster* (Akgül and Tu, 2004, 2007; Tang and Tu, 1995). The mechanism(s) by which PB may affect mRNA stability are not clear. Since cultured cells usually do not respond well, if at all, to psychoactive drugs like PB, we needed to develop a simple *in vivo* system to investigate the mechanism by which PB affects *gst*D21 mRNA stability. We chose the *Drosophila* system over the rodents because of the intronless nature of the *gst*D21 gene and the relative ease of maintenance and manipulation.

With the *Drosophila* system, we have at our disposal genetic and molecular tools such as transposition mutagenesis, siRNA technology, and an extensive collection of mutants generated by *p*–element insertions, classical mutagenesis, and other approaches (Rubin, 1988). On establishing an *in vivo* PB–responsive system for analyzing RNA decay, we can generate transformant flies with various designer (e.g., deletion and chimeric) constructs and, in these rendered contexts, identify in the mRNA sequences *cis*-acting elements important for RNA decay.

The major challenge to study RNA turnover in a whole organism is to devise method(s) that selectively and quickly activate transcription of the target gene to a very high level, followed by rapid shut-off of gene activation for monitoring the decay rate of the target mRNA under normal or well-controlled physiologic state of the organism. For the *Drosophila* system, we chose the *hsp*70 promoter to activate target transgene expression at 35 °C. When heat shock factor associates with the *hsp*70 promoter on heat shock (HS), the pausing RNA polymerase II complex at the promoter is immediately released for elongation. The promoter returns to the uninduced state within less than 60 min after removal of heat shock (Tang *et al.*, 2000), and the organism quickly returns to the normal physiologic state. These attributes make the HS-responsive *hsp*70 promoter suitable for use in studying RNA decay *in vivo*. In the following, we describe a system to investigate mRNA decay under the effects of PB using the *gst*D21 mRNA as the reporter. Furthermore, by incorporating the chimeric gene approach, we are able to dissect the D21 mRNA for the *cis*–acting elements responsible for drug-mediated stabilization of *gst*D21 mRNAs in *Drosophila* (Akgül and Tu, 2007).

2. MATERIALS AND METHODS

2.1. Materials

Bacteriologic media were purchased from Invitrogen/Life Technologies (Rockville, MD), and chemicals from ICN, Life Technologies or Sigma–Aldrich (St. Louis, MO). Sodium pentobarbital was purchased from Sigma–Aldrich. Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Radioactive nucleotides ([α-^{32}P] UTP, specific activity ∼800 mCi/μmol) were purchased from ICN (Irvine, CA). RPA III kits were purchased from Ambion (Austin, TX). Restriction enzymes were products of New England Biolabs (NEB) (Beverly, MA) or American Allied Biochemicals (Aurora, CO). *Pfu* DNA polymerase and Quick–change® site-directed mutagenesis kit were purchased from Stratagene (San Diego, CA). T4 DNA ligase, MMLV–reverse transcriptase, SP6 RNA polymerase and the plasmid vectors pGEM T Easy® and pSP64(A) for *in vitro* transcription were purchased from Promega (Madison, WI). Tobacco acid phosphatase was from Epicentre Technologies (Madison, WI). T7 RNA polymerase was a generous gift from Bi–Cheng Wang (University of Georgia, Athens, GA). *E. coli* DH5α competent cells and *Pfx* DNA polymerase were products of Life Technologies. The plasmid vector pCaSpeR–hs–act for *Drosophila* transformation was obtained from C. S. Thummel of the University of Utah (Thummel *et al.*, 1988). The △2–3 line {*P*[ry$^+$ 2–3](99B)} (Robertson *et al.*, 1988) 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 (University Park, PA). All glassware used in RNA work is baked at 180 °C overnight. All solutions except for Tris buffers are treated with 0.1% DEPC (Sigma) overnight under a hood and autoclaved for 15 min and placed under the hood at least for 5 h to evaporate the residual DEPC in the solutions. Because DEPC changes the chemical structure of Tris, the stock solutions are prepared first with DEPC-treated water and then filtered through a 0.22-μm Nalgene filter.

