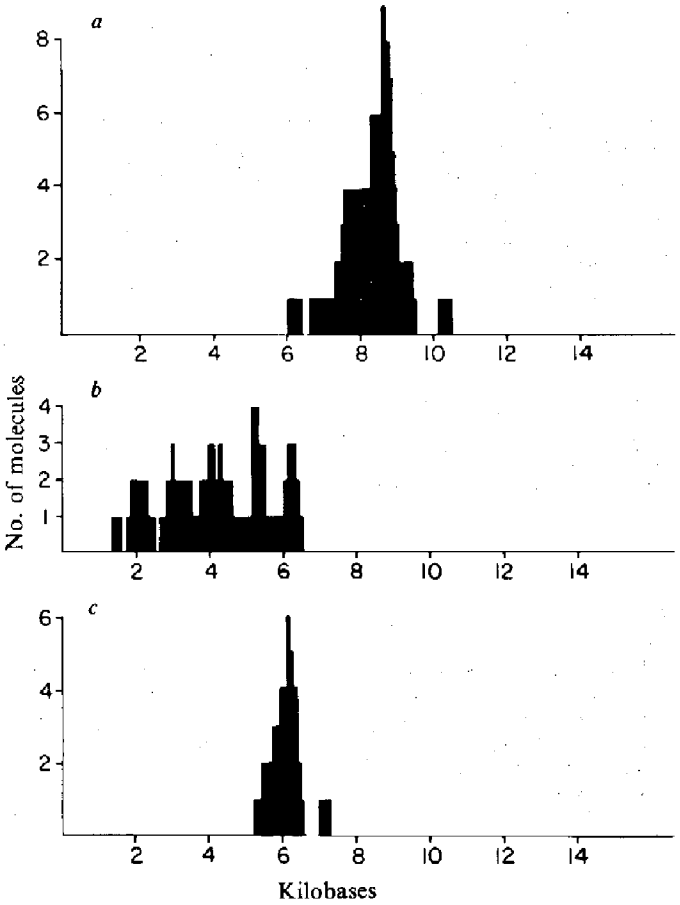

Fig. 5 Mapping of the replication start sites. The distance from the midpoint of the eye forms to the ends of each molecule was measured. A 3% error correction, determined by successive measurements of the standard, pBR322, was incorporated into each determination. *a*, The longer distance from the midpoint of the eyes to the *Kpn*I site on molecules between 10.8 and 13.8 kb; *b*, the shorter distance from the midpoint to the *Kpn*I site; *c*, the eye midpoint on molecules between 9 and 10.3 kb.

at the boundary between the 26S gene and the non-transcribed spacer, a distance that is 6.15 kb from the *Kpn*I site (Fig. 1).

The finding of a specific replication origin for the rDNA genes of *L. variegatus* is consistent with previous work in prokaryotes and the localization of origins in lower eukaryotic extrachromosomal elements. In addition to the finding of specificity, the suggested localization within the rDNA spacer is consistent with the assignment of the other mapped origins to non-transcribed regions. The amplified rRNA genes in *Tetrahymena*[6,22] and *Physarum*[7] have specific origins of replication mapping near the middle of the spacer separating the linear, palindromic genes. Using Miller spreads of ribonuclear complexes, the ribosomal genes of *Drosophila* showed a negative correlation between the centre of eye forms and transcription fibrils, suggesting that the origin resides in the spacer region[23]. In yeast, autonomously replicating plasmids (ARS) with specific chromosomal inserts have been characterized by a higher transformation frequency than uninserted vectors, a closed circular form, and a high rate of curing[9,24]. The putative origin of the yeast ARS element containing the *Trp1* gene maps in a non-transcribed region flanking the 3′ end of the gene [25,26]. The origin in *Xenopus* mitochondrial DNA also maps to a region in which no transcripts have been detected[27]. If origins in general are located in spacer regions, then the compelling need for an RNA primer to accompany replication must be resolved with the absence of spacer transcription. The promoters located in some spacers could serve a replication function[28-30].

