Identification of Critical Staphylococcal Genes Using Conditional Phenotypes Generated by Antisense RNA

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Comprehensive genomic analysis of the important human pathogen *Staphylococcus aureus* was achieved by a strategy involving antisense technology in a regulatable gene expression system. In addition to known essential genes, many genes of unknown or poorly defined biological function were identified. This methodology allowed gene function to be characterized in a comprehensive, defined set of conditionally growth-defective/lethal isogenic strains. Quantitative titration of the conditional growth effect was performed either in bacterial culture or in an animal model of infection. This genomic strategy offers an approach to the identification of staphylococcal gene products that could serve as targets for antibiotic discovery.

Gene disruption/inactivation technology remains an important tool for identifying essential genes for bacterial growth and pathogenesis. Various strategies have been used in bacterial systems to achieve functional inactivation of gene products. Most of these involve gene knockout methods that use insertional techniques, deletions (e.g., allelic replacement), and point mutation (*1–4*). Strains carrying null mutations in genes essential for growth are generally not recoverable for further analysis; thus, conditional lethal mutants have been used successfully to identify and characterize genes essential for viability. Temperature-sensitive mutations allow maintenance of the mutant strain because of the conditional nature of the phenotype, and despite its limitations some success has been achieved (*5*, *6*).

Controlled gene expression systems, which allow selective genes to be regulated (i.e., titrated up or down) and thereby functionally analyzed, have the potential to provide more quantitative data on the gene product. Regulated systems have been described for *Escherichia coli* (*7*, *8*) and *Bacillus subtilis* (*9*, *10*), and are now being developed for bacterial pathogens (*11–13*), although this approach cannot be applied readily to comprehensive genomic analyses.

Antisense technology has been used effectively to inhibit gene expression in a variety of eukaryotic systems (*14–18*), but not extensively in bacterial systems, despite antisense regulation being a well-known phenomenon in bacteria (*19–23*).

Here, we have combined regulated expression and antisense technology for rapid identification and characterization of essential (and growth-defective) genes from the human pathogen, *Staphylococcus aureus*. We created many defined strains exhibiting conditional growth phenotypes. These strains, in which specific genes are regulated by antisense RNA, were used to analyze the effect of staphylococcal gene products on growth and/or virulence in bacterial culture and in animal models of disease.

A tetracycline (tet) transcription regulatory system that we have adapted for *S. aureus* was used to regulate gene and antisense expression (*13*, *22*). Induced expression of an antisense RNA to the α-toxin gene essentially eliminates expression of the α-toxin virulence protein, in vitro and in vivo (*22*). We used the same gene control plasmid vector system to create a random cloned library of small staphylococcal DNA fragments (200 to 800 base pairs) derived by shearing genomic DNA. The blunt-ended, agarose gel–sized and purified fragments were inserted into the plasmid downstream of the tet regulatory region [Web fig. 1 (*24*)]. The library was transformed into *S. aureus* and screened by replica plating colonies in the presence or the absence of anhydrotetracycline (ATc; a weakly antibacterial analog of tetracycline with excellent inducer properties) [Web fig. 1 (*24*)]. Colonies that grew in the absence of ATc (i.e., without induction) but that were absent or growth-defective in the presence of ATc were selected (Fig. 1A). From 20,000 transformants screened in this manner [~500 to 1000 colony-forming units (CFU) per plate], 600 independent colonies (3%) were identified as growth-defective or lethal after induction with ATc. These phenotypes were confirmed by restreaking to single colonies, then purifying plasmid from each colony, transforming the plasmid DNA back into a wild-type *S. aureus* host, and retesting growth in the presence and absence of ATc (Fig. 1B). All the initially selected conditional growth phenotypes were reconfirmed.

