

Measuring the Rates of Transcriptional Elongation in the Female *Drosophila melanogaster* Germ Line by Nuclear Run-on

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We adapted the nuclear run-on method to measure changes in the rate of RNA polymerase II (pol II) transcription of repetitive elements and transposons in the female germ line of *Drosophila melanogaster*. Our data indicate that as little as an approximately 1.5-fold change in the rate of transcription can be detected by this method. Our nuclear run-on protocol likely measures changes in transcriptional elongation, because rates of transcription decline with time, consistent with a low rate of pol II re-initiation in the isolated nuclei. Surprisingly, we find that the retrotransposon *gypsy* and the repetitive sequence *mst40* are silenced posttranscriptionally in fly ovaries.

Eukaryotic genomes exist as chromatin (from the Greek *chroma*, meaning colored), a complex of DNA and histone proteins that facilitates efficient DNA packaging and differential regulation of gene expression. Cytologically, chromatin can be divided into heterochromatin, first identified by its dark staining, and euchromatin, which stains more lightly (Zacharias 1995). Heitz imagined euchromatin to be genetically active and rich in genes (Zacharias 1995). In contrast, he proposed heterochromatin to be genetically inert and gene-poor. Nearly eight decades later, his prescient assessment stands essentially uncorrected.

Constitutive heterochromatin (α -heterochromatin) comprises tandem arrays of repetitive DNA sequences (“satellite” sequences), punctuated occasionally by insertions of transposable elements. Constitutive heterochromatin is believed to organize specialized structures such as centromeres and telomeres that act to maintain genetic stability and ensure the segregation of chromosomes during mitosis and meiosis (Allshire et al. 1995; Nimmo et al. 1998; Bernard et al. 2001; Pidoux and Allshire 2005; Huang and Moazed 2006). Heterochromatic states are epigenetically inherited: The DNA packaging state is maintained after replication and mitosis, irrespective of the underlying DNA sequence (Elgin and Grewal 2003). At the molecular level, all heterochromatic regions contain lysine 9-methylated histone 3 and the evolutionarily conserved heterochromatic protein 1 (HP1; Swi6 in fission yeast) (Huisinga et al. 2006). Heterochromatin has other unusual properties: It replicates late in S phase, remains condensed throughout the cell cycle, resists recombination, and silences reporter genes inserted within or nearby, a property that underlies position-effect variegation (Henikoff 1992; Mahtani and Willard 1998; Copenhagen et al. 1999; Puechberty et al. 1999; Gilbert 2002; Schubeler et al. 2002). In contrast, facultative heterochromatin is transcriptionally active, but it can adopt the structural and functional characteristics of heterochromatin under special circumstances. Examples of facultative heterochromatin include the inactive X chromosome of mammals (which is

genetically identical to its active sister); the silenced rRNA genes in nucleolar dominance, a phenomenon in which rRNA genes of only one parent are active in animal and plant hybrids; and the developmentally regulated β -globin locus in animals (Weintraub et al. 1981; Forrester et al. 1989; Lewis and Pikaard 2001; Grummt and Pikaard 2003; Kim and Dean 2004; Lawrence and Pikaard 2004; Lawrence et al. 2004; Heard 2005).

Between constitutive heterochromatin and euchromatin lies β -heterochromatin, which is less condensed, largely deficient in tandem sequence arrays, filled with copies of numerous transposable element families, and contains few functional genes (Holmquist et al. 1998). Repetitive sequences account for the majority of all heterochromatic sequences in *Drosophila melanogaster*, whose genome is 30% heterochromatic, suggesting that they have a central role in heterochromatin assembly. Sequence comparison of the *D. melanogaster* fourth chromosome, which is largely heterochromatic, with the syntenic *Drosophila virilis* chromosome 6, which is euchromatic, suggests that the difference in chromatin packaging reflects the density and distribution of transposable elements (Riddle and Elgin 2006; Slawson et al. 2006).

The predominance of repetitive elements in heterochromatin suggests that these selfish genetic elements are silenced transcriptionally. Repetitive element silencing is essential for genome integrity, as their transposition is the major source of genome rearrangements (Kazazian 2004). In plants, animals, and fungi, RNA silencing has been implicated as a major defense against repetitive element transposition (Martienssen and Colot 2001; Sijen and Plasterk 2003; Kalmykova et al. 2005; Nolan et al. 2005; Savitsky et al. 2006). In the male and female germ line of *D. melanogaster*, retrotransposons and repetitive sequences are silenced by the repeat-associated small RNA (rasiRNA) pathway (Vagin et al. 2006), an RNA silencing mechanism distinct from both the RNA interference (RNAi) and microRNA (miRNA) pathways. Small silencing RNAs 24–30 nucleotides long, rasiRNAs not only are about 3–7 nucleotides longer than siRNAs and

miRNAs, but they are also chemically different, in that they lack one of the 3'-terminal hydroxyl groups characteristic of animal short interfering RNAs (siRNAs) and miRNAs. Consistent with this chemical difference, rasiRNAs may not be produced by either Dicer-1, which makes *Drosophila* miRNAs, or Dicer-2, which makes siRNAs. rasiRNA-directed silencing of repetitive genetic elements requires the putative helicases Spn-E, and Armitage as well as Piwi or Aubergine, members of the Piwi subclade of Argonaute family of proteins.

