Genetic Requirements for Inheritance of RNAi in *C. elegans*

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In *Caenorhabditis elegans*, the introduction of double-stranded RNA triggers sequence-specific genetic interference (RNAi) that is transmitted to offspring. The inheritance properties associated with this phenomenon were examined. Transmission of the interference effect occurred through a dominant extragenic agent. The wild-type activities of the RNAi pathway genes *rde-1* and *rde-4* were required for the formation of this interfering agent but were not needed for interference thereafter. In contrast, the *rde-2* and *mut-7* genes were required downstream for interference. These findings provide evidence for germ line transmission of an extragenic sequence-specific silencing factor and implicate *rde-1* and *rde-4* in the formation of the inherited agent.

Gene-silencing mechanisms function in regulating gene expression and cellular differentiation in a wide variety of organisms and are responsible for such diverse phenomena as chromosomal dosage compensation, genetic imprinting in mammals, virus resistance in plants, and transposon silencing in *Drosophila* (*1*–*4*). A variety of mechanisms underlie these diverse silencing phenomena, including apparent transcriptional blocks (*1*, *2*) and posttranscriptional interference (*3*, *5*). RNA signals have been implicated in the initiation of gene silencing in both natural (*1*, *5*) and experimental contexts (*6*). Recently, double-stranded RNA (dsRNA) has been shown to induce sequence-specific genetic interference in several organisms (*7*–*10*). This interference phenomenon has been named RNA interference, or RNAi. The current body of evidence favors a model in which RNAi blocks a posttranscriptional step in gene expression (*6*, *11*) and suggests possible similarities with posttranscriptional gene silencing (PTGS) phenomena previously described in plants (*12*) and *Neurospora* (*13*). In *C. elegans*, potent and long-lasting effects associated with RNAi have led to speculation that amplification of the interfering agent or modification of chromosomal targets might function in RNA interference (*6*, *14*). To gain insight into the nature of RNAi, we examined the inheritance properties associated with this phenomenon.

Transmission of RNAi from the injected hermaphrodite to the first generation (F_1) progeny has been observed for several genes (*6*, *11*, *15*). In most cases complete recovery of wild-type gene activity occurs in the second (F_2) generation after injection (*6*, *11*). However, in interference experiments targeting genes expressed in the maternal germ line, we observed interference in the F_2 generation and to a lesser extent in later generations (Fig. 1) (*16*). In genetic crosses, the interference effect was transferred with the sperm or oocyte as a dominant factor, resulting in genetic interference in the F_1 and F_2 generations up to 10 days after the injection of dsRNA (Fig. 1). The persistence of genetic interference raised the possibility that an active genetic process was required for the initiation and transmission of interference.

In other organisms, the inheritance of epigenetic effects can involve reversible alterations of the gene or of the associated chromatin. In some cases these effects can exhibit genetic dominance (*17*). We therefore examined whether the interference effect induced by RNAi exhibited linkage to the target gene. We constructed a strain such that the F_1 males that carry the RNAi effect also bear a chromosomal deletion that removes the target gene (Fig. 1B). We then investigated whether the sperm that inherit the deletion, and hence have no copies of the target locus, could carry the interference effect into the F_2 generation. The wild-type sperm and the deficiency-bearing sperm were able to transfer interference to the F_2 hermaphrodite progeny (Fig. 1B). Thus, the target locus was not needed for inheritance of the interference effect. Although males were sensitive to RNAi and could inherit and transmit RNAi acquired from their mothers (Fig. 1), direct injections into males did not cause transmission of RNAi to F_1 for several genes tested (*18*–*22*). Thus, the initial transmission of RNAi to F_1 progeny may involve a mechanism active only in hermaphrodites (*23*), whereas subsequent transmission to the F_2 progeny appears to involve a distinct mechanism that is active in both hermaphrodites and males.

