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Extrachromosomal DNA Transformation of *Caenorhabditis elegans*

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DNA was introduced into the germ line of the nematode *Caenorhabditis elegans* by microinjection. Approximately 10% of the injected worms gave rise to transformed progeny. Upon injection, supercoiled molecules formed a high-molecular-weight array predominantly composed of tandem repeats of the injected sequence. Injected linear molecules formed both tandem and inverted repeats as if they had ligated to each other. No worm DNA sequences were required in the injected plasmid for the formation of these high-molecular-weight arrays. Surprisingly, these high-molecular-weight arrays were extrachromosomal and heritable. On average 50% of the progeny of a transformed hermaphrodite still carried the exogenous sequences. In situ hybridization experiments demonstrated that approximately half of the transformed animals carried foreign DNA in all of their cells; the remainder were mosaic animals in which some cells contained the exogenous sequences while others carried no detectable foreign DNA. The presence of mosaic and nonmosaic nematodes in transformed populations may permit detailed analysis of the expression and function of *C. elegans* genes.

DNA transformation leads to the production of a heritable change in an organism through the introduction of exogenous DNA. Transformation of single cells is of proven utility in the genetic manipulation of bacteria (29), yeast (22), and cultured mammalian cells (for a review, see reference 41). Isolated and defined DNA sequences can be introduced, and their effects on the in vivo physiology of the cell can be assessed. For instance, DNA transformation has played a paramount role in the isolation and analysis of genes that alter the proliferative properties of cells (e.g., oncogenes; for a review, see reference 3) and genes that determine cell type (e.g., mating type in yeasts; for a review, see reference 34). Thus, methods for stably introducing DNA into single cells provide a new window into the molecular mechanisms of cellular behavior.

Similarly, techniques for DNA transformation of multicellular organisms facilitate the molecular analysis of developmental processes. DNA transformation of multicellular organisms has been achieved by microinjection of fertilized eggs of mice (6, 10, 18, 53), frogs (14), sea urchins (16), and *Drosophila melanogaster* (39). In *D. melanogaster* (17, 42, 45) and in several cases in mice (7, 19, 50) the reintroduced genes appear to be properly regulated during development. Thus, DNA transformation permits studies of the expression of isolated sequences in specific tissues at specific times. DNA transformation may also be used to identify and isolate other sequences that encode functions vital for determination and development.

The nematode *Caenorhabditis elegans* has been used widely for studies of developmental genetics. Its simple anatomy and life cycle have permitted the characterization of mutants defective in particular tissues, behaviors, and developmental pathways (5). The cell lineage of the entire worm is known (25, 48, 49), permitting detailed comparisons of mutant and wild-type developmental patterns. DNA transformation offers the promise of combining the molecular analysis of defined DNA sequences with the rich genetics and developmental physiology of the nematode. Here we describe the introduction of foreign DNA into the *C. elegans* genome by microinjection into the gonad. The exogenous DNA formed a high-molecular-weight concatamer which was extrachromosomal and heritable. These experiments, demonstrating DNA transformation of *C. elegans*, provide a foundation for studying transformation and expression of developmentally interesting genes.

MATERIALS AND METHODS

Strains and media. *C. elegans* var. Bristol N2 (5) or a strain lacking the major DNase of the worm [*nuc-1(el392)X*] (47) were used for the injections. DSB40 (alias *Escherichia coli* HB101) was used for bacterial transformation experiments. *C. elegans* was grown and manipulated as described by Brenner (5). To genetically manipulate the transformants, males were isolated from a population of transformed hermaphrodites that had been incubated at 30°C for 5 h. Individual males were mated with MT465 [*dpy-5(e61)I bli-2(e768)II; unc-32(e189)III*] and MT464 [*unc-5(e53)IV; dpy-11(e224)V; lon-2(e678)X*] provided by the *C. elegans* Genetic Stock Center. Bacteria were grown and manipulated as described by Davis et al. (11). Bacterial strains harboring plasmids used for injection were generously provided by P. Southern (pSV2neo) and R. Jefferson (pCEV70.3-neo). YRp17 has been described previously (46). YRp17-unc was constructed by inserting the 3.8-kilobase-pair (kpb) *Bgl*II fragment of the *unc-54* gene (24) into the *Bam*HI site of YRp17 by standard recombinant DNA techniques (30).

Microinjection of DNA. Plasmid DNA was prepared from bacterial strains by CsCl gradient centrifugation by standard methods (30). To ensure purity, two sequential CsCl gradients were used. Ethidium bromide was removed by isopropanol extraction, and the DNA was ethanol precipitated and suspended at 0.25 to 2 mg/ml in 5.0 mM KCl–0.1 mM $K_{1.5}PO_4$ (pH 7). Prior to injection, fluorescein isothiocyanate dextran (average molecular weight, 150,000; Sigma Chemical Company, St. Louis, Mo.) was added to 1 mg/ml.

Injection procedures described by Kimble et al. (26) were modified as follows. Young adult nematodes (1 to 12 h after the L4 adult molt) were anesthetized in 0.1% ethyl *m*-

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aminobenzoate methanesulfonic acid salt (Tricaine methanesulfonate) and 0.01% tetramisole (both purchased from Sigma) in M9 salts (5) for 10 to 30 min at room temperature. The worms were then transferred to an agar plate to remove excess moisture. A halocarbon oil (Voltalef 1S oil; Gallard/Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.; or Halocarbon oil, series 700; Halocarbon Products Corp., Hackensack, N.J.) was placed over the worms prior to transfer. A half-cover slip was positioned on a slide with a small quantity of distilled water. Three edges of the coverslip were sealed with petrolatum; the fourth edge was covered with the halocarbon oil (3S Voltalef oil equilibrated with M9 buffer or series 700 Halocarbon oil). Anesthetized nematodes (10 to 15) were transferred to the oil and then aligned with their vulvae in apposition to the edge of the cover slip. The nematodes remained viable and paralyzed at this water/oil interphase for several hours.