2.2. Transgenic constructs and nomenclature

We use small plasmid vectors pBluescript or pGEM T Easy (Promega) for initial cloning of PCR products and manipulation of gene segments before transferring the chimeric DNA constructs into the transformation vector pCaSpeR–hs–act (Thummel *et al.*, 1988). Site-directed mutagenesis and swapping of DNA fragments between homologous genes can be accomplished more easily with small plasmids. When we need a restriction site in the coding region to facilitate DNA fragment swapping, we introduce base changes that conserve the amino acid sequence to minimize any possible

effect of mis-sense mutation(s) on the stability of transgenic mRNA(s) (e.g., the swapping at nucleotide 567 in Fig. 15.1, D21–170–D1). We then use site-directed mutagenesis to restore some mutations back to the wild-type sequence. A typical reaction contains 30 to 50 ng of double-stranded template DNA, 300 nM of each of the two primers, 0.2 mM dNTPs, 1× *Pfu* DNA polymerase buffer, and 2.5 U *Pfu* DNA polymerase (Stratagene). PCR conditions are as follows: 1 cycle of 94 °C for 1 min; 18 cycles of 94 °C for 30 sec, 55 °C for 1 min, and 68 °C for 11 min. The PCR product is then digested with 1 U of *Dpn* I to remove the methylated template DNA, and an aliquot is used for transformation (Sambrook and Russell, 2001).

Once the desired constructs in pBluescript or pGEM T Easy are generated and their sequences confirmed, a pair of primers with appropriate restriction sites at the 5′-ends is used to amplify the inserted DNA for cloning into the pCaSpeR–hs–act transformation vector (Thummel *et al.*, 1988). Inclusion of a few additional nucleotides 5′-to the restriction sites on the PCR primers facilitates complete digestion of the amplified products (Moreira and Noren, 1995). We use commercial fragment isolation kits to purify the restriction enzyme-digested PCR products for further manipulations. The pCaSpeR–hs–act transformation vector has eight unique restriction sites in the multiple cloning site (MCS) sequences. We typically use the *Bam*HI and *Eco*RI sites for DNA insertions so that the extraneous MCS
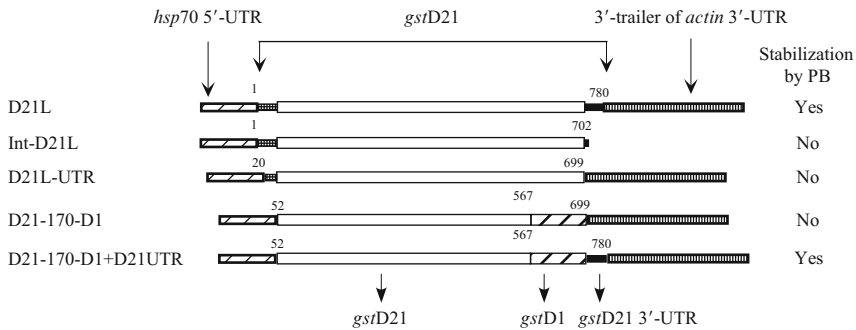
Figure 15.1 Transgenic constructs of *gst*D21. Nucleotides (nts) 1 to 780 represent the complete *gst*D21 (L) mRNA sequence (5′-UTR, nts 1 to 52; coding region, nts 53 to 699; 3′-UTR, nts 700 to 762 for S form and nts 700 to 780 for the L form) (Akgül and Tu, 2004). Each transgenic RNA should have the same heterologous UTR context, containing the 5′-UTR of *hsp*70 and the 3′-trailer of *actin* 3′-UTR. Open boxes represent the *gst*D21 protein–coding region. The native 5′-UTR of *gst*D21 mRNA is represented by a checkered box preceding the protein coding region, and the native 3′-UTR is specified by a solid box. The *gst*D1 (nt 567 to 699) sequence in the chimeric genes D21–170–D1 and D21–170–D1 + D21UTR are shown in cross-hatched boxes. Pentobarbital-mediated stabilization of a given D21 transgenic mRNA is indicated by a Yes or No.

sequences are absent in the final constructs. All transgenes have the same heterologous sequence context by having the *hsp*70 5′-UTR and the trailer region of the *actin*5C's 3′-UTR. The 5′-UTR of the *hsp*70 should afford optimum translation under heat shock conditions, and by doing so any potential effect of translation on the stability of the transgenic mRNAs may be avoided or minimized.