A previous study identified two sets of *C. elegans* genes required for RNAi (*15*). One phenotypic class comprised of the *rde-1* and *rde-4* mutants that are deficient in RNAi but have no other phenotypes, and a second class, which includes *rde-2*, *rde-3*, *mut-2*, and *mut-7*, was deficient in RNAi and exhibited transposon mobilization, reduced fertility, and a high incidence of chromosome loss. Our studies have shown that all mutants in both phenotypic classes are strongly deficient in RNA interference in both the F_1 and later generations (*15*, *24*). However, these experiments did not address whether the activities of these genes might be sufficient in the injected animals to initiate heritable RNAi or are required directly in the F_1 or F_2 animals themselves for interference, or both.

The activities of *rde-1*, *rde-2*, *rde-4*, and *mut-7* may be sufficient in the injected hermaphrodite for interference in the F_1 and F_2 generations. We designed crosses such that wild-type activities of these genes would be present in the injected animal but absent in the F_1 or F_2 generations (Figs. 2 and 3). To examine inheritance in the F_1 generation, we injected mothers heterozygous for each mutant, allowed them to produce self-progeny, and examined whether the homozygous mutant progeny exhibited genetic interference (Fig. 2A). The *rde-1* and *rde-4* mutant F_1 progeny exhibited robust interference, comparable to that exhibited by the wild type, whereas the *rde-2* and *mut-7* F_1 progeny did not (Fig. 2A). In control experiments, injection of dsRNA directly into the *rde-1* and *rde-4* mutant progeny of uninjected heterozygous mothers did not result in interference (Fig. 2B). Thus, injection of dsRNA into heterozygous hermaphrodites results in an inherited interference effect that triggers gene silencing in otherwise RNAi-resistant *rde-1* and *rde-4* mutant F_1 progeny, whereas *rde-2* and *mut-7* mutant F_1 progeny remain resistant.

To examine the genetic requirements for RNAi genes in the F_2 generation, we generated F_1 male progeny that carry the interference effect as well as one mutant copy of each respective locus, *rde-1*, *rde-2*, and *mut-7* (Fig. 3A). We then backcrossed each of these males with uninjected hermaphrodites homozygous for each corresponding mutation (Fig. 3A). The resulting cross progeny included 50% heterozygotes and 50% homozygotes that were distinguished by the presence of the linked marker mutations. The heterozygous siblings served as controls and in each case exhibited interference at a frequency similar to that seen in wild-type animals (Fig. 3A). The *rde-2* and *mut-7* homozygous F_2 progeny did not exhibit interference, indicating that the activities of these two genes are required for interference in the F_2 generation. In contrast, homozygous *rde-1* F_2 animals exhibited wild-type levels of F_2 interference (Fig. 3A). Control *rde-1* homozygotes generated through identical crosses were resistant to *pos-1::RNAi* when challenged de novo with dsRNA in the F_2 generation (*25*). Thus, *rde-1* activity in the preceding generations was sufficient to allow interference to occur in *rde-1* mutant F_2 animals, whereas the wild-type activ-

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ities of *rde-2* and *mut-7* were required directly in the F_2 animals for interference.

In the preceding experiments, the expression of *rde-1* (+) and *rde-4* (+) in the injected animal was sufficient for interference in later generations. In contrast, the wild-type activities of the *rde-2* and *mut-7* genes were required for interference in all generations assayed. Thus, *rde-2* and *mut-7* might be required downstream only or might also function along with *rde-1* and *rde-4*. To examine whether *rde-2* and *mut-7* activities function along with or downstream of *rde-1*, we designed genetic crosses in which the activities of these genes were present sequentially (Fig. 3B). For example, we injected *pos-1* dsRNA into *rde-1* (+); *rde-2* (–) animals and then crossed these to generate *rde-1* (–); *rde-2* (+) F_1 progeny. *rde-1* (+) activity in the injected animals was sufficient for F_1 interference even when the injected animals were homozygous for *rde-2* or *mut-7* mutations (Fig. 3B); however, it was not sufficient when the injected animals were homozygous for the *rde-4* mutation (Fig. 3B). Thus, *rde-1* can act independently of *rde-2* and *mut-7* in the injected animal, but *rde-1* and *rde-4* must function together. These findings indicate that *rde-1* and *rde-4* function in the formation of the inherited interfering agent, whereas *rde-2* and *mut-7* function at a later step.