Self-filling glass capillaries (Kwik-fil; W-P Instruments, Inc., New Haven, Conn.) were pulled with a Brown-Flaming micropipette puller (Sutter Instrument Co., San Francisco, Calif.). The needles were baked at 80°C for at least 1 h. The tips of the needles were filled with the fluorescent dextran-DNA solution by capillary action. The remainder of the needle was filled with a generic vegetable oil (King Soopers, Boulder, Colo.) with a pulled out Pasteur pipette. The needle was attached via a Leitz microtool holder to an oil-filled injection system. A 1-ml gas-tight syringe with a Luer tip and a threaded plunger (Hamilton Co., Pittsburgh, Pa.) was used to drive the DNA injections. Just before injection, the tip of the micropipette was broken by lightly touching it to the edge of the cover slip with a micromanipulator. Tip sizes varied: any tip that permitted easy flow of the DNA solution and was less than a few microns in diameter was successfully used.

Microinjection was performed with a Zeiss IM-35 inverted microscope equipped with differential interference contrast optics at approximately ×500 magnification. A region of the adult worm was picked in which the distal arm of the gonad (identified by its characteristic density of nuclei) lay against the cuticle (See Fig. 1). A micromanipulator was used to push the needle into the distal arm of the gonad. Pressure was exerted with the injection syringe until material visibly flowed into the worm. The needle was rapidly removed from the worm to avoid physical damage. In successful injections, the fluorescent dextran was seen to be confined to the distal arm of the gonad by brief viewing with epifluorescent illumination. We have not been able to correlate the amount of material injected (estimated visually to be 1 to 10 pl) with incorporation of DNA into progeny of the injected worm. After injection, the nematodes were placed on agar media at 20°C. As the injected worms laid their eggs, they were periodically transferred to fresh media. Transformants usually appeared after the first 10 and before the first 50 progeny were produced.

Hybridization screening of transformants. To avoid agar contamination in DNA preparations, worms were grown on the standard nematode growth medium, containing (or overlayed with) 1% agarose. After two or three generations of growth, the worms were harvested by washing the plates. The populations were cleaned of much of the bacteria by low-speed centrifugation in conical tubes and washing in M9 salts. After a final wash in 0.2 M Tris hydrochloride (pH 8)–0.1 M EDTA, the worms were collected from the bottom of the tube in 0.1 ml, placed in a 1.5-ml microfuge tube, and frozen. As the tube thawed, 0.1 ml of a proteinase K solution (0.2 M Tris hydrochloride [pH 8], 0.1 M EDTA, 2% sodium dodecyl sulfate (SDS) and 200 μg of proteinase K per ml) was added. The samples were subsequently incubated at 65°C for 30 min, 85 μl of 5 M potassium acetate was added, and the tubes were incubated on ice for at least 20 min. The SDS-potassium acetate precipitates were removed by centrifugation for 15 min in a microfuge at 4°C. The supernatant was poured into a new tube, 0.5 ml ethanol was added, and the tubes were immediately spun for 5 min in a microfuge. The precipitate was washed twice with 70% ethanol and air dried. After resuspension in 20 to 50 μl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA–10 μg of RNase A per ml, 5 μl was spotted onto duplicate nitrocellulose filters. The filters were then treated as Southern transfers (43) and hybridized with ^{32}P-labeled pBR322 or a λ-*C. elegans* hybrid DNA (gift of M. Krause) carrying a single-copy worm DNA sequence. Single-copy sequences could be detected easily in DNA preparations from a few hundred worms.

To analyze patterns of hybridizing fragments, rapidly prepared worm DNA was extracted with phenol and ethanol precipitated. Enough DNA was extracted from a few thousand worms for several restriction enzyme digests. DNA was also prepared from *C. elegans* larvae in the L1 stage as described previously (12). Fragments were sized by agarose gel electrophoresis (32), transferred to nitrocellulose paper (43), and hybridized with ^{32}P-labeled plasmid DNA. All such probes were prepared by nick translation (38). All hybridizations were carried out at 32°C in 50% formamide–0.825 M NaCl–0.1 M $Na_{1.5}PO_4$–5 mM Na_2EDTA–100 μg of denatured salmon sperm DNA per ml–0.2% SDS or under conditions with roughly equivalent midpoint temperatures. Filters were washed at 32°C in 10 mM $NaPO_4$ (pH 7.0)–0.2% SDS. Kodak X-AR5 film was exposed to the filters with Dupont Lightning-Plus intensifying screens at −70°C for various amounts of time.

Stability tests and phenotypic analysis. The stability of foreign sequences was determined by cloning and hybridization analysis as follows. Single hermaphrodites were propagated for three generations, and then one was identified as a transformant by hybridization analysis. A large number of the third-generation progeny of the transformants were transferred individually to separate plates. The percentage of these progeny which still carried exogenous DNA was determined by allowing them to propagate, preparing DNA from the resulting populations, and detecting foreign sequences by hybridization. The percentage of positive progeny was extrapolated to the stability per generation (see Table 1). In a similar fashion, half of the third-generation progeny of clone 1.1 were allowed to grow for another three generations. The foreign DNA continued to be lost at a constant rate. The 25 progeny of clone 1.1.3 were carefully examined for morphological aberrations and differences in development, growth rate, or brood sizes. No differences were seen among the 5 individuals that contained the exogenous sequences and the 20 that did not. To preclude the possibility that the foreign DNA caused defects that would only be apparent in progeny of the transformed worms (examples include defects in germ line development or maternal-effect abnormalities), we also individually picked 10 F2 progeny of each of the 25 original isolates. Again, no phenotype could be detected in the worms that were later shown to contain the foreign DNA. Clone 1.1.3.3 showed a higher stability than its progenitors. However, it gave rise to transformants that showed lower stabilities. No consistently stable transformant segregated from such clones in well over 20 generations.

The stability of exogenous DNA sequences in worms transformed by the injection of linearized YRp17-unc DNA was determined in a similar manner. The exogenous DNA was lost at a rate comparable to that observed for *C. elegans* transformed with supercoiled pSV2neo. No Unc phenotype has been observed in approximately 200 worms that contain the injected DNA. Clones that appeared to be more stable than average (e.g., 2.1.16; see Table 1) gave rise to clones of average stability (data not shown). Clones of lower than average stability (e.g. 2.1.3) often remained of low stability but did give rise to clones with average stability (data not shown).