To identify the specific cloned DNA fragments that resulted in loss of cell viability, the

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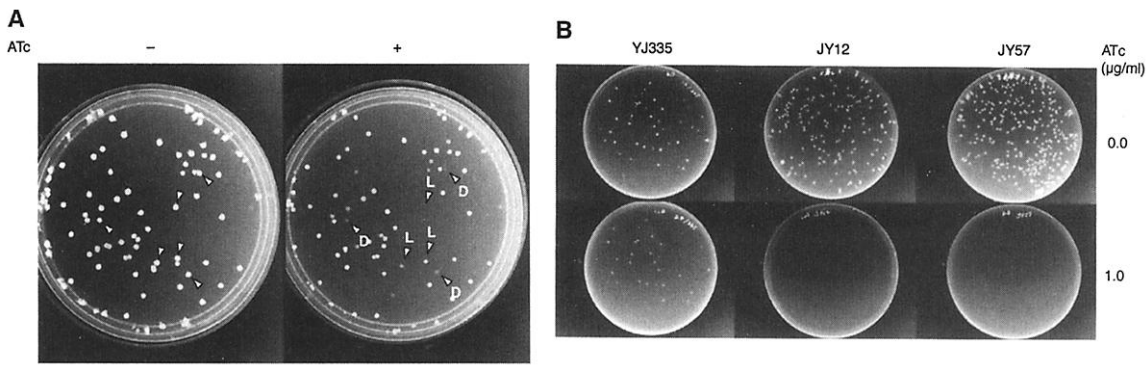

Fig. 1. (**A**) Screening growth-defective and lethal colonies. Colonies that grew normally on tryptic soy broth agar containing 5 μl/ml of Erm (TSA-Erm) plate, but appeared defective in growth or unable to grow on TSA-Erm with ATc, were identified and retested by streaking part of each colony onto the TSA-Erm-ATc and onto TSA-Erm after incubation overnight. L, lethal colony; D, defective colony. (**B**) Confirmation of growth defects and lethal events. Plasmid DNA was purified from *S. aureus* strains that carried different antisense DNA fragments and was electroporated into WCHU29-competent cells. Electrotransformants were selected on TSA-Erm and duplicated onto TSA-Erm plates containing various doses of ATc (0, 0.5, and 1.0 μg/ml).

plasmid inserts were amplified by polymerase chain reaction (PCR) with a common set of parent plasmid-specific primers (*25*) and DNA sequenced (*26*). Bioinformatic analysis (*27*) of the DNA sequences obtained indicated that about one-third of the selected clones (i.e., 1% of total clones plated) contained small, antisense-oriented fragments derived from different single open reading frame (ORF) regions. The remaining two-thirds of the clones contained sense-oriented ORF and non-ORF fragments, antisense non-ORF fragments, a mixture of sense- and antisense-oriented chimeric fragments, as well as sense and antisense fragments spanning multiple ORFs. It is likely that many of the sense-oriented and intergenic fragments represent either dominant-negative interfering sequences and/or important noncoding regulatory regions. However, we chose only those clones (~200) that represent single ORF antisense constructs for further analysis.

Our data indicated that random insertion of sheared, size-selected, blunt-ended fragments resulted in an overall efficiency of ~1 in 10^2 of generating conditional growth-defective events resulting from sub-ORF antisense induction. This efficiency is several orders of magnitude greater than that of any other conditional growth phenotypic selection procedure. Moreover, this procedure allows isolation and maintenance of the conditional strain, as well as rapid identification of the gene segment responsible for the conditional effect.

By this method we identified more than 150 critical staphylococcal genes where antisense ablation led to lethal or growth-inhibitory effects (Table 1). About 40% of these

Table 1. Characterization of antisense isolates. "Gene" denotes closest *Bacillus subtilis* homolog; "–" denotes no *B. subtilis* homolog; gene names in parentheses denote known *S. aureus* gene; asterisk denotes *Haemophilus ducreyi* 50S ribosomal protein homolog. Additional mutants can be found in Web table 1 (*24*).