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1. Jacob, F., Brenner, S. & Cuzin, F. *Cold Spring Harb. Symp. quant. Biol.* **28**, 329–348 (1963).
2. Kolter, R. & Helinski, D. R. *A. Rev. Genet.* **13**, 355–391 (1979).
3. Nathan, D. & Danna, K. *Nature new Biol.* **236**, 202–212 (1972).
4. Crawford, L. V., Syrett, C. & Wilde, A. *J. gen. Virol.* **21**, 515–521 (1973).
5. Chalberg, R. & Kelly, T. *J. molec. Biol.* **135**, 988–1012 (1979).
6. Robberson, D. L., Clayton, D. A. & Morrow, J. F. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4447–4451 (1974).
7. Truett, M. A. & Gall, J. G. *Chromosoma* **64**, 295–303 (1977).
8. Vogt, V. M. & Braun, R. *Eur. J. Biochem.* **80**, 557–566 (1977).
9. Struhl, K., Stinchcomb, D. T., Scherer, S. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1035–1039 (1979).
10. Harland, R. M. & Laskey, R. A. *Cell* **21**, 761–777 (1980).
11. Blin, N. *et al. J. biol. Chem.* **254**, 2716–2721 (1979).
12. Wilson, F. G., Blin, N. & Stafford, D. C. *Chromosoma* **58**, 247–253 (1976).
13. Edenburg, H. J. & Huberman, J. A. *A. Rev. Genet.* **9**, 255–284 (1975).
14. Wellauer, P. K., Dawid, I. B., Brown, D. D. & Reeder, R. H. *J. molec. Biol.* **105**, 461–486 (1976).
15. Arnheim, N. & Southern, E. M. *Cell* **11**, 366–370 (1977).
16. Dawid, I. B. & Wellauer, P. K. *Cold Spring Harb. Symp. quant. Biol.* **42**, 1185–1194 (1977).
17. Long, E. & Dawid, I. B. *A. Rev. Biochem.* **49**, 727–764 (1980).
18. Dressler, D., Wolfson, J. & Magazin, M. *Proc. natn. Acad. Sci. U.S.A.* **69**, 998–1002 (1972).
19. Huberman, J. A. & Riggs, A. D. *J. molec. Biol.* **32**, 327–341 (1968).
20. Kriegstein, J. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **71**, 135–139 (1974).
21. Weintraub, H. *Nature new Biol.* **236**, 195–197 (1972).
22. Cech, T. & Brehn, S. L. *Nucleic Acids Res.* **9**, 3531–3543 (1981).
23. McKnight, S. L., Bustin, M. & Miller, O. L. *Cold Spring Harb. Symp. quant. Biol.* **42**, 741–754 (1977).
24. Stinchcomb, D. T., Thomas, M., Kelley, J., Selkor, E. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4559–4563 (1980).

Suppression of an amber mutation by microinjection of suppressor tRNA in *C. elegans*

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Informational suppression by nonsense suppressor tRNAs has classically been a powerful tool for study of the mechanism of protein synthesis, to obtain conditional mutants and to demonstrate that a gene encodes a given protein product (for reviews see refs 1–3). In the nematode *Caenorhabditis elegans*, two genetically identified suppressors, *sup-5* and *sup-7* (refs 4, 5), have recently been shown to be amber suppressor tRNAs (N. Wills, R. F. Gesteland, J. Karn, L. Barnett, S. Bolten and R. H. Waterston, personal communication). We report here the microinjection of *sup-7* tRNA into the gonad of an animal bearing an amber allele of a maternal-effect mutant affecting sex determination (*tra-3*). We observe phenotypic suppression in the injected parent's offspring. tRNA from wild-type animals does not show this *in vivo* suppressor activity, and *sup-7* tRNA does not cause suppression of a non-amber allele of the same gene. *In vivo* suppression of an amber mutant by microinjection provides a new means of gene manipulation in *C. elegans*.