Reisolation of plasmids from *C. elegans* transformants. One microgram of DNA isolated from a transformed strain was digested to completion with *Eco*RI. *Eco*RI cut the injected plasmid pSV2neo once. The digested DNA was phenol extracted, ethanol precipitated, and then suspended to 10 μg/ml and ligated with T4 DNA ligase. The ligated sample was used to transform *E. coli* HB101 to ampicillin resistance (Amp^r) (29). Plasmid DNA was prepared from each of the Amp^r strains (30); restriction endonuclease digestion, agarose gel electrophoresis, and hybridization analysis permitted determination of the structure of each plasmid. All enzymes utilized in the above manipulations described above were purchased commercially and used according to the directions of the vendor.

Copy number determinations. The number of copies of the foreign DNA sequences in the transformed worm cells were measured by quantitative hybridization analysis. DNA preparations from transformed populations were spotted onto triplicate nitrocellulose filters. The filters were hybridized with ^{32}P-labeled pBR322 DNA, bacteriophage DNA containing a single-copy gene, and bacteriophage DNA containing a fourfold repetitive actin gene sequence (15) (generous gifts of M. Krause). After washing, the hybridization to individual spots was quantitated by liquid scintillation counting. To control for the different specific activities and hybridization efficiencies of the three probes, we also spotted known amounts of pBR322 hybrids containing the actin gene or the single-copy sequence on the filters. The ratio of pBR322/λ hybridization to these control spots was used to calculate the amount of pBR322 hybridization expected for each worm DNA preparation if the plasmid sequences were present in one copy per genome (see Table 2). The percentage of transformed worms in each population was used to derive the copy number per genome per transformant. It should be noted that the copy number is undoubtedly an underestimate. The actin sequence repeat was slightly underestimated, and many of the worms in a population did not carry DNA in all their cells.

Cytological analysis. Single adult hermaphrodites were picked from a transformed strain. The worms were first transferred to agar plates on which they laid 20 to 50 eggs. These same adults were then individually transferred to small drops of M9 salts on subbed slides (37), bisected, and gently squashed with a cover slip to display the gonads and the intestine. After the slide was frozen in liquid N_2, the cover slips were popped off, and the preparations were fixed in ethanol-acetic acid (3:1) at 4°C. The slides were rinsed in 95% ethanol and air dried. Staining was performed in phosphate-buffered saline with 1 μg of diamidinophenylindole (DAPI) per ml. The slides were rinsed in phosphate-buffered saline and mounted in Gelutol (Monsanto). Mature oocytes present in the preparations were examined for the presence of extrachromosomal DNA by epifluorescent illumination. In the meantime, the progeny of these adults were allowed to propagate, and DNA was prepared from the populations and screened for the presence of foreign DNA. Thirteen hermaphrodites were scored for both cytologically detectable extrachromosomal DNA and heritable foreign sequences. Oocytes from four of the adults contained DAPI-stained extrachromosomal material; these same four oocytes contained foreign DNA as determined by dot blots. One adult contained foreign DNA that was not cytologically detectable. Failure to observe extrachromosomal material in one transformant is not surprising given the instability of the high-molecular-weight array and the limited number of properly stained mature oocytes.

In situ hybridizations. The following procedure is a modification of the method described by Albertson (1). Bisected adult worms or eggs prepared by 1% NaOCl–1.0 N NaOH digestion of gravid adults were fixed as described above. To prepare the tissues for DNA-DNA hybridization, they were first digested with 10 μg of heat-treated RNase A per ml in 0.165 M NaCl–20 mM $Na_{1.5}PO_4$–1 mM EDTA (1× SPE) at 37°C for 1 h in a wet box. The slides were rinsed in 1× SPE and water and then denatured in 0.7 N NaOH for 1.5 min. After the slides were rinsed in an increasing series of ethanol concentrations, they were air dried. About 0.1 to 0.2 μg of ^{35}S-labeled DNA was prepared by nick translation (38) with 5 μM [^{35}S]dATP (New England Nuclear Corp., Boston, Mass.) and 1 mM dithiothreitol present in the standard reaction mixture (11). The reaction was terminated with 20 mM EDTA–0.2% SDS–2 mg of salmon sperm DNA per ml. After elution from a spin column (11) and ethanol precipitation, the probe was suspended in 0.1 ml of 1× SPE–50% formamide–5× Denhardt solution–10 mM dithiothreitol. The DNA was denatured by incubating at 70°C for 10 min. Salt concentrations were then increased to 5× SPE, and the probe was placed on ice. About 5 to 10 μl of this solution was placed directly on the dried tissue preparations. A small cover slip was carefully laid over the samples, and they were incubated in a wet box containing 5× SPE and 50% formamide at 37°C overnight. After hybridization, the slides were washed in five to six changes of 2× SPE, 1× SPE, and a series of increasing ethanol concentrations and air dried. The slides were coated with a 1:1 dilution of Kodak NTB-2 emulsion, exposed for 6 h to 3 days, developed, and stained with DAPI as described above. The in situ-hybridized tissues were examined under simultaneous dark-field and epifluorescent illumination.

RESULTS

Microinjection of DNA. Whole organism transformation requires the introduction of foreign DNA into the germ line. In *C. elegans*, the distal arm of the adult gonad is an ideal target for introduction of DNA by microinjection. Each *C. elegans* hermaphrodite contains two reflexed gonad arms (Fig. 1). The distal arm of each gonad arm is a syncytium: a common core of cytoplasm surrounded by germ line nuclei. Progressing around the bend in each gonad arm, cell membranes enclose the nuclei to form immature oocytes. Mature oocytes, arrested in prophase of the first meiotic division, pass through the spermatheca where they are fertilized. The fertilized eggs develop for a time before they are laid through the hermaphrodite's vulva. Thus, in the cytoplasm of the distal arm, many nuclei may be accessible to injected compounds, and any nuclei that take up foreign DNA may then be incorporated into fertilized eggs. Furthermore, microinjection of nucleic acids into the gonad has been