2.2.1. Microinjection and establishment of transgenic lines

We follow the procedure of Thummel *et al.* (1998) for *Drosophila* transformation, which is a modification of the initial approach demonstrated by Rubin and Spradling (1982 and 1983). We will limit the discussion of *Drosophila* transformation to those that apply to our results. Plasmid DNAs (chimeric genes on pCaSpeR–hs–act vector) should be of the highest possible purity for decent transformation efficiency. We use DNA preparations (Qiagen Midi Plasmid DNA preparation kit) at 0.5 mg/ml in ddH$_2$O and with an A$_{260}$/A$_{280}$ ratio of $\geq$1.8 for transformation. We collect embryos at room temperature of 23 to 25 °C for 45 min, but microinjection is carried out in an 18 °C room, and the procedure is completed within 45 min after the embryo harvest. The glue used to line up the embryos on the microscope cover slip is prepared by vortex-mixing double-sided Scotch adhesive tape in n-hexane. The hexane supernatant is then empirically adjusted to the appropriate viscosity in 1.5-ml microcentrifuge tubes before use. We avoid the use of any dye marker in the DNA solution to optimize transformation efficiency; the injection can be easily visualized by a slight postinjection disturbance at the posterior end of the embryo. We minimize the number of nontransformants by use of the injection needle to smash the aged (cellularized) or uninjected embryos.

The newly eclosed G$_0$ flies are crossed individually to *yw* to remove any background transposase. All the female flies used in crosses are virgins. Yellow-eyed to red–eyed G$_1$ progeny with longer body bristles (Sb⁻) are recrossed with *yw*. Each stable line is established through sibling crosses of colored-eyed G$_2$ flies. On average, for each DNA construct, we inject 300 embryos, of which 10% reach adulthood with 25% being transformants. We typically maintain at least three separate lines for each transgene construct.

By our design, we use heat shock to induce the transgenes driven by the *hsp*70 promoter, whereas the endogenous *gst*D21 gene is induced by PB (Akgül and Tu, 2004). Therefore, increases in the level of transgene-derived mRNAs upon simultaneous treatment with HS and PB (HSPB-treated) that are detected beyond that by HS alone, could be attributed to stabilization of the transgenic gstD21 mRNA by PB. When combined treatment is intended, it should be ensured that the endogenous gene is not induced by heat shock.

2.3. Pentobarbital and heat shock treatments

Pentobarbital (PB) regulates *gst* gene expression at both the transcriptional and posttranscriptional levels (Tang and Tu, 1995). Because of PB's water solubility, we chose this xenobiotic as a model drug to identify *cis*-acting determinants responsible for drug-induced changes of *gst*D21 mRNA stability. We discuss the procedure for PB and heat shock treatment of flies in this section.

Adult flies maintained on a standard cornmeal medium (Tang and Tu, 1994) (2- to 3-day-old, ∼150 to 200 flies per bottle) are starved for 5 h in clean milk bottles with foam plugs at room temperature. We place a strip (3 × 10 cm) of 3MM paper saturated (∼800 μl solution per strip) with either a 5% sucrose solution (control flies) or 200 mg/ml PB in 5% sucrose (PB-treated flies) in each bottle for the treatment for 2 h at room temperature. We avoid the use of dripping strips because the flies become stuck to the bottle wall (thus lower recovery). For a time-course analysis of RNA decay (e.g., calculation of the half-life of a given chimeric mRNA), flies are treated with PB for 2 h and then transferred to clean milk bottles containing a 3MM paper strip saturated with a 5% sucrose solution for 0 (control), 0.25, 0.5, 1, 2, 3, 4, 8, 10, and 12 h.