What is the physiological function of such inherited interfering agents? The *rde-1* and *rde-4* mutations appear to be simple loss-of-function mutations and do not exhibit overt phenotypes, except for a nearly complete absence of interference in response to dsRNA (*15*). However, *rde-2*, *mut-7*, and other RNAi pathway genes have several additional phenotypes, most notably a mobilization of the normally silent transposons in the germ line (*15, 26*). Because the *rde-1* and *rde-4* appear to initiate RNAi in response to dsRNA but are not required for transposon silencing, other stimuli may act upstream of *rde-2* and *mut-7* to initiate transposon silencing. The *rde-1* gene is a member of a highly conserved gene family with 22 homologs in *C. elegans* as well as numerous homologs in plants, animals, and fungi (*15*). The *Drosophila* gene *sting* encodes a *rde-1* homolog involved in a PTGS-like silencing mechanism that acts on the transcripts of the repetitive *X*-linked *Stellate* locus (*27*). Perhaps gene silencing mediated by *sting* and other *rde-1* homologs involves upstream stimuli distinct from dsRNA (Fig. 4). These distinct upstream stimuli might in turn lead to the formation of secondary extragenic agents similar to those induced by

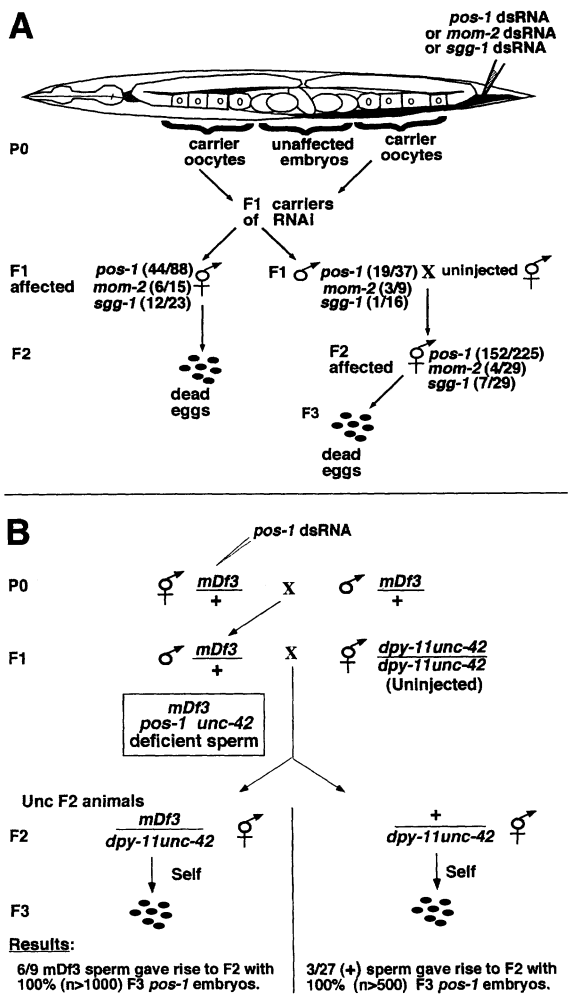

Fig. 1. Maternal establishment and paternal transmission of RNAi. (**A**) Schematic diagram showing a wild-type hermaphrodite, P0, receiving an injection of dsRNA. The needle is illustrated inserted in the intestine (the normal target for RNAi injection). (In subsequent figures, the injection of dsRNA is indicated by a similar schematic needle shown above the genotype of the recipient worm.) The three different species of dsRNA named above the needle were delivered into worms in independent experiments. The hermaphrodite gonad with its symmetrical anterior and posterior U-shaped arms is shown. Several fertilized eggs are shown in the centrally located uterus (white ovals). Rectangular mature oocytes (carrier oocytes) are shown queued up in the gonad arms most proximal to the uterus. The embryos present at the time of injection give rise to unaffected F_1 progeny. Oocytes in the proximal arms of the gonad inherit the RNAi effect