tRNA was introduced into *C. elegans* embryos by injection into the hermaphrodite gonad (Fig. 1). In this technique, a micropipette penetrates the adult cuticle and body wall to enter the distal arm of the gonadal tube (~400 μm long and 30 μm in diameter, Fig. 1*a*). This region of the gonad consists primarily of immature germ cells connected by bridges to a central core of cytoplasm[6] (Fig. 1*b*). Studies with a fluorescein-tagged D-amino acid dodecapeptide (synthesized and kindly provided by D. Weisblatt) demonstrate that small injected macromolecules

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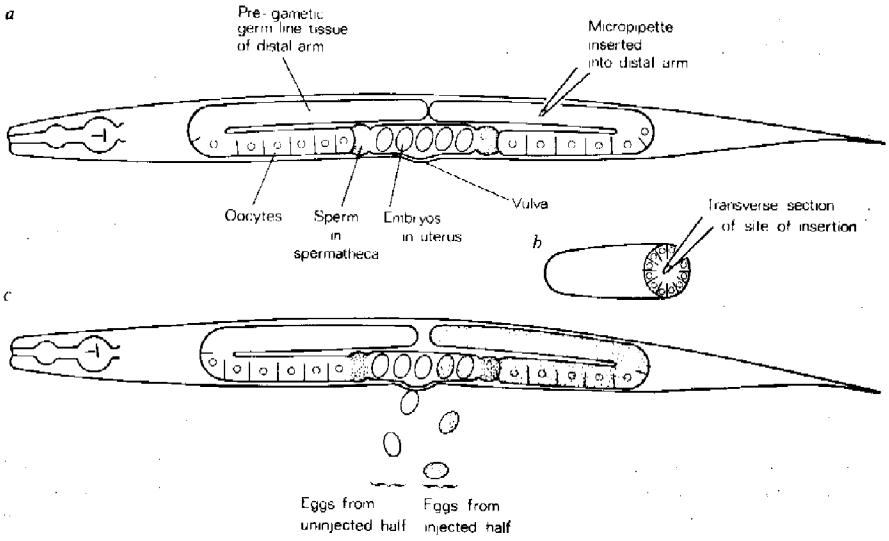

Fig. 1 Introduction of tRNA into embryos by microinjection into the parental gonad. The hermaphrodite gonad consists of two symmetrical and equivalent U-shaped tubes[6]. Each half produces oocytes and sperm; embryos are made in assembly-line fashion as oocytes pass, one by one into the spermatheca to be fertilized, and then enter the uterus to begin embryogenesis. A micropipette (4–6 μm diameter at the tip) is inserted into the germ-line tissue in the distal arm of the gonad of an animal anaesthetized with 0.01% tetramisole and 0.1% tricaine mounted against the edge of a coverslip under Voltalef oil (BDH), and inspected using a Wild M8 dissecting microscope at ×50 magnification. 5–10 pl tRNA (20 mg ml^{-1}) is injected using an oil-filled syringe connected to the micropipette. Each injected worm is examined after 4–8 h to ensure that the injected half-gonad is still producing embryos. Embryos made by the uninjected half-gonad serve as an internal control.