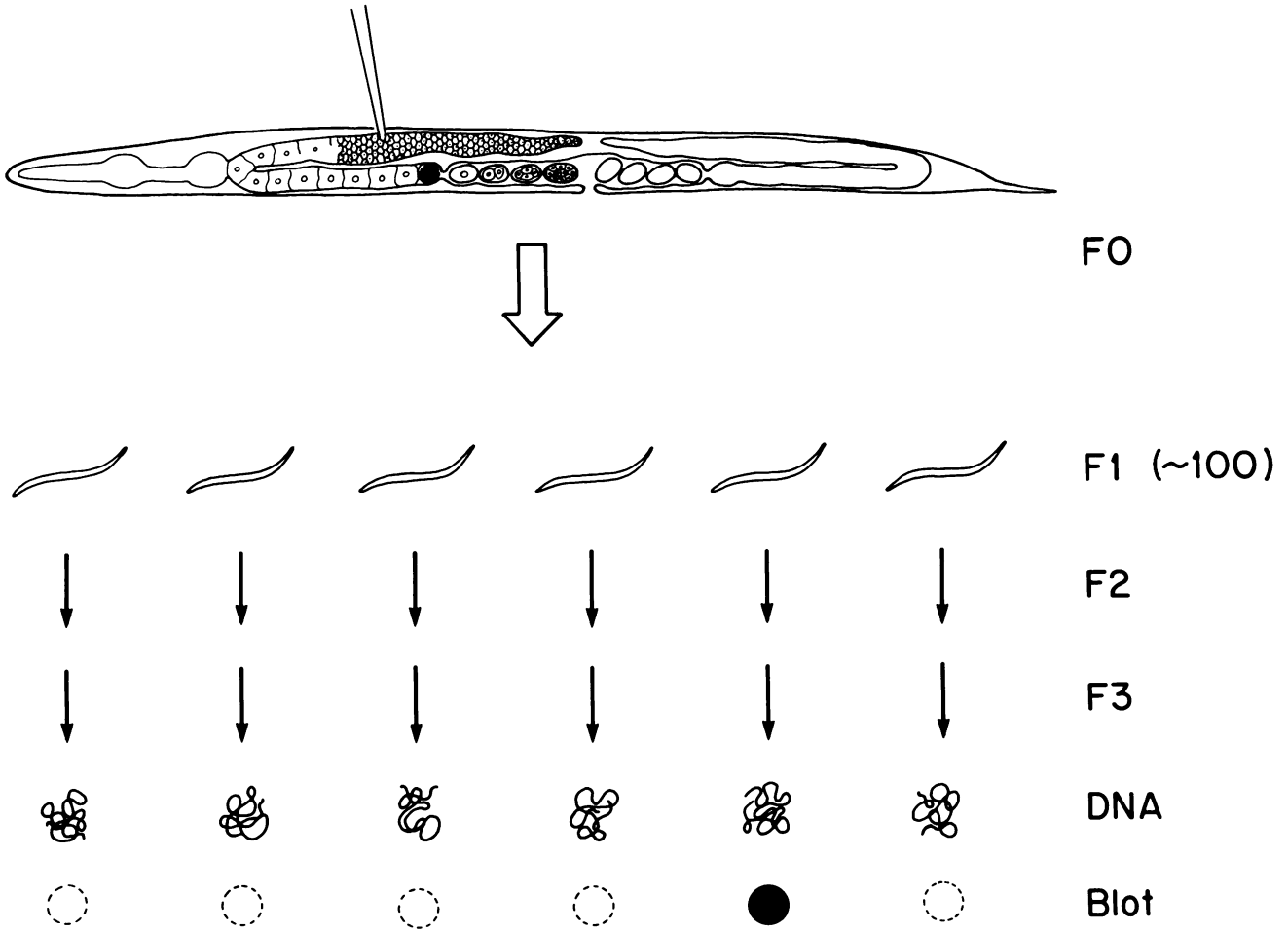


FIG. 1. Microinjection of DNA. Anesthetized nematodes were lined up against a cover slip under an oxygen-permeable halocarbon oil. The tip of a glass capillary needle was filled with 0.2 to 2.0 mg of DNA per ml and 1 mg of fluorescein-conjugated dextran per ml. After the cuticle and gonad were pierced by micromanipulation, 1 to 20 pl of the solution was injected. Successful injection was monitored by fluorescence of the coinjected dextran. Progeny of the injected worms were transferred to individual plates and allowed to propagate for two to three generations. These populations were harvested, and their DNA was prepared and spotted onto nitrocellulose filters. The presence of injected sequences was ascertained by hybridization analysis (see the text). One or a few populations arising from the F1 progeny were found to maintain the injected DNA.

shown to be feasible. Injection of tRNA from a *sup-7* strain into the syncytium transiently suppresses an amber mutation (26).

The techniques for microinjection of DNA (Fig. 1) involved only minor modification of the procedures of Kimble et al. (26). Worms were anesthetized, lined up along the edge of a cover slip, and covered with an oxygen-permeable halocarbon oil. Hermaphrodites remained viable and immobile for several hours under these conditions. A freshly broken glass needle was used to inject several picoliters of a solution of DNA (0.2 to 1 mg/ml) and fluorescein-conjugated dextran. Successful injection was monitored by epifluorescent illumination. Up to a dozen worms were injected with DNA in the course of a few hours. Greater than 80% of the worms survived and produced progeny from the injected gonad.

DNA transformation: heritability of microinjected DNA. The injected worms were transferred to agar plates and allowed to lay eggs. The first 100 progeny of the injected worms were individually transferred to fresh media and propagated for two generations. The populations were harvested (usually in batches of 5 to 10), and DNA was prepared and spotted onto nitrocellulose filters. The DNA we injected contained portions of the bacterial plasmid pBR322 (4). Thus, the foreign DNA was detected in the progeny by hybridization with ^{32}P-labeled plasmid sequences (Fig. 1). In roughly 10% of the injections, exogenous plasmid sequences were maintained in the progeny. Only a few (usually one or two) of the F1 progeny from a single injection gave rise to populations that contained the foreign DNA.

If F1 progeny from the injected worm were truly transformed, the exogenous plasmid sequences should have been maintained and passed on to future generations. Individual members of a population carrying the foreign DNA were placed on single plates (Fig. 1). These individuals were removed by three generations from the original F1 parent. After the nematodes were allowed to propagate, the populations were harvested; and DNA was prepared, spotted onto nitrocellulose filters, and hybridized with ^{32}P-labeled pBR322. Again, worms were found that retained the pBR322 sequences. We maintained such stocks by periodically identifying individuals that carried the exogenous DNA. The foreign sequences were heritable over dozens of generations.