but also carry a functional maternal mRNA (F_1 carriers of RNAi). After a clearance period, during which carrier and unaffected F_1 progeny are produced, the injected P0 begins to produce exclusively dead F_1 embryos with the phenotype corresponding to the inactivation of the gene targeted by the injected RNA (*19, 22, 29*). Potential F_1 and F_2 carriers of the interference effect were identified within the brood of the injected animal. In the case of hermaphrodites, carriers were defined as "affected" if the animals produced at least 20% dead embryos with phenotypes corresponding to maternal loss of function for the targeted locus. Male carriers were defined as animals whose cross progeny included at least one affected F_2 hermaphrodite. The total number of carriers identified in each generation for each of the three dsRNAs injected is given in parentheses as a fraction of the total number of animals assayed. Black ovals, F_2 and F_3 dead embryos from the carriers. (**B**) Extragenic inheritance of RNAi. Illustration of a genetic scheme to generate F_1 males that carry both *pos-1* (RNAi) and a chromosomal deficiency for the *pos-1* locus. F_2 progeny of the carrier male include two genotypes: phenotypically wild-type animals that inherit the (+) chromosome, and phenotypically uncoordinated (Unc) progeny that inherit the mDf3 chromosome. The fraction shown (in this and all subsequent figures) represents the number of RNAi-affected F_2 hermaphrodites over the total number of cross progeny scored for each genotype class.

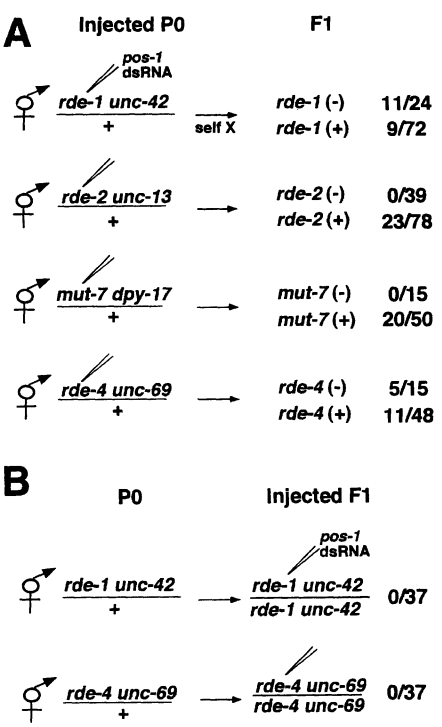

Fig. 2. Genetic schemes to determine whether the wild-type activities of *rde-1*, *rde-2*, *mut-7*, and *rde-4* are sufficient in the injected animal for interference among the F_1 self progeny. (**A**) Heterozygous hermaphrodites from each genotype class (*30*) were injected with *pos-1* dsRNA. In each case, two types of F_1 self-progeny (right), distinguished by virtue of the linked marker mutations, were scored for interference. (**B**) Homozygous F_1 progeny from heterozygous (uninjected) mothers were directly injected with *pos-1* dsRNA. The fractions indicate the number of affected animals out of the total number of animals of each genotype scored.