Heat shock of flies is carried out in milk bottles (∼150 to 200 flies per bottle) at 35 °C for 1 h in a hybridization oven (Model 2000, Micro-hybridization incubator, Robbins Scientific Company, Sunnyvale, CA). A 3MM paper strip saturated with a 5% sucrose solution (∼800 μl) is placed in the bottles during the HS treatment to prevent dehydration of the flies. Two strips are used per bottle when HS treatment is performed at elevated temperatures (e.g., 37 °C). (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.) The extent of chimeric mRNA induction can be adjusted by controlling HS temperatures (e.g., elevated expression of transgenes at 37 °C relative to 35 °C). To minimize variations of temperature for HS treatment, different lines of transgenic flies to be compared in the same experiment are heat-shocked at the same time. To concurrently overexpress D21 transgene (heat shock–inducible) and endogenous *gst*D21 (PB-inducible) mRNAs, the flies are treated with PB at room temperature for 1 h and then at 35 °C for the second hour (Akgül and Tu, 2002). For a time-course study, the flies are transferred, after the treatment, into clean milk bottles containing 3MM paper strips saturated with a 5% sucrose solution kept at room temperature. Flies are collected in liquid nitrogen–treated 250-ml wide-mouth centrifuge bottles, snap-frozen with copious amounts of liquid nitrogen, and stored in 15-ml polypropylene tubes at −80 °C until use. We avoid the use of polystyrene tubes for storing flies as they easily crack in liquid nitrogen.

2.4. RNA isolation and RNase Protection Assays

Despite the availability of many rapid RNA isolation kits, we have consistently obtained excellent yield with high–quality RNA from both embryos and adults by following the procedure of Ullrich *et al*. (1977). Up to 2 mg of total–fly RNA can be isolated from 1 g of frozen flies. We prefer to use guanidine hydrochloride over guanidine isothiocyanate in the homogenization buffer because the former is easier to dissolve. This procedure is not appropriate for isolating RNAs smaller than 200 nt, because they do not efficiently cosediment with the high–molecular–weight RNAs (www.ambion.com). Typically, 0.5 to 1 g of frozen flies or embryos are homogenized in 7 ml of lysis buffer (4 M guanidinium hydrochloride, 1 M β–mercaptoethanol, 0.1 M NaOAc, pH 5, and 0.01 M EDTA) in a 15–ml Douce homogenizer first with Pestle B ($\sim$20 strokes) and then with Pestle A ($\sim$10 strokes). CsCl (1.05 g) is added per ml of the homogenate until it is fully dissolved at room temperature. The solution ($\sim$8 ml) is then transferred into a clear ultracentrifuge tube underlaid with 2 ml of DEPC–treated, cushion solution (saturated CsCl solution containing 10 mM EDTA and 50 mM NaOAc, pH 5). The mixture is then centrifuged at room temperature in a Beckman Ti70.1 rotor at 139,000g for 25 h. The flaky RNA visible to the naked eye below a non–UV–absorbing thick band is drawn with a 1-ml syringe with an 18-gauge needle. The RNA in CsCl solution $\sim$1 ml per tube is diluted with 3 volumes of DEPC–treated water before ethanol (2.5 volumes) precipitation in a Corex tube (15 ml or 30 ml). The RNA is collected by centrifugation at 13,000 rpm at 4 $^{\circ}$C in a swinging bucket rotor (e.g., Sorvall HB4). The RNA dissolved in DEPC–treated water is aliquoted to prevent subsequent multiple freeze-and-thaw cycles. The quality of RNA prepared by this method survives long–term storage (-80 $^{\circ}$C) without any degradation for up to 3 y.