Isolate	Gene	Function	Phenotype on TSA-Erm with ATc	Isolate	Gene	Proposed function	Phenotypes on TSA-Erm with ATc
YJ77-9	(DNAA)	DNA helicase	Lethal	YJ41-7	yitJ	Global transcription termination	Defect
JY143	DNAC	DNA helicase	Lethal	YJ64-4	ybeK	Nucleoside hydrolase	Lethal
JY12	(pcrA)	DNA helicase	Lethal	YJ69-3	yqeT	Probable methyltransferase	Defect
YJ2-8	(rpoB)	RNA polymerase	Lethal	JSB442	yhdO	Acyltransferase	Defect
YJ65-7	(rpoA)	RNA polymerase	Lethal	JSB162	ykpA	ABc transporter	Defect
YJ70-3	(rpoC)	RNA polymerase	Lethal	JSB319	yciP	Transporter	Defect
JY57	rpsC	30S ribosomal protein	Lethal	JSB147	obg	GTP binding protein	Lethal
JSB204	rpsD	30S ribosomal protein	Lethal	YJ79-1	ysxB	Ribosomal protein	Lethal
JY87	rpsF	30S ribosomal protein	Lethal	JSB19	ytiA	Ribosomal Protein	Lethal
YJ49-1	rpsI	30S ribosomal protein	Lethal	JSB433	*	Ribosomal protein	Defect
JSB20	rpsJ	30S ribosomal protein	Lethal	YJ49-12	ygaE	Unknown	Lethal
JY16	rpsM	30S ribosomal protein	Lethal	YJ50-7	ywbM	Unknown	Defect
YJ72-2	rpsR	30S ribosomal protein	Lethal	YJ53-2	yxdM	Unknown	Lethal
YJ3-5	rplB	50S ribosomal protein	Lethal	YJ55-1	ybcD	Unknown	Lethal
JSB237	rplC	50S ribosomal protein	Lethal	JY80	ytwP	Unknown	Lethal
JSB447	rplJ	50S ribosomal protein	Lethal	JSB4	yddR	Unknown	Lethal
JSB194	(rplK)	50S ribosomal protein	Lethal	JSB52	ywfL	Unknown	Lethal
YJ49-1	(rplM)	50S ribosomal protein	Lethal	JSB178	yurX	Unknown	Lethal
JSB168	rplN	50S ribosomal protein	Defect	JSB202	ywfL	Unknown	Lethal
JSB22	(rplO)	50S ribosomal protein	Lethal	JSB206	ywbN	Unknown	Lethal
JSB161	rplP	50S ribosomal protein	Lethal	JSB407	ykqC	Unknown	Lethal
JSB181	rplR	50S ribosomal protein	Lethal	JSB422	ywfI	Unknown	Lethal
JSB185	rplT	50S ribosomal protein	Lethal	YJ68-4	–	Unknown	Lethal
JSB453	rplV	50S ribosomal protein	Lethal	YJ7-7	–	Unknown	Lethal
YJ82-8	rplX	50S ribosomal protein	Lethal	YJ47-6	–	Unknown	Defect
JSB312	rpmA	50S ribosomal protein	Lethal	YJ49-11	–	Unknown	Defect
JSB11	rpmI	50S ribosomal protein	Lethal	YJ65-2	–	Unknown	Lethal
YJ58-5	valS	tRNA synthetase	Lethal	YJ67-4	–	Unknown	Lethal
JY34	serS	tRNA synthetase	Lethal	YJ68-17	–	Unknown	Defect
JSB150	(proS)	tRNA synthetase	Lethal	YJ69-6	–	Unknown	Defect
JY260	cysS	tRNA synthetase	Lethal	YJ69-15	–	Unknown	Defect
JY474	alaS	tRNA synthetase	Lethal	JY156	–	Unknown	Defect
JSB335	pheS	tRNA synthetase	Lethal	JY188	–	Unknown	Defect
JSB369	sporC	Peptidyl-tRNA dehydrolase	Defect	JY349	–	Unknown	Lethal
JSB115	tsf	Elongation factor	Lethal	JY367	–	Unknown	Lethal
JY61	tufA	Elongation factor	Lethal	JY511	–	Unknown	Defect
JY365	(fus)	Elongation factor	Lethal	JSB59	–	Unknown	Lethal
YJ78-3	(secA)	Preprotein translocase	Defect	JSB60	–	Unknown	Lethal
JSB193	(secY)	Preprotein translocase	Lethal	JSB91	–	Unknown	Lethal
YJ77-2	(murB)	UDP-*N*-acetylmuramate dehydrogenase	Lethal	JSB174	–	Unknown	Defect
YJ60-6	adk	Adenylate kinase	Defect	JSB187	–	Unknown	Lethal
JY27	pyrC	Dihydrooratase	Lethal	JSB196	–	Unknown	Lethal
YJ68-14	ftsH	Cell division protein	Defect	JSB290	–	Unknown	Lethal
YJ72-3	furA	Transporter	Defect	JSB291	–	Unknown	Lethal
YJ84-8	qoxB	Quinol oxidase	Defect	JSB313	–	Unknown	Defect
YJ84-12	ampS	Aminopeptidase	Defect	JSB316	–	Unknown	Defect
YJ45-5	yufD	NADH dehydrogenase	Defect	JSB399	–	Unknown	Defect
YJ69-1	(wapA)	Fibronectin binding protein	Defect	JSB414	–	Unknown	Lethal
YJ15-9	(yomI)	Virulence extracellar factor	Defect	JSB459	–	Unknown	Lethal
JY348	(yvrC)	Hemin-binding periplasmic protein	Defect				