Transformation of *C. elegans* does not require any particular worm sequences in the injected DNA. Sequences from several plasmids that totally lacked worm DNA were successfully injected and were inherited by the progeny. To date, we have transformed *C. elegans* with plasmids that share only 2,297 base pairs (bp) of pBR322 sequences (52). Foreign DNA has been detected in the progeny after injection of either supercoiled plasmids or linear DNA into either wild-type or nuclease-deficient [*nuc-1(e1392)X*] (47) worms. The data are insufficient to determine relative frequencies.

Stability of the foreign DNA. The results from a series of experiments, in which the heritability of sequences of the injected supercoiled plasmid pSV2neo (44) and a linearized plasmid YRp17-unc were analyzed, are presented in Table 1. In the case of the pSV2neo transformant, the hybridization screen was repeated three times over 12 generations. The exogenous DNA was found in 30 to 90% of the F1 progeny of a given transformant. The specific level of stability was not heritable: worms showing a higher stability of the foreign DNA gave rise to transformants with lower stabilities and vice versa. Transformants obtained after injection of linear DNA showed similar properties (Table 1).

Although the exogenous DNA was heritable, it segregated abnormally. On average, each transformant passed the foreign DNA on to approximately half of its progeny. If the exogenous DNA had integrated into one of the *C. elegans* chromosomes, then after one generation, three-fourths of the progeny would contain the foreign sequences. After three generations, 9/16 or 54% would retain the DNA. After six generations approximately 50% of the animals would contain the injected DNA and the transformants would be almost entirely homozygous. Our results show that the stability of the foreign DNA is lower than expected for Mendelian segregation of integrated DNA. The exogenous sequences continued to be lost over many generations, and transformed strains that are completely stable have not been obtained.

TABLE 1. Stability of foreign DNA[a]

DNA injected	Isolated worm	No. of generations	No. positive/ total	Stability/ generation (%)
pSV2neo (supercoiled)	1	3	1/24	35
	1.1	3	7/48	53
	1.1	6	1/48	52
	1.1.1	3	6/50	49
	1.1.2	3	9/48	57
	1.1.3	3	5/25	59
	1.1.3.2	3	4/25	54
	1.1.2.3	3	18/25	90
YRp17-unc (linear)	2	3	6/50	49
	2	6	1/50	50
	2.1.3	1	8/25	32
	2.1.5	1	15/25	60
	2.1.16	1	25/25	100
	2.1.16	2	9/12	87

[a] The stability of foreign DNA sequences in a *C. elegans* strain transformed by injection of supercoiled pSV2neo DNA was assessed by measuring the number of transformants left in populations grown from a single worm. The numbering system reflects the ancestry of a given worm. For instance clone 1.1 is a single positive clone picked from a population generated by clone 1; clones 1.1.1, 1.1.2, and 1.1.3 are siblings picked from the population generated by clone 1.1. The transformants obtained after the injection of linear DNA are numbered similarly. For a detailed description of these experiments, see the text.

The abnormal segregation of exogenous sequences during propagation of a transformed population could have been due to a deleterious phenotype imparted by the foreign DNA. In the stability experiments described above, both first- and second-generation progeny of transformed worms were carefully screened for any detectable reduction in brood size, growth rate, or fertility. Those progeny with the exogenous sequences and those without showed no observable differences in their growth characteristics (Table 1).

If the foreign DNA is transmissible by males, the exogenous sequences could be genetically manipulated with ease. We obtained 19 males from a strain transformed by the injection of supercoiled plasmids. The males were individually mated to hermaphrodites that were homozygous for several morphological markers. Seven heterozygous progeny of each cross were isolated and allowed to propagate. The populations were harvested and tested for the presence of foreign DNA as described above. Of the 19 males, 2 carried the foreign DNA; 4 of the 14 progeny from these males (29%) were transformed. In the subsequent generation, we assessed the segregation of the foreign DNA relative to a marker on each of the six *C. elegans* linkage groups. The foreign DNA in this particular transformed strain segregated randomly with respect to *dpy-5 I*, *bli-2 II*, *unc-32 III*, *unc-5 IV*, *dyp-11 V*, and *lon-2 X* (data not shown); the exogenous DNA was not tightly linked to the center of any of the *C. elegans* linkage groups. Thus, foreign DNA can be propagated through the male germ line, and strains can be easily constructed that contain the foreign DNA.

Structure of the transforming DNA. DNA isolated from transformed worm populations was subjected to electrophoresis to determine the molecular structure of the exogenously added DNA. Without prior endonuclease digestion, the foreign DNA was found to be of high molecular weight (Fig. 2A, lane 1) and comigrated with *C. elegans* chromosomal DNA. When the DNA from the transformed strains was digested with a restriction enzyme that cleaved once in the original injected plasmids, the exogenous sequences migrated heterogeneously (Fig. 2A, lane 2). However, a predominant band in the worm DNA comigrated with a linear version of the original injected plasmid (Fig. 2B, compare lanes 1 through 4 with lane 5). Partial digestion of DNA from the transformants produced strongly hybridizing fragments at mobilities corresponding to multiples of the injected sequence (monomer, dimer, and trimer molecular weights; data not shown). Thus, multiple copies of the injected plasmid are colinear, suggesting that most of the foreign DNA is present in a large head-to-tail tandem array(s) of plasmid sequences.