To monitor changes in the expression of a specific chimeric *gst* mRNA, we have reliably used the ribonuclease protection assay (RPA) (Calzone *et al*., 1987). It is feasible to assess the quantity, as well as the ends of a transcript (e.g., alternatively polyadenylated mRNAs) (Fig. 15.2A) by this method. In addition, we can simultaneously analyze multiple RNA species with cRNA probes of different lengths (e.g., Akgül and Tu, 2002; 2004) by following Ambion's instructions for RPA assays. Riboprobes (cRNAs) are prepared from linearized plasmid DNAs containing the target sequences in the antisense orientation cloned downstream of a T7 or SP6 RNA polymerase promoter. We obtain excellent radiolabeled *in vitro* transcripts with 0.5 μg linearized plasmid DNA according to Promega's instructions. Only the mRNA sequences perfectly complementary to the radioactive riboprobe would be protected from the subsequent RNase digestions. We prepare riboprobe clones from DNAs of the chimeric plasmids used for
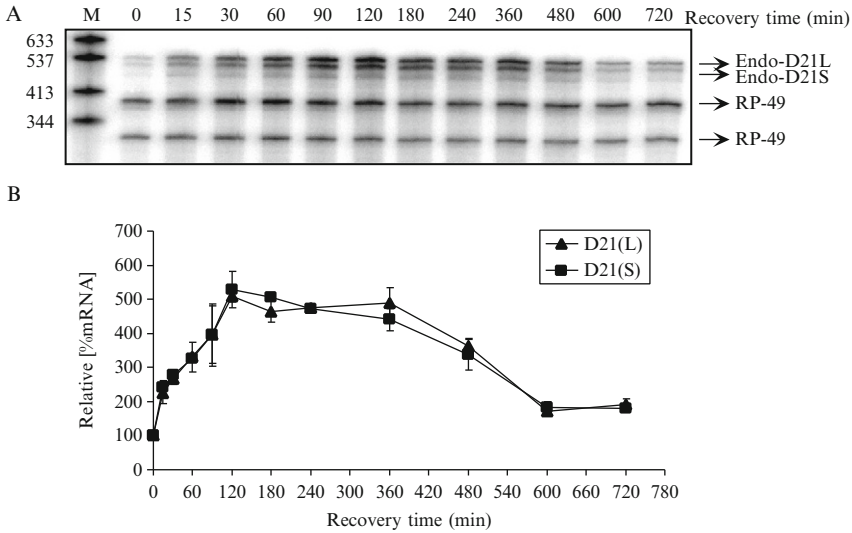
Figure 15.2 RNase protection assay analysis of pentobarbital-stabilized D21 transcripts. Pentobarbital treatment and RPA analysis were performed as indicated in "Pentobarbital Treatment" and "RNA Isolation and RPA Analyses" sections. The RPA analysis was carried out with 40 μg of total-fly RNA and a radiolabeled D21.AS. *Sma*I riboprobe. (A) The riboprotected D21 fragments were resolved in an 8% denaturing polyacrylamide gel. M, a radiolabeled *in vitro*–transcribed RNA markers, with sizes shown to the left of the panel. Endo-D21L and Endo-D21S, the L (long) and the S (short) forms of the endogenous *gst*D21 mRNAs, respectively; RP-49, the endogenous control ribosomal protein 49 mRNA. (B) mRNA amounts in (A) were normalized against the level of RP-49 mRNA (upper band) and expressed relative to the control level (100%).

establishing the transgenic lines. All plasmid DNAs used for riboprobe preparation must be correctly sequenced, because a single nucleotide difference would result in unhybridized bases that could be subjected to RNase cleavage and generation of partially ribo-protected fragments.

We typically use 20 to 40 μg of total-fly RNA in each RPA reaction to analyze *gst*D21 mRNA decay. However, less RNA would suffice if the expression level of the mRNA is relatively higher. The riboprobe-protected products are analyzed by denaturing polyacrylamide gel electrophoresis (0.75-mm thick, 8%, 19:1), followed by exposure in a PhosphorImager cassette for 1 to 5 h at room temperature depending on the intensity of the signal(s). The relative amount of each mRNA is then quantified. PhosphorImager signals are normalized against an endogenous reference mRNA (e.g., RP-49) (O'Connell and Rosbash, 1984). There is no need for drying the gel, because riboprobes with high specific activity are generated through *in vitro* transcription in the presence of [α-^{32}P] UTP (800 mCi/μmol). The cRNA probe is purified through a 6% denaturing polyacrylamide gel and extraction buffer (Ambion) and is included at levels threefold to fourfold (determined

empirically) in excess to the target mRNA. Although one preparation of radioactive probes is sufficient for more than 100 reactions, prolonged storage at $-80\,°C$ over 2 days results in undesirable smears in RPA signals, presumably because of radiation–induced breakage of the probe.

## 2.5. Determining 5′- and/or 3′-ends and decay intermediates of *gst*D21 mRNAs

This method is based on circularization of decapped mRNAs followed by RT–PCR (Couttet *et al,* 1997) (Also see Chapter 22 by Grange). Although several methods are available to identify the 5′- or 3′-end of an mRNA individually, we have successfully used circular RT–PCR to simultaneously map both ends of various *gst*D21s at the nucleotide level (Akgül and Tu, 2007). It has been particularly useful in mapping multiple *gst*D21 decay intermediates at the nucleotide level. We first use a nested set of radioactive probes in RPA analyses to roughly estimate the ends of the intermediates (Akgül and Tu, 2007). These results are then used as a guide in designing the appropriate pair of primers (∼50 nt from the ends) for the subsequent RT–PCR experiment. This procedure can also be used to determine the length of the poly(A) tail of an mRNA, if desired (Couttet *et al,* 1997).