Supporting the view that rasiRNAs act to silence repetitive sequence by reducing their rate of transcription, mutations in *spn-E*, *aubergine*, and *piwi* are reported to cause loss of lysine-9 methylation of histone H3 on silenced genes (Pal-Bhadra et al. 2004). Historically, heterochromatin has been regarded as transcriptionally inert, but new evidence in fission yeast suggests that heterochromatin can be transcribed by RNA pol II and then silenced by the posttranscriptional destruction of the nascent transcript, perhaps at its site of transcription (Volpe et al. 2002; Djupedal et al. 2005; Kato et al. 2005; Buhler et al. 2006).

What mechanism silences repetitive elements in the germ line of any organism is unknown. Mutations in *armi* increase the steady-state concentration of repetitive element mRNA in the fly germ line (Vagin et al. 2006). Here, we examine the transcriptional rates of the silenced and desilenced retrotransposon *gypsy* and the repetitive sequence *male-specific transcript 40 (mst40)* in the *D. melanogaster* female germ line. Contrary to expectation, we find that loss of *gypsy* or *mst40* silencing caused by loss of the Armi protein is not accompanied by a change in the rate of transcription of either of these selfish genetic elements.

MATERIALS AND METHODS

Isolation of nuclei. The rate of transcriptional elongation was determined by nuclear run-on analysis essentially as described previously (So and Rosbash 1997). Briefly, ovaries from 50–100 females were dissected with needles into *Drosophila* Ringer's solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl₂, 10 mM Tris-HCl at pH 7.5) and stored on ice in a 1.5-ml homogenization tube (Kontes, Vineland, New Jersey) during isolation. Isolated tissues were centrifuged at 2000g at 4°C, the supernatant discarded, and then homogenized with 20–30 strokes of the pestle in 300 µl of homogenization buffer (10 mM HEPES-KOH at pH 7.5, 10 mM KCl, 0.8 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), and 100 µg/ml yeast tRNA (Ambion, Austin, Texas) containing one tablet of Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) for each 10 ml. An additional 200 µl of homogenization buffer was used to rinse the tube. The rinse and the homogenate were pooled and then filtered through a Bio-Spin column (Bio-Rad).

The filtrate was overlaid on a 500-µl cushion of 1 M sucrose dissolved in 10 mM HEPES (pH 7.5), 10 mM KCl, 10% glycerol, and 1 mM EDTA plus one tablet of Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) for each 10 ml and centrifuged at 10,000g for 10 minutes at 4°C. After discarding the supernatant, the

nuclei were resuspended in 500 µl of nuclear resuspension (NR) buffer (40 mM HEPES-KOH at pH 8.0, 25% glycerol, 5 mM magnesium acetate, 1 mM DTT, and 0.1 mM EDTA containing for each 10 ml one tablet of Complete Mini EDTA-free Protease Inhibitor Cocktail [Roche]) and centrifuged for 2 minutes at 8000g at 4°C.

Labeling of nascent transcripts. The pellet was resuspended in 195 µl of NR buffer, and the reaction was initiated by adding 50 µl of 5x reaction mixture (5 mM magnesium acetate; 750 mM KCl; 2.5 mM each ATP, CTP, and GTP; 10 mM DTT; 1 µl of RNasin [Promega]; 50 mM of creatine phosphate; and 60 µg/ml creatine kinase) and 5 µl of [α -³²P]UTP (6000 Ci/mmol, 40 µCi/µl; MP Biomedicals, Irvine, California). After incubation for 30 minutes at 23°C, the reaction was stopped by adding 25 µl of RNase-free DNase (RQ1, Promega) and incubating for 5 minutes at 37°C. Proteins were digested by adding 20 µl of 15x proteinase buffer (7.5% SDS, 150 mM EDTA) and 3 µl of 10 mg/ml proteinase K and then incubating for 30 minutes at 37°C. After extraction with phenol:chloroform, the RNA was precipitated with three volumes of ethanol. The precipitate was recovered by precipitation, washed with 80% (v/v) ethanol, air-dried, and resuspended in 50 µl of H₂O. Unincorporated nucleotides were removed by three consecutive rounds of purification using mini Quick Spin Columns for RNA (Roche) according to the manufacturer's instructions and then used for hybridization as described below.