When transformed into yeasts, linear molecules homologously recombine with yeast chromosomal sequences (36). To test whether linear molecules homologously integrate into the *C. elegans* genome, we injected linear DNA into the gonad. A plasmid carrying a fragment of the *unc-54* gene (YRp17-unc) was cut at a unique site within the nematode gene. After injection, a transformed strain was obtained as described above. If the linear molecule had recombined homologously, it would have disrupted the *unc-54* locus resulting in a distinctive uncoordinated phenotype; no such phenotype was observed among the transformants or their progeny. The structure of the foreign DNA carried in the transformants was different from the structures observed after injection of supercoiled molecules. When subjected to electrophoresis without digestion, the hybridizing sequences were of high molecular weight. However, when the foreign DNA was digested with a restriction

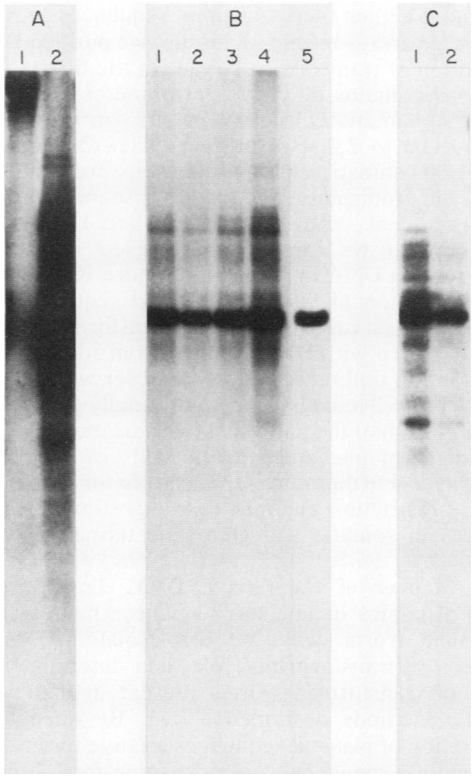


FIG. 2. Structure of foreign DNA after injection of supercoiled molecules. DNA isolated from populations of transformed nematodes was separated by size by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pBR322 DNA. These strains arose from a single worm injected with the supercoiled plasmid pCEV70.3-neo (R. Jefferson and D. Hirsh, unpublished data). Autoradiographs of the washed and hybridized filters are shown here. (A) DNA was subjected to electrophoresis without prior endonuclease digestion (lane 1). When uncut, the hybridizing DNA comigrated with the nematode chromosomal DNA. The sample was digested with the restriction endonuclease *Hin*dIII which cut the injected plasmid once; when cut, a heterogeneous spectrum of DNA fragments hybridized (lane 2). (B) DNA samples prepared from four progeny of a single transformed worm were digested with *Hin*dIII (lanes 1 through 4). The patterns of hybridization were identical, even on longer exposure of the autoradiograph. The major fragment that hybridized comigrated with the linear DNA which was produced by digesting the supercoiled plasmid with *Hin*dIII (lane 5). (C) Hybridization to DNA isolated from L1 larvae (lane 1) compared with the hybridization to DNA from adults (lane 2). The patterns are identical.

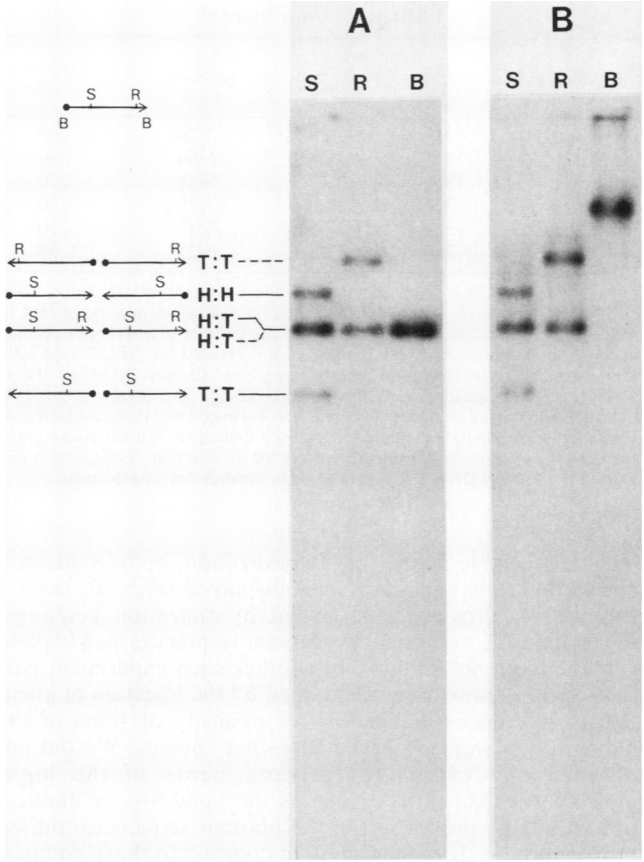


FIG. 3. Structure of foreign DNA after injection of linear molecules. The plasmid YRp17-unc was linearized by digestion with *Bam*HI and was injected into *nuc-1(el392)X* hermaphrodites. The structure of the injected DNA is diagrammed to the left of the figure. The unique *Bam*HI (B), *Sal*I (S), and *Eco*RI (R) cleavage sites of the molecule define its orientation. As a control for random ligation, the linear DNA was ligated in vitro and was digested with the three restriction endonucleases. The digested DNA was sized by agarose gel electrophoresis, transferred to nitrocellulose, hybridized with ^{32}P-labeled YRp17-unc DNA, and visualized by autoradiography. (A) Digestion with either *Sal*I (lane S) or *Eco*RI (lane R) produced bands corresponding to the tail-to-tail, head-to-tail, and head-to-head ligated fragments as shown to the left of the figure (T, tail; H, head). (The head-to-head *Eco*RI fragment was faint and could be observed on longer exposure; data not shown). As expected, digestion with *Bam*HI produced a single band corresponding to the original linear DNA. DNA isolated from a nematode transformed by injection of the foreign DNA was digested and analyzed on the same agarose gel as the controls. (Panel B) Digestion with *Sal*I (lane S) produced three bands; the mobility of the bands was identical to that of the in vitro-ligated products. The mobility of the two bands observed by digestion with *Eco*RI (lane R) were also identical to those of the controls. Digestion of the foreign DNA with *Bam*HI produced a band of higher mobility than expected. Longer exposure of these digests of foreign DNA uncovered other bands; their identity is being investigated.

enzyme that cut once in the injected molecule, three predominant bands were observed (Fig. 3A and B). The bands corresponded to those expected for head-to-tail, head-to-head, and tail-to-tail ligations of the injected linear molecule. The fragments produced by digestion of the exogenous DNA were identical in size to the fragments produced by digestion of the in vitro-ligated controls, within 100 bp. However, the single-stranded cohesive ends of the injected molecules were altered in *C. elegans*: the restriction site at the cohesive end was not reconstructed after ligation (Fig. 3B, lane B). Thus, injection of linear DNA produces transformed nematodes carrying high-molecular-weight arrays constructed by random ligation. However, except for these differences in sequence organization, the high-molecular-weight arrays behaved identically in the experiments we describe, whether they were produced by injection of linear DNA or the injection of supercoiled molecules (Table 1).