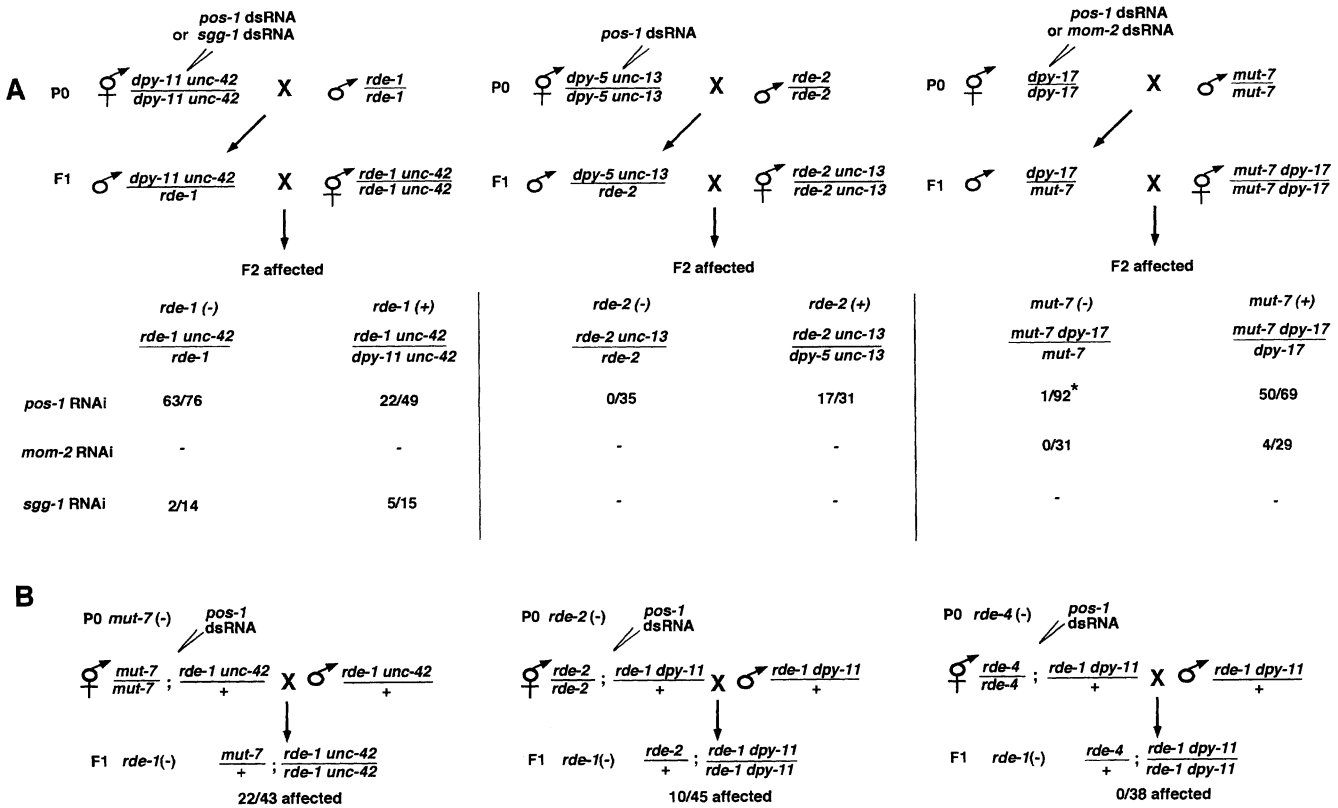

Fig. 3. Genetic crosses designed to follow the requirements for *rde-1*, *rde-2*, *rde-4*, and *mut-7* in (**A**) F_2 and (**B**) F_1 interference. (A) The dsRNAs injected are listed above the schematic needle. Recipient hermaphrodites were marked with visible mutations closely linked to wild-type alleles of each RNAi pathway gene. F_1 carrier males heterozygous for each mutation were crossed with the homozygous mutant hermaphrodites of the genotype shown. Two types of cross progeny were analyzed for F_2 interference. The results are tabulated with the injected dsRNA listed at the left and the genotype inferred from the linked visible marker mutations listed above each column. The fractions indicate the number of affected animals out of the total number of animals of each genotype scored. The asterisk indicates that the *dpy-17* gene is located 2.7 map units away from *mut-7* whereas *unc-42* and *unc-13* markers are each about 0.1 map units from *rde-1* and *rde-2*, respectively. Thus, recombination between *dpy-17* and *mut-7* is likely in F_1 males and may explain the occurrence of a single carrier F_2 animal (1/92). (B) Genetic crosses to determine whether *rde-1* activity is sufficient to initiate RNAi in injected animals that lack the wild-type activities of *rde-2*, *mut-7*, or *rde-4*. Animals with the genotypes shown were injected with *pos-1* dsRNA and then crossed to generate F_1 hermaphrodites homozygous for *rde-1*. The fraction illustrates the number of F_1 affected hermaphrodites out of the total number of animals of each genotype scored.

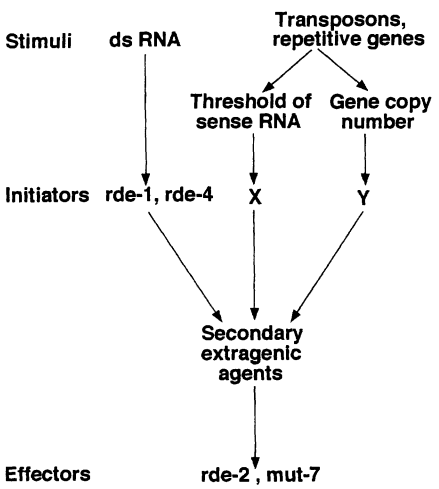

Fig. 4. Model for RNAi and other PTGS-like silencing pathways in *C. elegans*.

dsRNA injection (Fig. 4). Molecules similar to the small 25 nucleotide RNAs recently found in silenced transgenic plants (*28*) may constitute the sequence component that confers specificity on these hypothetical secondary interfering agents (Fig. 4).

References and Notes

1. B. Panning and R. Jaenisch, *Cell* **93**, 305 (1998).
2. M. A. Surani, *Cell* **93**, 309 (1998).
3. F. Ratcliff, B. D. Harison, D. C. Baulcombe, *Science* **276**, 1558 (1997).
4. S. Jensen, M.-P. Gassama, T. Heidmann, *Nature Genet.* **21**, 209 (1999).
5. F. G. Ratcliff, S. A. MacFarlane, D. C. Baulcombe, *Plant Cell* **11**, 1207 (1999).