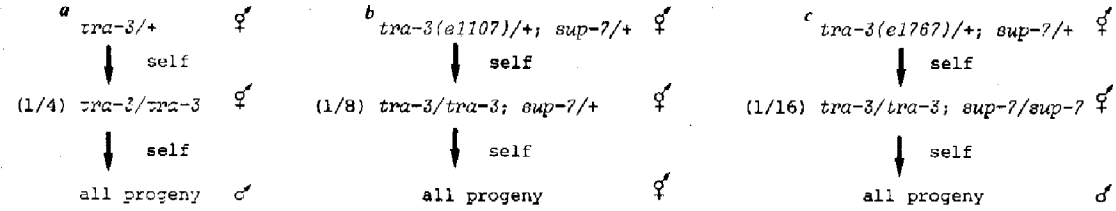

Fig. 2 *a*, Maternal effect of *tra-3*[7]. Homozygous *tra-3/tra-3* XX animals are hermaphrodite (♀) if born from a heterozygous *tra-3/+* parent, but are pseudomale (♂) if born from a homozygous *tra-3/tra-3* parent. Thus, expression of the wild-type gene in the heterozygous parent rescues the phenotype of its homozygous progeny. In addition, a wild-type gene introduced by cross-fertilization by a wild-type male produces hermaphrodite cross-progeny, again rescuing the *tra* phenotype. *b*, One allele of *tra-3*, *e1107*, is suppressed. Here, we show that one maternal dose of *sup-7* is sufficient for suppression. Other crosses have shown that one copy of *sup-5* or *sup-7* in either the hermaphrodite parent or the zygote can rescue the *e1107* phenotype. *c*, Another allele of *tra-3*, *e1767*, is not suppressed. Two copies of *sup-7* in both parent and zygote cannot rescue the *e1767* phenotype.

can diffuse within minutes throughout the U-shaped gonadal tube and that oocytes and embryos produced by this tube contain injected tracer (data not shown). Thus, this procedure provides a means of injecting several embryos by a single injection into a target tissue that is large and easy to inject compared with the embryo (Fig. 1*c*).

The mutation selected for attempted rescue by microinjection of *sup-7* tRNA is in the *tra-3* locus[7]. Normally in *C. elegans*, sex is determined by the X:autosome ratio[11]. In a wild-type diploid strain, XX animals are self-fertilizing hermaphrodites and X0 animals are male. However, recessive mutations in one of three autosomal *transformer* genes (*tra-1*, *tra-2* and *tra-3*) divert XX animals from the hermaphrodite to the male pathway of development[7].

tra-3 was chosen for mutant rescue by injection of suppressor tRNA for two reasons. First *tra-3* exhibits a maternal effect (Fig. 2*a*). The wild-type gene product expressed by a heterozygous (*tra-3/+*) XX hermaphrodite rescues its homozygous (*tra-3/tra-3*) self progeny. The self progeny of these *tra-3/tra-3* hermaphrodites, however, all develop as morphologically distinct pseudomales (Fig. 3*a*). The first sign of sexual dimorphism is midway during embryogenesis (J. Sulston, personal communication). Thus, the *tra-3* gene product is likely to be present in oocytes, and must function during embryogenesis.

Second, both amber and non-amber alleles of *tra-3* are available. Genetic tests with *sup-5* and *sup-7*, both amber suppressors, have shown that *e1107* is an amber allele of *tra-3* and that *e1767* is not (Fig. 2*b*,*c*). (Alleles are given lettered numbers, for example, *e1107*, according to the place and time isolated[8].) Even a single copy of the *sup-5* gene, the weaker of the two suppressors[5], is sufficient to rescue the *tra-3* phenotype of *e1107* progeny (Fig. 2*b*). Conversely, two copies of the stronger suppressor, *sup-7*, do not rescue the *tra-3* phenotype of *e1767* progeny (Fig. 2*c*).

sup-7 tRNA (for extraction procedure see Table 1 legend) was microinjected into the distal arm of *e1107/e1107* hermaphrodite gonads, and the progeny of each injected parent examined for suppression. The tail, vulva and gonad morphology of a hermaphrodite is easily distinguishable from that of the expected *tra-3* pseudomale. Ten animals continued to produce embryos in the half-gonad injected. Of these, six produced some progeny that were clearly hermaphrodite (Table 1). The presence of any hermaphrodites among progeny of a

Table 1 Results of suppressor tRNA microinjection in *C. elegans*

Allele injected	tRNA injected	No. injected*	No. progeny†	No. hermaphrodites
e1107	*sup-7*	10	1,023	21
e1767	*sup-7*	10	1,106	0
e1107	Wild type	13	1,127	0
e1107	—	—	4,000	0

Wild-type (*C. elegans* Bristol strain, N2) and *sup-7*-bearing [RW2108 = *unc-15(e1214)*I; *dpy-18(e364)*III; *sup-7(st5)*X] animals grown in liquid culture[9] were suspended in 0.05 M disodium napthalene disulphonate together with an equal volume of phenol saturated with 1% aqueous 8-hydroxyquinoline and disrupted in a French pressure cell at 2,000 p.s.i. RNA was extracted according to the method of Kirby[10] and fractionated on a DEAE-cellulose (Whatman DE52) column. The fraction eluting between 0.25 M and 1.0 M NaCl, containing 4S, 5S and small RNAs, was assayed for tRNAs by aminoacylation with ^{14}C-labelled amino acids, using a crude nematode aminoacyl tRNA synthetase preparation. Using a mixture of 10 ^{14}C-labelled amino acids (each 100 cmol^{-1}), 300–500 pmol per A_{260} unit of both wild-type and *sup-7* crude tRNAs were charged.