Stability of the arrays during propagation. To determine if the arrays of exogenous sequences are altered during their propagation, we compared the pattern of restriction enzyme-generated fragments of foreign DNA in several progeny that arose from a single transformant. The progeny showed

TABLE 2. Copy number[a]

Population	Copy no.		No. positive/ total	Copy no./ transformant
	Actin	pBR322		
pSV2neo-1	2.8	8.4	1/24	200
−1.1.1	4.5	36.3	6/50	300
−1.1.2	3.1	32.7	9/48	170
−1.1.3	2.4	32.8	5/25	160
−1.1.3.2	3.1	37.5	4/25	230
−1.1.3.3	2.1	57.7	18/25	80

[a] We purified DNA from the populations characterized in Table 1 for hybridization analysis. By quantitating the hybridization relative to that of single-copy sequences, we could calculate the number of copies per genome in the entire population. These numbers are presented for pBR322 and actin sequences. There are four actin genes in *C. elegans*; the average obtained here was only 3.0, suggesting a routine underestimate. The levels for pBR322 hybridization ranged from 8 to 58 copies per worm genome in the populations. However, each population contained a low percentage of transformed worms. We used the stability test results to correct for the fraction of worms that still carried the foreign DNA to obtain the copy number per transformant.

indistinguishable patterns of hybridization, both in quantity and in the array of minor bands displayed (Fig. 2B, lanes 1 through 4). Progeny that lacked hybridization contained fewer than 0.1 copies of exogenous sequences per haploid genome (data not shown). In another such experiment performed on a transformant obtained by the injection of linear DNA, differences in the relative intensity of some of the minor bands were observed (data not shown). We did not observe any extensive rearrangements of the high-molecular-weight arrays (such as the rapid loss or duplication of a large proportion of the plasmid sequences) during propagation in the nematodes. In contrast to these comparisons of foreign DNA in progeny of the same transformant, DNA isolated from different transformants shared few, if any, of the minor bands.

Somatic tissue contains high-molecular weight arrays. We know that foreign DNA sequences are present in the germ line of transformed worms because of their heritability. To determine whether the exogenous sequences are also present as large arrays in somatic tissue, we purified DNA from a population of transformed larvae. In the first larval stage, *C. elegans* contains only two germ line cells and approximately 500 somatic cells (48). Adults, on the other hand, contain 1,000 to 2,000 germ line nuclei (23) and approximately 1,000 somatic cells (48). If there were major differences in the propagation of exogenous sequences in somatic versus germ line cells, differences should have been observed between the foreign DNA present in larval worms and the foreign DNA in the adults. Figure 2C (lane 1) shows the hybridization of bacterial plasmid sequences to DNA isolated from larval worms of a transformed population. When compared with DNA isolated from adults of the same population, no differences could be observed in the relative intensity (lane 2 contained proportionally less DNA than lane 1 [Fig. 2C]) or the pattern of hybridization. We conclude that both germ line and somatic cells of *C. elegans* are transformed. Furthermore, the pattern of fragments produced by restriction endonuclease digestion of the foreign sequences in somatic and germ line tissue appears to be identical.

Copy number of the foreign DNA. To determine the number of copies of injected sequences maintained in the transformed worm cells, we first isolated DNA from a population of transformants. We then determined the percentage of transformed worms present in that population using the methods described above. By quantitating the hybridization of plasmid sequences relative to that of single-copy genomic sequences and correcting for the number of transformed worms in the population, we could estimate the plasmid copy number per genome in an average transformed worm. Purified worm DNA was spotted onto triplicate nitrocellulose filters and hybridized with excess ^{32}P-labeled pBR322 DNA, a single-copy worm sequence, and a fourfold repeated worm sequence (15). Hybridization was quantitated by scintillation counting and standardized for the hybridization efficiencies of the different probes. The transformants showed high copy numbers of 80 to 300 foreign

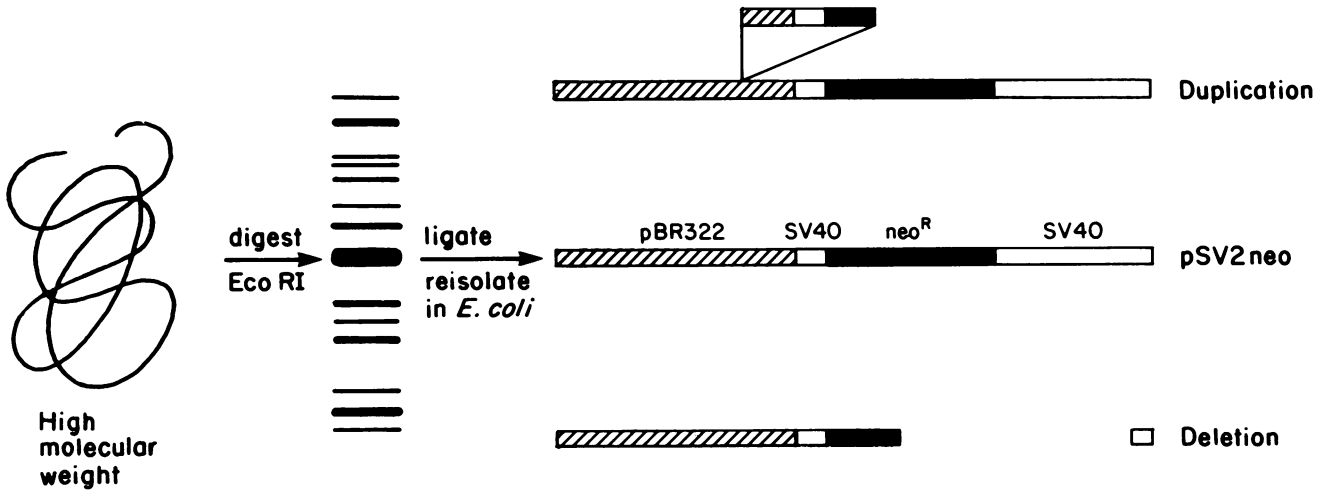


FIG. 4. Reisolation of transforming sequences. To ascertain the cause of the fragment heterogeneity in the transformed worms (Fig. 2) we reisolated the plasmid sequences. DNA was isolated from a transformant produced by injection of supercoiled pSV2neo. The DNA was digested, circularized by ligation, and used to transform *E. coli* to ampicillin resistance. Fourteen Amp^r colonies were obtained. The amp^r strains harbored three classes of plasmids; their structure is diagrammed here. The heterogeneity of fragment sizes was accounted for by duplications and deletions of injected sequences (see the text).