For cRT–PCR reactions, we treat 10 µg of total RNA with 1 U of RNase-free DNase (Promega) for 20 min at 37 °C in 50 µl of 50 mM Tris-HCl, pH 7.5, and 10 mM MgCl$_2$ to remove any residual genomic DNA contamination. After phenol–chloroform (Ambion) extraction twice and ethanol (RNase-free absolute alcohol) precipitation and a 70% ethanol wash, half of the recovered RNA is treated with 2.5 U of TAP (tobacco acid pyrophosphatase) in the presence of 20 U of RNasin, 50 mM NaOAc, pH 6, 1% β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100 in 20 µl final volume for 60 min at 37 °C. The decapped RNA is phenol–chloroform extracted twice, ethanol–precipitated, and circularized with 20 U of T4 RNA ligase (Pharmacia), 50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 20 mMDTT, 100 µM ATP, 100 µg/ml acetylated BSA, and 20 U of RNasin in 400 µl volume at 16 °C for 16 h. The circularized RNA is extracted with phenol–chloroform twice and ethanol precipitation as before. Then, cDNA is prepared by reverse transcription with MLV–RT and 10 ng of a gene-specific 18-mer primer. The resulting cDNA reaction mixture should be boiled for 5 min before digestion with a mixture of RNase A and RNase T1. The treated cDNA is recovered by phenol–chloroform extraction once and ethanol precipitation. One percent of the recovered cDNA is used for PCR amplification. The PCR product is gel-purified and cloned into pGEM T Easy vector (Promega). Cloned plasmid DNAs are randomly selected for sequence analysis to determine the 5′- and/or 3′-ends of the mRNAs. We sequenced ∼100 clones to obtain a distribution of the ends of RNA decay intermediates of *gst*D21 mRNA (Akgül and Tu, 2007).

2.5.1. Determination of *gst*D21 mRNA half-lives

D21 mRNA levels are normalized relative to the endogenous RP-49 mRNA levels. All values are subsequently determined relative to the ratio obtained in the control lines (e.g., no HS and/or PB treatment), which is designated as 100% expression. Because PB remains in the gut of the flies and thus continues to induce *gst*D21 expression more than 2 h after the cessation of the PB treatment (Fig. 15.2B), we use the time points between 3 and 10 h for half-life calculations. The log of the relative (% mRNA) level is plotted against time after treatment to obtain a linear regression trend line (Fig. 15.3). The half-life is then calculated from the formula $t_{1/2} = -0.693/k$ (min), where $k = -2.303(m)$. The slope, m, is obtained from the trend line ($y = mx + b$). An example of such a calculation is presented in Fig. 15.3, where log (% mRNA) of HS-induced D21–170–D1 mRNA are plotted against recovery time (in min). The $t_{1/2}$ for the D21–170–D1 chimeric RNA is calculated to be 3.5 h.

To assess whether the native D21 3′-UTR has a *cis*-acting element for stabilizing D21 mRNA in the presence of PB, we prepare two constructs with and without the UTR (e.g., D21–170–D1 vs D21–170–D1 + D21UTR in Fig. 15.1). The $t_{1/2}$ of both transgenic D21 mRNAs is subsequently calculated under HS and HSPB conditions. If the D21 native UTR contains a *cis*–acting stabilizing element, the $t_{1/2}$ is expected to be similar for the reporter transcript without the UTR (D21–170–D1 in Fig. 15.1), whereas the reporter transcript with the UTR (D21–170–D1 + D21UTR
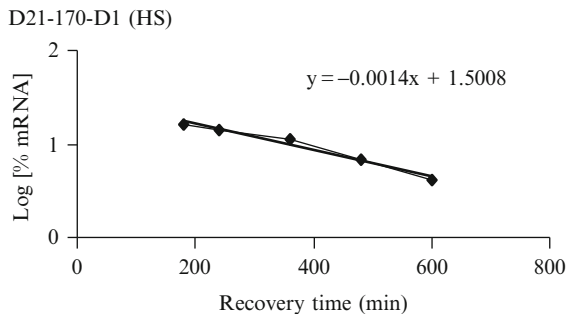
Figure 15.3 Calculations of mRNA half-lives from the time–course data. On the basis of RPA analyses (Fig. 15.2A), we plot log[% mRNA] vs minutes recovery time, defining the value at zero time as 100%. We used linear regression to find a best–fit straight line ($y = mx + b$; m is the slope) for data obtained between 3 and 10 h. The half-life ($t_{1/2}$) is derived from the equation: $t_{1/2} = -0.693/k$, where $k = -2.303m$. This is a representative plot for calculating the $t_{1/2}$ of the HS-induced D21–170–D1 transgenic mRNA (Fig. 15.1). From the best-fit regression line, $t_{1/2} = -0.693/(-2.303)(0.0014) = 213$ (min), where m is equal to 0.0014. We typically use at least three different fly populations for each treatment (e.g., heat shock or combined heat shock and PB treatment) and carry out three RPA analyses from each of two independent RNA isolations (i.e., a total of six RPA analyses).