genes are orthologs or homologs of known essential bacterial genes.

About 30% of the staphylococcal genes that we identified appear to be homologs of bacterial genes with proposed functions, including members of known key pathways (e.g., transcription, translation, and metabolism), but also including genes with presumed biochemical activities (e.g., guanosine triphosphate binding/hydrolysis and methyl/acyl transferases) whose pathway functions remain unclear.

The remaining 30% of staphylococcal genes identified represent apparent critical genes of unknown function. Many of these have homologs in other bacteria, but as yet, little or no functional information is known.

Two distinct phenotypic classes were identified by our selection criteria. One class exhibits a no-growth (i.e., essential) phenotype after antisense induction. The second class exhibits a slow- or reduced-growth phenotype, but does eventually give rise to definitive small colonies (i.e., defective, but not lethal). Several of these defective isolates are known virulence factors, such as fibronectin binding protein (YJ69-1), virulence extracellular factor (YJ15-9), and an ABC transporter (JSB162). The slower growth of these strains in vitro may not have been detected in previous studies or, alternatively, may result from a more generalized growth defect caused by the antisense mechanism of inhibition on secreted gene products. The procedure allows identification of certain important nonessential genes by virtue of their detectable growth-inhibitory effects during selection. We cannot rule out that some of the growth-defective isolates may represent essential genes, because a suboptimal antisense effect would not inhibit expression sufficiently to obtain the lethal phenotype. Likewise, definitive identification of essential genes is problematic for genes configured in polycistronic operons. We do not know the potential effects of defined sub-ORF antisense RNA induction on the expression of genes upstream or downstream of the mRNA region to which the antisense fragment is made. In no case was a known nonessential gene identified as a conditional lethal phenotype in our analysis.

The vector system used to generate the antisense RNAs is both inducible and titratible (*22*). The growth of the various *S. aureus* antisense isolates in vitro was induced by ATc in a dose-dependent way (Fig. 2) (relative to the positive control, wild-type strain carrying the parent plasmid YJ335). Hence, quantitative evaluation of the relevance of each gene product to cell viability can be obtained by selective titration of any gene product.

To demonstrate the titration of essential genes in vivo, we chose a murine model of hematogenous pyelonephritis, because it represents a localized kidney infection from which bacteria can be readily recovered (*22*). As a control we used the wild-type *S. aureus* strain carrying the parent vector, YJ335. In the presence or absence of ATc induction, infection persisted in the animal, and ~5 × 10^5 CFU were recovered from the kidneys at 72 hours after infection (Fig. 3). Similarly, when the same *S. aureus* strain was used, but carrying different inducible antisense gene segments (YJ2-8 and YJ3-5), ~5 × 10^5 CFU were recovered from infected kidneys of animals not treated with the inducer ATc. When the animals were treated with increasing concentrations of ATc, a dose-dependent effect was observed on recoverable bacteria. At the highest ATc dose (500 ng/g), no bacteria were recovered, and the infection completely resolved (Fig. 3). Thus, our defined set of growth-defective/essential gene functions can be studied in the context of a titratible, conditional phenotype in relevant models of infection. This presents the prospect of examining the importance of a gene product when it is switched off after infection has been established.