Hybridization of labeled transcript to filter-immobilized probes. Labeled RNA from isolated nuclei was hybridized to immobilized, strand-specific, in-vitro-transcribed RNA probes for firefly luciferase (*Photinus pyralis*; *Pp luc*), *roo*, *l-element*, *HeT-A*, *mst40*, *gypsy*, *rp49*, and *act5C*. RNA probes were transcribed with T7 RNA polymerase from PCR templates prepared with the oligonucleotides reported in Table 1. *Pp luciferase* was transcribed from the pGL-2 control vector (Promega) using the primers: 5'-gcg taa tac gac tca cta tag GAG AGG AAT TCA TTA TC-3' and 5'-GAA GAG ATA GCC CTG GTT CCT G-3'. Each in-vitro-transcribed RNA probe (5 µg) was denatured in 500 µl of ice-cold 10 mM NaOH and 1 mM EDTA, transferred to Hybond-XL membrane (Amersham) using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad) according to manufacturer's instructions, and immobilized on the membrane by UV irradiation (200 µjoules/cm; Stratelinker, Stratagene). The membrane was prehybridized in Church buffer (Church and Gilbert 1984) for 1 hour at 65°C and hybridization was carried out overnight at 65°C. After hybridization, membranes were washed twice with 2x SSC/0.1% (w/v) SDS for 30 minutes at 65°C and analyzed by phosphorimager (Fuji, Tokyo, Japan).

RESULTS

Nuclear Run-on Measures Relative Rates of Transcription

Weiss (1960) demonstrated for the first time that isolated eukaryotic nuclei retain the ability to synthesize RNA. Such RNA synthesis results from transcript elongation by

Table 1. Primers Used to Generate T7 RNA Polymerase Transcription Templates for Production of Antisense RNA Probes

Element or gene	Primer sequence (5' to 3')	
<i>roo</i>	5' primer	taatagcactactattagggagaccacGGAGGGTTTGATTTAGGGACAGTG
	3' primer	AGCAGAAGCAGCAACAGCAGTAG
<i>HeT-A</i>	5' primer	taatagcactactattagggagaccacGGAGAAGATCGCTGTTCTG
	3' primer	GACACGCGAAAAGCGAAC
<i>I-element</i>	5' primer	taatagcactactattagggagaccacTTGGCCTTTAGTTTTGATGC
	3' primer	CATCAACACAGCCCAATTGAC
<i>act5C</i>	5' primer	gcgtaatagcactactatagggTGTAGGTGGTCTCGTGGATGC
	3' primer	GGCCACCGTGAGAAGATGAC
<i>mst40</i>	5' primer	taatagcactactattagggagaccacAACGATCGCTTGCATCTAC
	3' primer	TCAAATCAGACGAAAGTTCAAGG
<i>rp49</i>	5' primer	gcgtaatagcactactatagggTFACTCGTTCTTTGAGAACG
	3' primer	GACCATCCGCCAGCATAACAG

RNA polymerases, rather than initiation of transcription after nuclear isolation (Cox 1976). These experiments led to the establishment of “nuclear run-on” as the standard method for measuring the relative rates of gene transcription (McKnight and Palmiter 1979; Swaneck et al. 1979). In a prototypical nuclear run-on experiment, intact nuclei are isolated by centrifugation and then incubated with exogenous ATP, GTP, CTP, and [α - 32 P]UTP, incorporating radiolabeled nucleotide into transcripts initiated prior to cell lysis but elongated in the isolated nuclei. Radiolabeled nuclear RNA is isolated and then hybridized to strand-specific RNA probes immobilized on a nylon filter. The specificity of hybridization is established by comparing the hybridization signals for the genes of interest to those of control genes whose transcription is presumed to be invariant under the conditions compared (Fig. 1).

We sought to apply the nuclear run-on method to germline tissue from flies. To begin our study, we first tested whether run-on of nuclei isolated from dissected *D. melanogaster* ovaries accurately reflects changes in tran-

scriptional rates. We compared the apparent transcriptional rates of the *RpL32* gene (commonly known as *rp49*) in nuclei isolated from ovaries of wild-type flies containing two copies of *rp49* (*2x rp49*) to that in mutant *Df(3R)L127/TM6; Dp(3;1)B152/Dp(3;1)B152* flies in which one copy of the *rp49* locus on the third chromosome is deleted and each X chromosome contains an additional copy of the locus, for a total of three copies of *rp49* (hereafter, *3x rp49*). As a control, we used the *act5C* gene. Hybridization to the firefly luciferase (*Pp Luc*) antisense transcript, whose sequence shares little similarity with any gene in *D. melanogaster*, provided a measure of the non-specific background. The steady-state mRNA level in the ovaries of the *3x rp49* flies was greater by a factor of 1.84 ± 0.39 (average \pm standard deviation) than in the ovaries of the *2x rp49* flies (Fig. 2A). Transcription of *rp49* in the ovaries with three copies of the gene was greater by a factor of 1.75 ± 0.24 (average \pm standard deviation for three independent trials) than in the ovaries from flies bearing two copies of the *rp49* gene (Fig. 2B). We conclude that