* Only animals that continue to produce embryos in the half-gonad injected are included.

† Only half the number listed arise from the injected half-gonad.

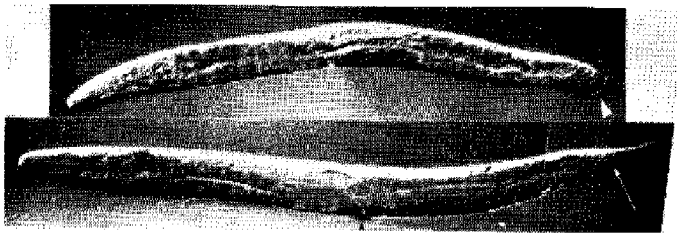

Fig. 3 Comparison of the unrescued *tra-3* phenotype, a pseudomale (*a*), and a rescued hermaphrodite (*b*). Both animals were derived from a *tra-3/tra-3* XX hermaphrodite injected with *sup-7* tRNA. The pseudomale possesses a vestigial male tail (white arrowhead); the hermaphrodite has a whip-like tail (arrow). The pseudomale either has no vulva, as shown here, or a grossly malformed vulva; the hermaphrodite has a normal vulva (small black arrowhead). The pseudomale gonad is intersexual; the rescued gonad is indistinguishable from a hermaphrodite gonad, except that oocytes are made later than normal. Only one hermaphrodite has laid eggs, so some defect in the egg-laying system is probable. The injected animal carries a mutation in *dpy-4* (*e1166*) which is closely linked to *tra-3* on chromosome IV; this distinguishes the *tra-3* homozygote among progeny of a heterozygous parent. In addition, the animal is homozygous for a mutation in *nuc-1* (*e1392*), which encodes the major endodeoxyribonuclease.

homozygous *tra-3* animal is particularly striking as no hermaphrodites were observed among the progeny of control animals (see below). Thus, 60% of the injected animals showed the effects of suppression. It is unclear, however, whether the injected RNA acts before or after fertilization. The genotype of the injected animals was confirmed by their production of pseudomales (expected to arise from the uninjected half gonad). The genotype of the rescued hermaphrodite progeny produced after injection was verified in turn by examining their self progeny; in all cases, only *tra-3* pseudomales were produced, confirming that these phenotypic hermaphrodites were homozygous *tra-3* animals.

As controls, *sup-7* tRNA was injected into animals homozygous for the non-amber allele, *e1767*, and wild-type tRNA was injected into animals homozygous for the amber allele, *e1107*. In addition, 4,000 progeny of uninjected *e1107/e1107* hermaphrodites were examined. In both injected and uninjected control animals, no hermaphrodite progeny were produced (Table 1). Therefore, production of hermaphrodites after injection of *sup-7* tRNA into *e1107/e1107* animals must result from *in vivo* suppression.

The small number of hermaphrodites made by each injected parent may result from the relative impurity of the tRNA injected, or it may mean that the injected molecules are concentrated into a few oocytes. Experiments are in progress to determine more precisely when the rescued embryos are made after injection, and what the effects of injecting a more purified tRNA preparation might be on the efficiency of suppression.

In vivo suppression by *sup-7* tRNA provides a convenient means of assaying and manipulating gene function during embryogenesis. Injection of *sup-7* tRNA into individual blastomeres after fertilization should generate precisely engineered genetic mosaics for subsequent analysis of tissue-specific developmental effects. In addition, suppression of *tra-3* or other genes with suppressible alleles may prove useful as a bioassay to detect transcripts of a cloned DNA encoding the *sup-7* gene *in vivo*.