In conclusion, the antisense system described offers a comprehensive genomic approach to readily identify and characterize growth-critical gene functions in the clinically important human pathogen, *S. aureus*. Each gene identified is maintained as one of a collection of conditional growth-defective/lethal isogenic strains. This set of isolates allows titratible phenotypic control over the expression of the gene's function in bacterial culture and in relevant models of infection.

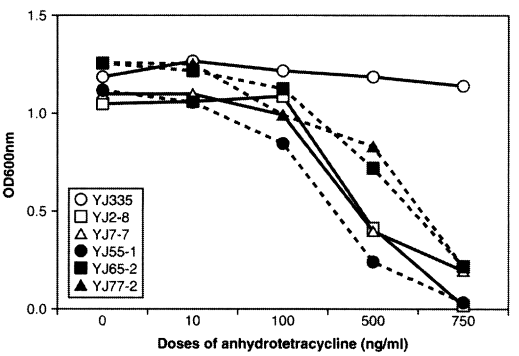

Fig. 2. Titration of inhibition of growth by induced antisense RNA. *Staphylococcus aureus* strains carrying inducible antisense RNA were incubated in tryptic soy broth (TSB) containing 5 μg/ml of Erm with various doses of ATc at 37°C, and incubated with shaking overnight. The density of cells was measured at an absorbance of 600 nm.

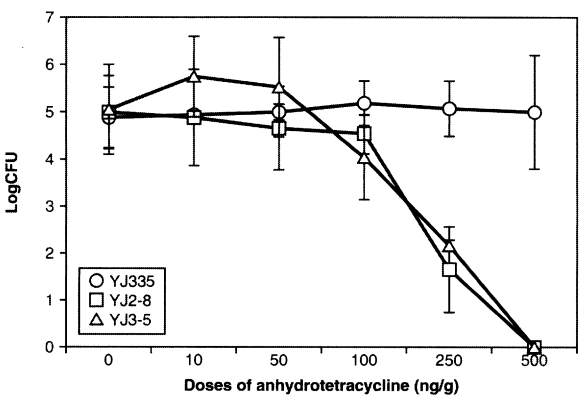

Fig. 3. Recovery of *S. aureus* from infected kidneys. CD-1 female mice (25 g) obtained from Charles River Laboratories were used for in vivo titration. *Staphylococcus aureus* strains YJ335, YJ2-8, and YJ3-5 were harvested from 1 ml of stationary-phase culture, washed once with 1 ml of phosphate-buffered saline (PBS), and diluted to an absorbance at 600 nm (A_{600}) of 0.2. These bacterial suspensions were diluted and plated onto TSA-Erm plates for determination of viable CFU. Five mice per group were infected with about 10^7 CFU of bacteria through an intravenous injection of 0.2 ml of bacterial suspension into the tail vein by using a tuberculin syringe. Various doses of ATc were given orally in 0.2-ml doses (containing 5 μg of Erm per gram of mouse body weight) to infected mice on days 1, 2, and 3 after infection. The mice were killed by carbon dioxide overdose 2 hours after the last dose of ATc induction. Kidneys were aseptically removed and homogenized in 1 ml of PBS for enumeration of viable bacteria.