in Fig. 15.1) would have a greater *t* (i.e., stabilization) under HSPB conditions compared with HS conditions (Akgül and Tu, 2004; 2007).

Our previous results suggest that PB stabilizes the *gst*D21 mRNA (Tang and Tu, 1995). To identify the *cis*-acting determinant(s) responsible for PB-mediated stabilization, we first deleted the 3′-UTR of the *gst*D21 mRNA (Fig. 15.1, D21L-UTR) and found that this particular chimeric D21 transcript is unresponsive to PB treatment (i.e., no stabilization by PB) (Akgül and Tu, 2004). Reinsertion of the native 3′-UTR of *gst*D21 restored the PB-mediated stabilization (Akgül and Tu, 2004). We also detected a series of decay intermediates, Int-D21L (Fig. 15.1), which lacked the 3′-UTR as mapped by cRT-PCR. These putative decay intermediates were not stabilized by PB, supporting the notion that a PB-responsive element is present in the 3′-UTR of the *gst*D21 mRNA (Akgül and Tu, 2007).

We then used a reporter transcript (Fig. 15.1, D21-170-D1, originally constructed to study translational regulation of *gst*D21 mRNA) to test the effect of the *gst*D21 3′-UTR on the stability of the resulting chimeric D1-D21 mRNA (Fig. 15.1, D21-170-D1 + D21UTR) in the presence of PB. RPA analyses showed that insertion of the *gst*D21 3′-UTR conferred PB-responsiveness to the otherwise PB-nonresponsive D21-170-D1 reporter mRNA (Akgül and Tu, 2007). Although the preceding approach is specifically focused on identifying the PB-responsive cis element in the *gst*D21 mRNA, a similar strategy could be implemented to isolate *cis* elements in any mRNA.

3. CONCLUDING REMARKS

Much of our current understanding of the pathways of mRNA degradation in eukaryotic cells came from studies in *S. cerevisiae* and in mammalian cells (Caponigro and Parker, 1996; Isken and Maquat, 2007; Jacobson and Peltz, 1996; Ross, 1995). Commonly used methods for measuring mRNA half-lives include approach to steady-state method, transcriptional pulse-chase, drug inhibition of transcription, inhibition of transcription by regulated promoters, and short-term promoter activation. *In vitro* systems for mRNA turnover have also been developed (Ross, 1993). None of these systems are applicable to investigating mechanisms of drug-induced changes in mRNA stability.

By use of *hsp*70 promoter-based transgenes and transgenic lines, we have established a *Drosophila* system to investigate PB-mediated changes in mRNA decay using the *gst*D21 mRNA. This approach has been quite useful not only in dissecting the *gst*D21 RNA for *cis*-acting element(s) responsible for the changes in mRNA stability by PB treatment but also in explaining alternative polyadenylation of *gst*D21 mRNA (Akgül and

Tu, 2002; 2004; 2007). Our analysis suggests that the *cis*-acting element responsible for the PB-mediated stabilization resides in a 59 nucleotide sequence within the *gst*D21 mRNA 3′-UTR (Akgül and Tu, 2007). Further dissection of this PB-responsive region will allow the identification of *trans*-acting factors essential for the drug's effect on *gst*D21 mRNA at the level of RNA turnover.

ACKNOWLEDGMENT

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