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1. Gorini, L. *A. Rev. Genet.* **4**, 107–134 (1970).
2. Korner, A. M., Feinstein, S. I. & Altman, S. in *Transfer RNA* (ed. Altman, S.) 105–135 (MIT Press, Cambridge, Massachusetts, 1978).
3. Celis, J. E. & Smith, J. D. (eds) *Nonsense Mutations and tRNA Suppressors* (Academic, London, 1979).
4. Waterston, R. H. & Brenner, S. *Nature* **275**, 715–719 (1978).
5. Waterston, R. H. *Genetics* **97**, 307–325 (1981).
6. Hirsh, D., Oppenheim, D. & Klass, M. *Devl Biol.* **49**, 200–219 (1976).
7. Hodgkin, J. A. & Brenner, S. *Genetics* **86**, 275–287 (1977).
8. Horvitz, H. R., Brenner, S., Hodgkin, J. & Herman, R. K. *Molec. gen. Genet.* **175**, 129–133 (1979).
9. Sulston, J. E. & Brenner, S. *Genetics* **77**, 95–104, (1974).
10. Kirby, K. S. *Meth. Enzym.* **12B**, 87–98 (1968).
11. Madl, J. E. & Herman, R. K. *Genetics* **93**, 393–402 (1979).

When do carcinogen-treated 10T1/2 cells acquire the commitment to form transformed foci?

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A major advance in analysing the mechanisms of chemical carcinogenesis has been the development of cell culture systems in which normal rodent cells can be transformed as a result of *in vitro* exposure to specific chemicals or radiation[1–6]. An unexpected finding in these systems is that the frequency of transformation is often much greater than that of induction of drug-resistant mutants[3,6–10]. In addition, studies using the technique of cell spreading have suggested that the latent period between exposure of cell cultures to carcinogens and the emergence of cells having the heritable ability to form transformed foci is also much greater than that for the induction of drug-resistant mutants[8,11,12]. In most of the above transformation studies, the cells were exposed to carcinogens at low cell densities and, therefore, mutation-like events that might be induced with a low frequency could have been missed. Thus, we have now analysed the kinetics of cell transformation of C3H 10T1/2 cells when exposed to the potent ultimate carcinogen (±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BPDE) in conditions of high cell density. We report here that in these conditions acquisition of the ability to form transformed foci and acquisition of ouabain resistance occur with similar kinetics and within 2 days of carcinogen exposure.

To determine when carcinogen-treated C3H 10T1/2 cells acquire the heritable property of ouabain resistance or the ability to form morphologically transformed foci we used the protocol described in Fig. 1 legend. Replicate plates of cells were trypsinized and replated at the same density at various times after exposure to the carcinogen, and then the secondary plates were incubated and scored for the number of ouabain-resistant clones or the number of foci of transformed cells. The rationale is that if colonies of committed cells have already begun to form on the donor plates, then the individual cells of such colonies will be dispersed on the secondary plates where they will form multiple colonies or foci. Thus, the time after carcinogen treatment at which reseeding expands the number of colonies or foci indicates, approximately, the time at which the treated cells have become committed or clonogenic for the property being measured. Since the plating efficiency during the replating is $<100\%$, the number of colonies or foci on the secondary dishes may actually be a lower limit of the number of committed cells. We used the same initial cell density (50,000 cells per 6 cm plate) to study the induction by BPDE of both ouabain resistance and transformation.

Figure 1*a* shows representative plates from BPDE-treated and control cultures that were used to select for ouabain-resistant mutants, with or without the replating procedure. It is obvious that replating markedly increases the number of ouabain-resistant colonies. This effect was apparent within 2 days of BPDE treatment and reached a plateau within 5 days, at which time the cells on the primary plates were confluent (Fig. 2*a*). The replating procedure led to about an eightfold increase in the number of colonies growing in the presence of ouabain. This increase was less than the 16-fold increase one would expect if the cells had undergone approximately four doublings before reaching confluency. This difference may be

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