6. A. Fire *et al.*, *Nature* **391**, 806 (1998).
7. P. M. Waterhouse, M. W. Graham, M. B. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13959 (1998).
8. H. Ngo, C. Tschudi, K. Gull, E. Ullu, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14687 (1998).
9. J. R. Kennerdell and R. W. Carthew, *Cell* **95**, 1017 (1998).
10. A. Sanchez-Alvarado and P. Newmark, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5049 (1999).
11. M. K. Montgomery, S. Xu, A. Fire, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15502 (1998).
12. D. C. Baulcombe, *Plant Mol. Biol.* **32**, 79 (1996).
13. C. Cogoni and G. Macino, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10233 (1997).
14. P. A. Sharp, *Genes Dev.* **13**, 139 (1999).
15. H. Tabara *et al.*, *Cell* **99**, 123 (1999).
16. dsRNA was synthesized in vitro with T3 and T7 polymerases. Template DNA was removed from the RNA samples by DNase treatment (30 min at 37°C). Equal amounts of sense and antisense RNAs were then mixed and annealed to obtain dsRNA. dsRNA (1 to 5 mg/ml) was injected into the intestine of animals. In control experiments, a mixture of linearized template DNA plasmids (0.2 mg/ml) used for synthesizing RNA did not induce interference in P0, F_1, or F_2 when injected into the intestine of hermaphrodites.
17. Reviewed in S. Henikoff and L. Comai, *Cell* **93**, 329 (1998).
18. Wild-type males were injected with dsRNA targeting body muscle structural gene *unc-22*, cuticle collagen gene *sqt-3*, and maternal genes *pos-1* and *sgg-1*. Males of *pes-10::gfp* strain were injected with *gfp* dsRNA. Injected males were affected by *unc-22* and *gfp* dsRNA to the same extent as injected hermaphrodites. No RNAi interference was detected in F_1 progeny of injected males (40 to 200 F_1 animals scored for each RNA tested).
19. H. Tabara, R. J. Hill, C. C. Mello, J. R. Priess, Y. Kohara, *Development* **126**, 1 (1999).
20. D. G. Moerman, G. M. Benian, R. H. Waterston, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2579 (1986).
21. H. van der Keyl, H. Kim, R. Espey, C. V. Oke, M. K. Edwards, *Dev. Dyn.* **201**, 86 (1994).
22. Embryonic lethal phenotype resulting from inactivation of *sgg-1* in the injected mother will be described elsewhere (Y. Bei, in preparation).
23. After injection of dsRNA into homozygous mutant *rde-1*, *rde-2*, and *mut-7* hermaphrodites, interference