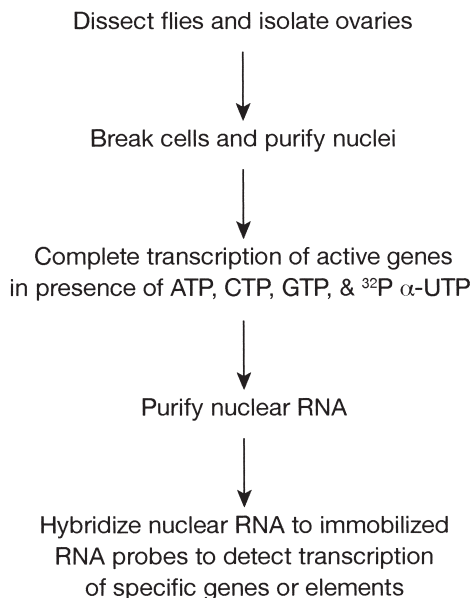


Figure 1. Schematic of nuclear run-on analysis.

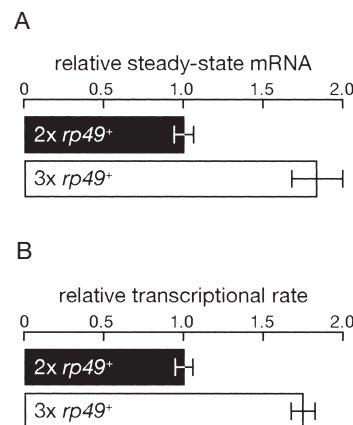


Figure 2. Nuclear run-on analysis can detect small changes in transcriptional rates. (A) Relative steady-state mRNA concentration in ovaries from flies with two or three copies of the *rp49* gene. (B) Nuclear run-on assay in ovaries from flies with two or three copies of the *rp49* gene. The assay readily detected the approximately 1.5-fold change in the transcription of *rp49* between the wild-type *rp49/rp49* (*2x rp49*) and the *Df(3R)L127/TM6; Dp(3;1)B152/Dp(3;1)B152* (*3x rp49*) flies.

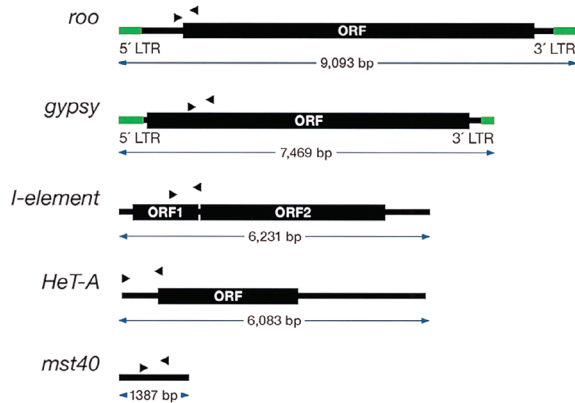


Figure 3. Position of primers used to prepare T7 RNA polymerase PCR templates to synthesize the antisense RNA probes used to detect repetitive elements.

the nuclear run-on assay readily detected an approximately 1.5-fold change in the transcriptional rate of a housekeeping gene in *D. melanogaster* ovaries.

A central assumption in this assay is that only transcripts initiated by RNA pol II prior to cell lysis are extended during the radiolabeling reaction. If this assumption is justified, the amount of [α - 32 P]UTP incorporated in an elongating transcript should decline with time, as individual molecules of RNA pol II complete transcription but do not initiate new rounds of RNA synthesis *in vitro*.

Labeled transcripts were isolated and hybridized to strand-specific RNA probes for four different repetitive sequences: two long terminal repeat (LTR) retrotransposons (*roo* and *gypsy*) and two non-LTR retrotransposons (*I-element* and *HeT-A*). Probes for two single-copy genes, *act5C* and *rp49*, served as controls. The positions of the primers used to make polymerase chain reaction (PCR) templates for RNA probe transcription by T7 RNA polymerase are diagrammed in Figure 3. If no reinitiation occurs during the run-on reaction, transcription rates should saturate when there are no more unfinished transcripts left to label. We find that after about 40 minutes, transcriptional rates saturated, consistent with RNA pol II having elongated all available transcripts without reinitiating new rounds of transcription (Fig. 4).