References and Notes

1. A. L. Cheung, C. Wolz, M. R. Yeaman, A. S. Bayer, *J. Bacteriol.* **177**, 3220 (1995).
2. M. Hensel *et al.*, *Science* **269**, 400 (1995).
3. B. J. Akerley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8927 (1998).
4. F. Arigoni *et al.*, *Nature Biotechnol.* **16**, 851 (1998).
5. M. B. Schmid, N. Kapur, D. R. Isaacson, P. Lindroos, C. Sharpe, *Genetics* **123**, 625 (1989).
6. P. K. Martin, T. Li, D. Sun, D. P. Biek, M. B. Schmid, *J. Bacteriol.* **181**, 3666 (1999).
7. L. M. Guzman, D. Belin, M. J. Calson, J. Beckwith, *J. Bacteriol.* **177**, 4121 (1995).
8. C. A. Rappleye, J. R. Roth, *J. Bacteriol.* **179**, 5827 (1997).
9. M. Geissendorfer, W. Hillen, *Appl. Microbiol. Biotechnol.* **33**, 657 (1990).
10. V. Vagner, E. Dervyn, S. D. Ehrlich, *Microbiology* **144**, 3097 (1998).
11. V. de Lorenzo, L. Eltis, B. Kessler, K. N. Timmis, *Gene* **123**, 17 (1993).
12. M. Stieger, B. Wohlgensinger, M. Kamber, L. Rolf, W. Keck, *Gene* **226**, 243 (1999).
13. L. Zhang *et al.*, *Gene* **255**, 297 (2000).
14. M. C. Costa de Beauregard, E. Pringault, S. Robine, D. Louvard, *EMBO J.* **14**, 409 (1995).
15. S. Agrawal *et al.*, *Proc. Natl. Acad. Sci. U.S.A* **94**, 2620 (1997).
16. A. Fire *et al.*, *Nature* **391**, 806 (1998).
17. T. Mizuta *et al.*, *Nature Biotechnol.* **17**, 583 (1999).
18. M. D. De Backer *et al.*, *Nature Biotechnol.* **19**, 235 (2001).
19. E. G. H. Wagner, R. W. Simons, *Annu. Rev. Microbiol.* **48**, 717 (1994).

20. T. Parish, N. G. Stoker, *FEMS Microbiol. Lett.* **154**, 151 (1997).
21. L. Good, P. E. Nielsen, *Nature Biotechnol.* **16**, 355 (1998).
22. Y. Ji, A. Marra, M. Rosenberg, G. Woodnutt, *J. Bacteriol.* **181**, 6585 (1999).
23. S. Brantl, E. G. H. Wagner, *Mol. Microbiol.* **35**, 1469 (2000).
24. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/293/5538/2266/DC1.
25. The specific DNA fragments leading to a lethal or growth-defective event after induction were amplified by PCR with plasmid-specific primers tetRfor1399 (5′-CAATACAATGTAGGCTGC-3′) and catUrev (5′-AGTTCATTTGATATGCCTCC-3′), and PCR SUPERMix (GiBCO-BRL life technologies). All PCRs were performed in a Perkin-Elmer 9600 thermal cycler. After addition of template DNA, the PCR mixture was denatured at 94°C for 2 min, followed by the thermal cycle (30 s at 94°C, 30 s at 51°C, and 1.5 min at 72°C).
26. DNA sequences of all antisense segments contained in strains listed in tables are available upon request, and identification numbers of unknown genes can be found on Web table 2 (*24*).
27. S. F. Altschul *et al.*, *Nucleic Acids Res.* **25**, 3389 (1997).
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A Role for the RNase III Enzyme DCR-1 in RNA Interference and Germ Line Development in *Caenorhabditis elegans*

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An early event in RNA interference (RNAi) is the cleavage of the initiating double-stranded RNA (dsRNA) to short pieces, 21 to 23 nucleotides in length. Here we describe a null mutation in *dicer-1* (*dcr-1*), a gene proposed to encode the enzyme that generates these short RNAs. We find that *dcr-1*(−/−) animals have defects in RNAi under some, but not all, conditions. Mutant animals have germ line defects that lead to sterility, suggesting that cleavage of dsRNA to short pieces is a requisite event in normal development.