is observed among their heterozygous *rde* (+) or *mut* (+) F_1 cross-progeny. This type of inheritance occurs only in hermaphrodites and may reflect a passive transfer of the injected material into the maternal germ line. The activities of *rde-1* and *rde-4* genes are necessary in the ensuing generation for the initiation of interference in response to this inherited material.

24. Homozygous hermaphrodites of *rde-1* and *rde-2* strains were allowed to mate with males of the same strains and then injected with *pos-1* or *mom-2* dsRNA (5 mg/ml). More than 400 F_1 hermaphrodites from each strain were picked (10 worms per plate) and their broods were examined for the occurrence of inviable *pos-1*–like or *mom-2*–like embryos. Similarly, 300 F_2 animals from these injections were analyzed. Finally, 60 F_1 males from each strain were mated and 300 of their F_2 progeny were examined for affected embryos. No *pos-1* affected embryos were observed in any generation.
25. Thirty-five *rde-1* homozygous animals generated through crosses shown in Fig. 3A were tested by feeding bacteria expressing *pos-1* dsRNA and 21 similar animals were tested by direct injections of *pos-1* dsRNA; all animals tested were resistant to *pos-1* (*RNAi*).
26. R. Ketting *et al.*, *Cell* **99**, 133 (1999).
27. A. Schmidt *et al.*, *Genetics* **151**, 749 (1999).
28. A. J. Hamilton and D. C. Baulcombe, *Science* **286**, 950 (1999).
29. C. E. Rocheleau *et al.*, *Cell* **90**, 707 (1997).
30. The alleles of RNAi-deficient mutants used in this study were as follows: *rde-1(ne300) unc-42*, *rde-1(ne219)*, *rde-2(ne221)*, *rde-4(ne299)*, and *mut-7(pk204)*.
31. We thank T. H. Shin, M. Soto, A. Fire, and members of our laboratory for many discussions; Y. Bei and T. Shin for help in scoring RNAi mutant embryos; G. Seydoux for the *pes-10::gfp* strain; and the anonymous reviewer for helpful suggestions. Some strains were obtained from the *C. elegans* Genetic Stock Center, which is funded by a grant from the NIH National Center for Research Support. Supported in part by a Pew scholarship to C.C.M. and by NIH grant GM58800.

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Single-Molecule Study of Transcriptional Pausing and Arrest by *E. coli* RNA Polymerase

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Using an optical-trap/flow-control video microscopy technique, we followed transcription by single molecules of *Escherichia coli* RNA polymerase in real time over long template distances. These studies reveal that RNA polymerase molecules possess different intrinsic transcription rates and different propensities to pause and stop. The data also show that reversible pausing is a kinetic intermediate between normal elongation and the arrested state. The conformational metastability of RNA polymerase revealed by this single-molecule study of transcription has direct implications for the mechanisms of gene regulation in both bacteria and eukaryotes.