Measuring the Rates of Repetitive Element Transcription

Hybridization-based nuclear run-on was conceived to measure the relative transcriptional rates of single-copy genes (McKnight and Palmiter 1979). Because repetitive elements are multicopy, we were concerned that their high aggregate rates of RNA synthesis might saturate the immobilized RNA probes, preventing our detecting changes in transcriptional rates. To exclude this possibility, we performed nuclear run-on using *armi* homozygous mutant ovaries, in which transposon silencing is depressed; 100%, 50%, 25%, and 10% of the 32 P-radiolabeled RNA was hybridized to 5 μ g of immobilized RNA probes for the transposons *roo*, *gypsy*, *I-element*, and *HeT-A*, the repetitive sequence *mst40*, and the single-copy

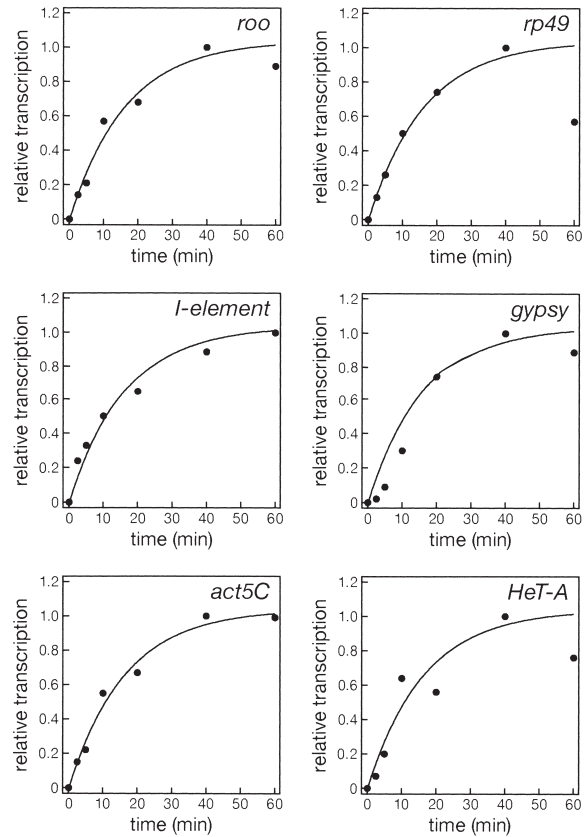


Figure 4. Nuclear run-on likely measures the rate of transcription without substantial reinitiation by RNA pol II, because transcriptional rates saturate with time.

gene *act5C*. For all six genes, the rate of transcription decreases linearly with dilution, even for *HeT-A* retrotransposon, whose steady-state transcript levels rise approximately 170-fold in the absence of *Armi* (Fig. 5) (Vagin et al. 2006). We conclude that our experimental conditions can accurately measure repetitive element transcriptional rates despite their high copy number.

Repetitive Elements Are Transcribed by RNA pol II

To establish that our assay detects RNA synthesis, we conducted the run-on in the presence of three RNA chain terminators: 3'-deoxy ATP, 3'-deoxy CTP, and 3'-deoxy GTP. Consistent with RNA synthesis, run-on transcription decreased dramatically in the presence of the RNA chain terminators for all six genes examined, *act5C*, *rp49*, *gypsy*, *roo*, *I-element*, and *HeT-A* (Fig. 6).

In *D. melanogaster*, 10 μ g/ml α -amanitin inhibits transcription by RNA pol II, but not by RNA pol I or III. To test whether RNA pol II predominantly transcribes repetitive elements in *D. melanogaster*, the nuclear run-on assay was conducted on nuclei preincubated with 10 μ g/ml α -amanitin for 10 minutes on ice. Hybridization signals were compared with those from mock-preincubated nuclei. α -Amanitin reduced repetitive element transcription by an amount comparable to the reduction observed

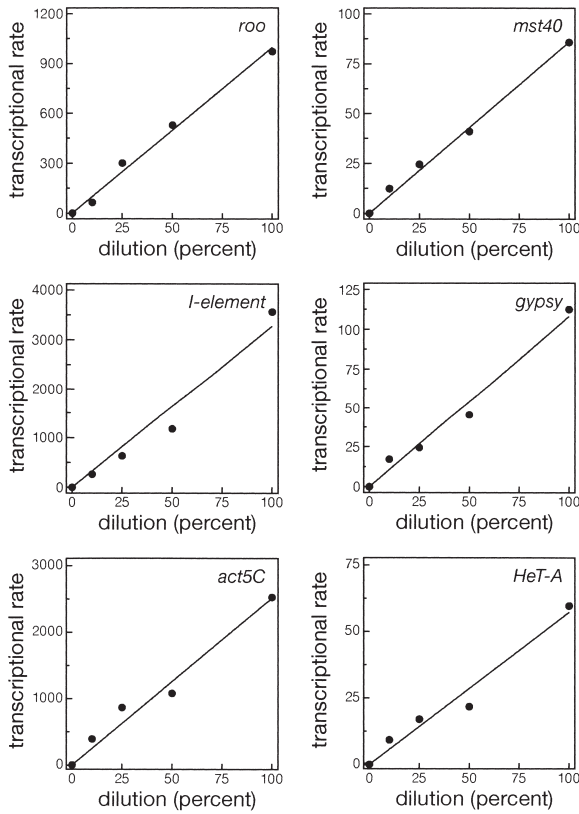


Figure 5. Nuclear run-on can detect the rates of repetitive element transcription. Dilution analysis detects no saturation of signal when registering repetitive element transcription in a nuclear run-on assay.