In many organisms, dsRNA initiates a potent posttranscriptional gene-silencing phenomenon known as RNAi (*1*, *2*). First discovered in *Caenorhabditis elegans* in 1998 (*3*), RNAi is closely related to posttranscriptional gene silencing in plants (*4*–*6*), and quelling in fungi (*7*, *8*) and likely represents a conserved, ancient pathway. In *C. elegans*, a number of genes are known to be required for efficient RNAi (*9*–*12*). Among these, *rde-1* and *rde-4* act early in the pathway and, by an unknown mechanism, facilitate the formation of a heritable, extragenic agent that can transmit RNAi to offspring (*9*, *13*). Several genes required for RNAi also have important roles in other cellular processes. For example, *rde-2* and *mut-7*, in addition to acting downstream of *rde-1* and *rde-4*, are necessary for transposon silencing and cosuppression (*10*, *14*). A subset of the *smg* genes are important for the persistence of silencing by RNAi and are also involved in nonsense-mediated decay (*12*). Mutations in *ego-1*, which encodes a protein with homology to an RNA-dependent RNA polymerase, disrupt RNAi for some germ line genes and lead to defects in germ line development (*11*). Although these genes and others are clearly important for RNAi, as yet, their precise role in the pathway remains unknown.

An important clue in regard to the mechanism of RNAi came with the discovery that RNAi generates 21- to 23-nucleotide (nt) pieces (sense and antisense), called small interfering RNAs (siRNAs) (*15*–*18*). siRNAs originate from the initiating dsRNA and are required for targeting the cognate message for degradation. On the basis of the characteristics of these pieces and the requirements of RNAi, it was proposed that a protein with sequence similarity to the dsRNA nuclease, RNase III, as well as an RNA helicase, was involved in the process (*2*). In support of this idea, siRNAs have been shown to have 3′ overhangs, 2 nt in length, as expected of an RNase III–like activity (*18*). Further, immunoprecipitation of the *Drosophila* RNase III/helicase enzyme (called Dicer) from extracts yields an immunoprecipitate that can degrade dsRNA into siRNAs, and decreased Dicer levels in vivo correlate with decreased gene silencing (*19*).

To obtain direct evidence that Dicer is involved in gene silencing, we examined the effectiveness of RNAi in a *C. elegans* strain containing a null mutation in the Dicer homolog (*dcr-1*). DCR-1 is encoded by an 8165–base pair (bp) gene in *C. elegans* and contains an NH_2-terminal DExH/DEAD-box type RNA helicase domain, two RNase III–like domains, and a COOH-terminal dsRNA binding motif (Fig. 1A). Animals with a deletion in *dcr-1* that removes a 2470-bp fragment spanning a region from exon 13 to intron 18 (Fig. 1A) were obtained from the *C. elegans* gene knockout consortium. The deletion removes the NH_2-terminal portion of the first RNase III domain and is also predicted to introduce multiple stop codons into the reading frame. We observed that *dcr-1*(−/−) animals were sterile, suggesting that DCR-1 has an essential role in vivo and also emphasizing that the deletion creates a loss-of-function allele.

In *C. elegans*, RNAi is typically initiated by injecting or feeding dsRNA, and gene silencing is subsequently observed in the F_1 progeny (*3*, *20*, *21*). Because *dcr-1*(−/−) animals were sterile and did not give rise to progeny, we used a transgenic line in which we could monitor RNAi in individual animals, without waiting for subsequent generations. The line was constructed by microinjecting DNA encoding green fluorescent protein (GFP) (*sur5::GFP*), as well as a previously described vector (*22*) containing an RNA hairpin matching the GFP sequence, under the control of a heat shock promoter [*hsp16-2$_p$GFP(IR)*]. In the transgenic line we isolated, heat shock produced an easily discernable RNAi phenotype in heat-shocked animals, so it was not necessary to analyze progeny (Fig. 1B). We mated our transgenic line with *dcr-1*(+/−) animals and examined *dcr-1*(−/−) progeny for RNAi resistance after heat shock. Whereas wild-type animals exhibited robust RNAi interference measured by a loss in GFP fluorescence (98% had substantially reduced GFP fluorescence, n = 120), animals homozygous for the *dcr-1* deletion were RNAi defective and continued to exhibit a strong fluorescence (Fig. 1B and Table 1). These results are consistent with the idea that *dcr-1* is required for RNAi.

Gene silencing by RNAi is known to in-

Table 1. *dcr-1* mutants are RNAi defective.

Trial	Percentage of *dcr-1(ok247)* animals exhibiting fluorescence
1	94*(n=33)
2	95(n=19)
3	94*(n=16)

*The remaining 6% displayed an intermediate phenotype.

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