Transcription elongation is a key step in the regulation of gene expression in cells. During elongation, RNA polymerase (RNAP) is known to move discontinuously, spending proportionally more time at some template positions, known as pause sites, than at others (*1–3*). These pauses are important for regulation and may precede other elongation events such as termination and arrest (*4*). Regulatory molecules in both bacteria and eukaryotes can switch RNAP to a pause- and termination-resistant form, but the chemical nature of this switch is not understood at present. Most of these dynamics have been characterized on complexes artificially halted by nucleotide starvation. These experiments indicate that during elongation, the 3′ end of the RNA can be displaced from the enzymes' active site either inducing pausing or extensive backtracking along the template, leading to arrest (*5–8*). Evidence of the conversion among these different states has not been obtained from molecules transcribing without interruption. To characterize these transcriptional dynamics and to establish the link between backtracking, pausing, and arrest by RNAP during continued elongation, we set out to follow directly the translocation of individual RNAP molecules along the DNA strand. This approach reveals important dynamics that are averaged out in bulk experiments. Although single-molecule experiments have already revealed several aspects of interactions between *E. coli* RNAP and DNA (*9–16*), single-molecule kinetics of transcriptional pausing have not been reported.

We followed transcription by single molecules of RNAP (n_{tot} = 87) in real time, using an integrated optical-trap/flow-control (OTFC) video microscope (Fig. 1) (*17*). There are three main advantages to this microscope. First, translocation of RNAP and pausing events can be directly observed in real time over long template lengths [1.1 ± 0.7 kilobase pairs (kbp)], because laser radiation damage is minimized. Second, by using hydrodynamic flow forces far below the stalling force, spatial and temporal resolutions are obtained that are better than assays based on Brownian motion (*12, 15, 18*). Third, pausing and other transcription events can be probed not only as a function of solution conditions, but also as a function of applied load force.

Figure 2 shows two examples of the shortening of the DNA tether between the two beads because of transcription activity by individual RNAP molecules (*19, 20*). Several features are immediately apparent: The rate of tether shortening is variable, RNAP sometimes pauses temporarily (Fig. 2, arrows), and the enzyme eventually stops permanently (Fig. 2, asterisk). The transcription rates are determined from the slopes of these plots and can be related to the position of the molecules on the template (Fig. 2, insets). The peak transcription rates appear in this graph as local maxima, and the temporary pauses as local minima (*21*).

The peak transcription rates displayed by each polymerase molecule between pauses were determined and averaged. This average peak rate varied significantly from molecule to molecule [2 to 11 bp/s for 0.2 mM nucleotide triphosphates (NTPs)]. To determine whether this variation was caused by different load forces applied to the various molecules, the mean of the average peak transcription rates for all molecules transcribing at a given force was determined and plotted against force (Fig. 3A). We found that the mean of the average peak transcription rates did not vary with force in the range of 0 to 15 pN, consistent with recent observations (*14*). The mean of the average peak transcription rate for all molecules (n = 38) over the complete force range was 7.3 ± 3 bp/s, and the mean of the average rates (i.e., including pauses) was 4.3 ± 2 bp/s for 0.2 mM NTPs and 14.5 ± 4 bp/s and 8.0 ± 3 bp/s, respectively, for 1 mM NTPs. These rates are comparable to those observed under similar conditions in solution (13 to 20 bp/s for 1 mM NTPs) (*12*) and to rates reported for single-molecule experiments on RNAP (5 to 15 bp/s for 1 mM NTPs) (*12–15*). Moreover, no transcriptional activity was observed beyond 15 pN, in agreement with reported stall force measurements on RNAP under slow force modulation conditions (*13, 14*).

The peak transcription rates for any given RNAP molecule also varies markedly along the template (2 to 27 bp/s). These variations, how-

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