for the single-copy control genes *act5C* and *rp49* (Fig. 6). We conclude that in flies, repetitive elements, like protein-coding genes, are transcribed by RNA pol II.

Repetitive Elements in *D. melanogaster* Ovaries Are Silenced Posttranscriptionally

In *armi* homozygous mutants, the steady-state RNA concentration increases by a factor of three for the LTR-

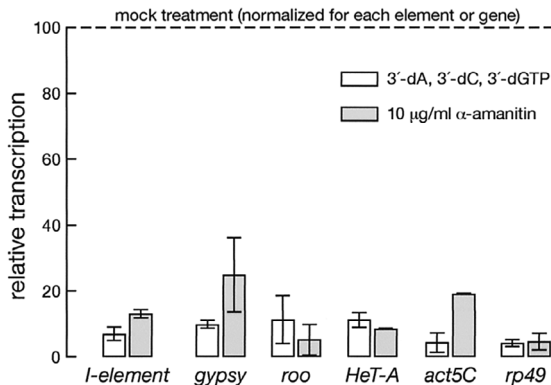


Figure 6. Repetitive element transcripts are largely RNA pol II products.

retrotransposon *gypsy* and more than 8-fold for *mst40* (Vagin et al. 2006). *mst40* sequences are located in region 40, at the base of chromosome 2L, close to or within the β -heterochromatin (Russell and Kaiser 1994). *gypsy* element insertions cause frequent mutations in *D. melanogaster* (Peifer and Bender 1988). Interestingly, studies of reversion of *gypsy*-induced mutations demonstrated that it is not simply the insertion of *gypsy* DNA that causes the mutant phenotype, because most *gypsy*-induced phenotypes can be suppressed by mutations in *suppressor of Hairy wing* [*su(Hw)*] (Peifer and Bender 1988). The 5'UTR (untranslated region) of *gypsy* contains an insulator sequence that binds Su(Hw) protein and is the only part of *gypsy* required to block the interaction of enhancers with the promoters they regulate. Insertion of this insulator sequence between an enhancer and a promoter by insertion of a *gypsy* element uncouples the enhancer from the promoter (Gause et al. 2001).

The *gypsy* element can only be mobilized in male and female progeny of mothers that both contain active copies of *gypsy* and are homozygous for permissive *flamenco* (*flam*) alleles (Chalvet et al. 1998; Sarot et al. 2004). What protein-coding genes, if any, reside in *flam* is unknown.

To determine the mechanism by which *Armi* silences *mst40* and *gypsy*, we analyzed ovaries from heterozygous and homozygous *armi* mutant females by nuclear run-on (Fig. 7). We found no significant change in transcription between *armi*^{+/+} and *armi*/*armi* ovaries for either *mst40* or *gypsy*. We conclude that in the female germ line of *D. melanogaster*, *Armi* acts to silence these two repetitive elements posttranscriptionally, rather than transcriptionally.

CONCLUSIONS

We have shown that nuclear run-on can be used to measure changes in transcriptional rates in the female *D. melanogaster* germ line. Our data indicate that as little as an approximately 1.5-fold change in the rate of transcription can be detected by this method. Our nuclear run-on protocol likely measures changes in transcriptional elongation, because rates of transcription decline with time, consistent with a low rate of pol II reinitiation in the isolated nuclei. Our data support the view that the nuclear run-on technique can be applied not only to single-copy genes, but

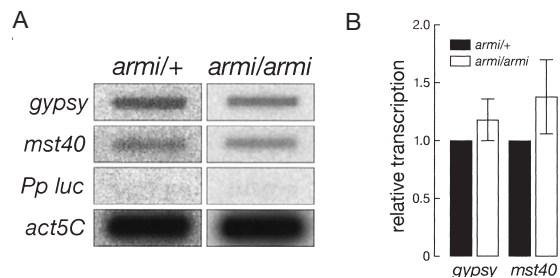


Figure 7. (A) A nuclear run-on experiment representative of the data used for the quantitative analysis in B. (B) The transcriptional rates of the retrotransposon *gypsy* and the repetitive sequence *mst40* do not change significantly when silencing is lost in homozygous mutant *armi* ovaries. The average (\pm standard deviation) relative to the transcriptional rate of *act5C* is presented.

also to multicopy transcriptional units such as repetitive elements and transposons. Surprisingly, we find that the retrotransposon *gypsy* and the repetitive sequence *mst40* are silenced posttranscriptionally in fly ovaries. Whether repetitive elements in general are silenced posttranscriptionally in the fly germ line remains to be established.

ACKNOWLEDGMENTS

We thank members of the Zamore lab for helpful discussions and comments on the manuscript and Alicia Boucher for assistance with fly husbandry. P.D.Z. is a W.M. Keck Foundation Young Scholar in Medical Research. This work was supported in part by grants from the National Institutes of Health to P.D.Z. (GM62862 and GM65236).

REFERENCES

- Allshire R.C., Nimmo E.R., Ekwall K., Javerzat J.P., and Cranston G. 1995. Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. *Genes Dev.* **9**: 218.
- Bernard P., Maure J.F., Partridge J.F., Genier S., Javerzat J.P., and Allshire R.C. 2001. Requirement of heterochromatin for cohesion at centromeres. *Science* **294**: 2539.
- Buhler M., Verdel A., and Moazed D. 2006. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* **125**: 873.
- Chalvet F., di Franco C., Terrinoni A., Pelisson A., Junakovic N., and Bucheton A. 1998. Potentially active copies of the gypsy retroelement are confined to the Y chromosome of some strains of *Drosophila melanogaster* possibly as the result of the female-specific effect of the flamenco gene. *J. Mol. Evol.* **46**: 437.
- Church G.M. and Gilbert W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci.* **81**: 1991.
- Copenhaver G.P., Nickel K., Kuromori T., Benito M.I., Kaul S., Lin X., Bevan M., Murphy G., Harris B., Parnell L.D., et al. 1999. Genetic definition and sequence analysis of *Arabidopsis* centromeres. *Science* **286**: 2468.
- Cox R.F. 1976. Quantitation of elongating form A and B RNA polymerases in chick oviduct nuclei and effects of estradiol. *Cell* **7**: 455.
- Djupeadal I., Portoso M., Spahr H., Bonilla C., Gustafsson C.M., Allshire R.C., and Ekwall K. 2005. RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes Dev.* **19**: 2301.
- Elgin S.C. and Grewal S.I. 2003. Heterochromatin: Silence is golden. *Curr. Biol.* **13**: R895.
- Forrester W.C., Novak U., Gelinis R., and Groudine M. 1989. Molecular analysis of the human beta-globin locus activation region. *Proc. Natl. Acad. Sci.* **86**: 5439.
- Gause M., Morcillo P., and Dorsett D. 2001. Insulation of enhancer-promoter communication by a gypsy transposon insert in the *Drosophila* cut gene: Cooperation between suppressor of hairy-wing and modifier of mdg4 proteins. *Mol. Cell. Biol.* **21**: 4807.
- Gilbert D.M. 2002. Replication timing and transcriptional control: Beyond cause and effect. *Curr. Opin. Cell Biol.* **14**: 377.
- Grumt I. and Pikaard C.S. 2003. Epigenetic silencing of RNA polymerase I transcription. *Nat. Rev. Mol. Cell Biol.* **4**: 641.
- Heard E. 2005. Delving into the diversity of facultative heterochromatin: The epigenetics of the inactive X chromosome. *Curr. Opin. Genet. Dev.* **15**: 482.
- Henikoff S. 1992. Position effect and related phenomena. *Curr. Opin. Genet. Dev.* **2**: 907.
- Holmquist G.P., Kapitonov V.V., and Jurka J. 1998. Mobile genetic elements, chiasmata, and the unique organization of beta-heterochromatin. *Cytogenet. Cell Genet.* **80**: 113.
- Huang J. and Moazed D. 2006. Sister chromatid cohesion in silent chromatin: Each sister to her own ring. *Genes Dev.* **20**: 132.
- Huisinga K.L., Brower-Toland B., and Elgin S.C. 2006. The contradictory definitions of heterochromatin: Transcription and silencing. *Chromosoma* **115**: 110.
- Kalmykova A.I., Klenov M.S., and Gvozdev V.A. 2005. Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. *Nucleic Acids Res.* **33**: 2052.
- Kato H., Goto D.B., Martienssen R.A., Urano T., Furukawa K., and Murakami Y. 2005. RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**: 467.
- Kazazian H.H.J. 2004. Mobile elements: Drivers of genome evolution. *Science* **303**: 1626.
- Kim A. and Dean A. 2004. Developmental stage differences in chromatin subdomains of the beta-globin locus. *Proc. Natl. Acad. Sci.* **101**: 7028.
- Lawrence R.J. and Pikaard C.S. 2004. Chromatin turn ons and turn offs of ribosomal RNA genes. *Cell Cycle* **3**: 880.
- Lawrence R.J., Earley K., Pontes O., Silva M., Chen Z.J., Neves N., Viegas W., and Pikaard C.S. 2004. A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Mol. Cell* **13**: 599.
- Lewis M.S. and Pikaard C.S. 2001. Restricted chromosomal silencing in nucleolar dominance. *Proc. Natl. Acad. Sci.* **98**: 14536.
- Mahtani M.M. and Willard H.F. 1998. Physical and genetic mapping of the human X chromosome centromere: Repression of recombination. *Genome Res.* **8**: 100.
- Martienssen R.A. and Colot V. 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* **293**: 1070.
- McKnight G.S. and Palmiter R.D. 1979. Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.* **254**: 9050.
- Nimmo E.R., Pidoux A.L., Perry P.E., and Allshire R.C. 1998. Defective meiosis in telomere-silencing mutants of *Schizosaccharomyces pombe*. *Nature* **392**: 825.
- Nolan T., Braccini L., Azzalin G., De Toni A., Macino G., and Cogoni C. 2005. The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*. *Nucleic Acids Res.* **33**: 1564.
- Pal-Bhadra M., Leibovitch B.A., Gandhi S.G., Rao M., Bhadra U., Birchler J.A., and Elgin S.C. 2004. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**: 669.
- Peifer M. and Bender W. 1988. Sequences of the gypsy transposon of *Drosophila* necessary for its effects on adjacent genes. *Proc. Natl. Acad. Sci.* **85**: 9650.
- Pidoux A.L. and Allshire R.C. 2005. The role of heterochromatin in centromere function. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **360**: 569.
- Puechberty J., Laurent A.M., Gimenez S., Billault A., Brun-Laurent M.E., Calenda A., Marçais B., Prades C., Ioannou P., Yurov Y., and Roizes G. 1999. Genetic and physical analyses of the centromeric and pericentromeric regions of human chromosome 5: Recombination across 5cen. *Genomics* **56**: 274.
- Riddle N.C. and Elgin S.C. 2006. The dot chromosome of *Drosophila*: Insights into chromatin states and their change over evolutionary time. *Chromosome Res.* **14**: 405.
- Russell S.R. and Kaiser K. 1994. A *Drosophila melanogaster* chromosome 2L repeat is expressed in the male germ line. *Chromosoma* **103**: 63.
- Sarot E., Payen-Groschene G., Bucheton A., and Pelisson A. 2004. Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* flamenco gene. *Genetics* **166**: 1313.
- Savitsky M., Kwon D., Georgiev P., Kalmykova A., and

- Gvozdev V. 2006. Telomere elongation is under the control of the RNAi-based mechanism in the *Drosophila* germline. *Genes Dev.* **20**: 345.
- Schubeler D., Scalzo D., Kooperberg C., van Steensel B., Delrow J., and Groudine M. 2002. Genome-wide DNA replication profile for *Drosophila melanogaster*: A link between transcription and replication timing. *Nat. Genet.* **32**: 438.
- Sijen T. and Plasterk R.H. 2003. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* **426**: 310.
- Slawson E.E., Shaffer C.D., Malone C.D., Leung W., Kellmann E., Shevchek R.B., Craig C.A., Bloom S.M., Bogenpohl J.N., Dee J., et al. 2006. Comparison of dot chromosome sequences from *D. melanogaster* and *D. virilis* reveals an enrichment of DNA transposon sequences in heterochromatic domains. *Genome Biol.* **7**: R15.
- So W.V. and Rosbash M. 1997. Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. *EMBO J.* **16**: 7146.
- Swaneck G.E., Nordstrom J.L., Kreuzaler F., Tsai M.J., and O'Malley B.W. 1979. Effect of estrogen on gene expression in chicken oviduct: Evidence for transcriptional control of ovalbumin gene. *Proc. Natl. Acad. Sci.* **76**: 1049.
- Vagin V.V., Sigova A., Li C., Seitz H., Gvozdev V., and Zamore P.D. 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**: 320.
- Volpe T.A., Kidner C., Hall I.M., Teng G., Grewal S.I., and Martienssen R.A. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**: 1833.
- Weintraub H., Larsen A., and Groudine M. 1981. Alpha-Globin-gene switching during the development of chicken embryos: Expression and chromosome structure. *Cell* **24**: 333.
- Weiss S.B. 1960. Enzymatic incorporation of ribonucleoside triphosphates into the interpolynucleotide linkages of ribonucleic acid. *Proc. Natl. Acad. Sci.* **46**: 1020.
- Zacharias H. 1995. Emil Heitz (1892–1965): Chloroplasts, heterochromatin, and polytene chromosomes. *Genetics* **141**: 7.