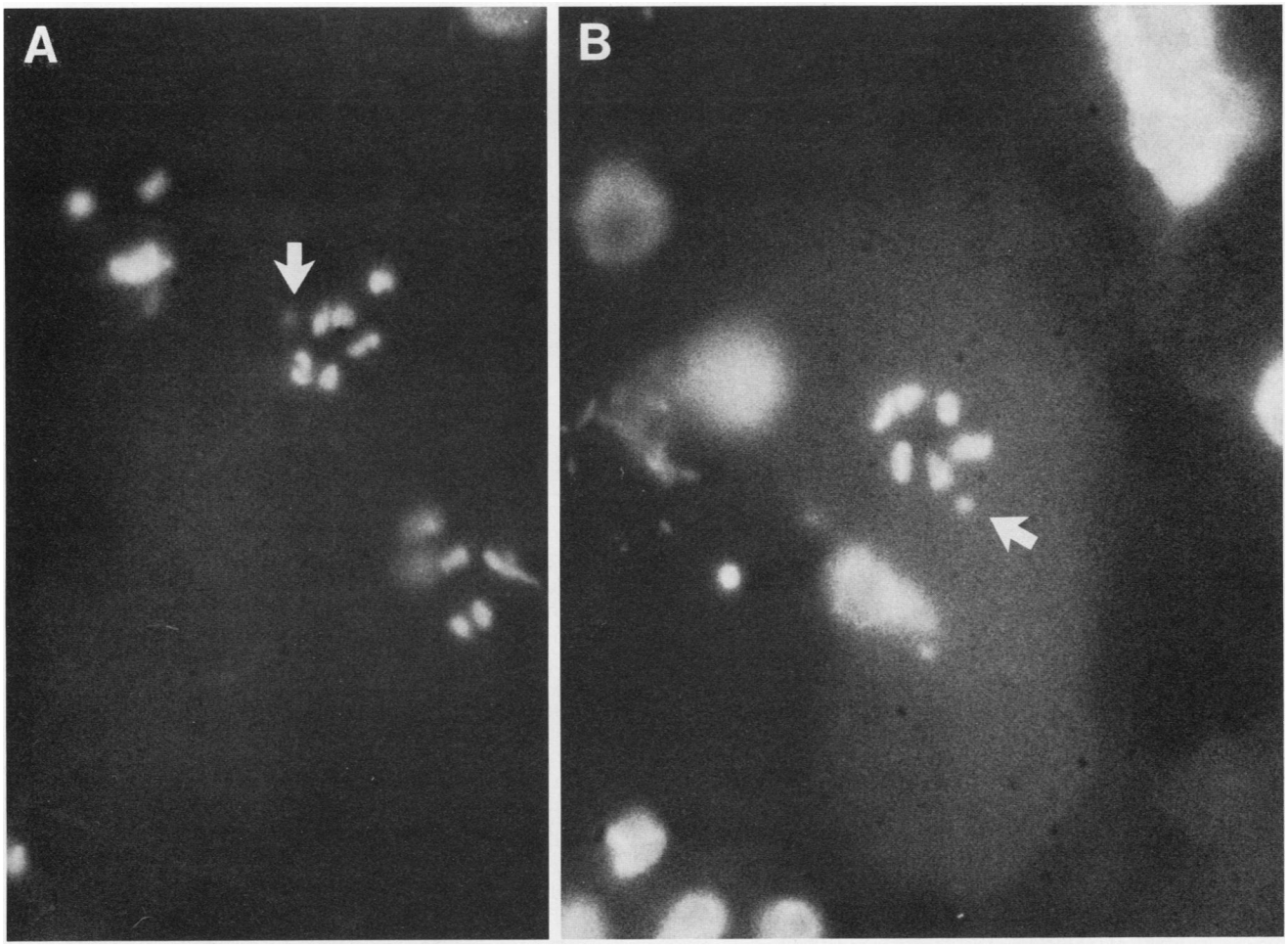


FIG. 5. Extrachromosomal arrays. (A and B) Oocytes of transformed adult hermaphrodites stained with DAPI (see the text). The oocyte chromosomes are seen as six brightly staining pairs of dots. The arrows indicate extrachromosomal material.

sequences per haploid genome (Table 2). Transformants produced by injection of linear DNA also carried many copies of the exogenous DNA; two different transformants showed copy numbers of approximately 100 to 200 per haploid genome (data not shown).

Reisolation of the foreign DNA. To analyze further the structure of the exogenously added DNA, we isolated the plasmid sequences from transformed worms. DNA was purified from populations transformed by microinjection of the supercoiled plasmid pSV2neo. The DNA was digested with *Eco*RI, an enzyme that cleaved the injected plasmid only once. The digested DNA was then diluted and circularized by ligation. The mixture of circularized worm and plasmid sequences was used to transform *E. coli* to ampicillin resistance (pSV2neo carries the Amp^r gene and colE1 origin of replication of pBR322 [44]). Fourteen Amp^r transformants were obtained; the plasmids they harbored were analyzed by restriction enzyme digestion, agarose gel electrophoresis, and hybridization analysis (Fig. 4). Two of the plasmids that were reisolated in *E. coli* carried direct duplications of parts of the vector sequences. One duplicated 1.2 kbp of pBR322 sequences, while the other duplicated 1.3 kpb of pBR322, simian virus 40, and sequences for neomycin resistance (Neo^r). A third plasmid we reisolated carried a 2.2-kpb deletion of Neo^r and simian virus 40 sequences (Fig. 4). In other experiments, duplications and deletions involving other regions of pBR322 were observed (data not shown). The duplications and deletions appeared to be created at random with no regard for sequence homology. Of the 14 plasmids that we reisolated, 6 showed patterns of restriction enzyme sites that were identical to those of the injected plasmid, indicating that the foreign sequences are maintained intact. The remaining 5 of the 14 plasmids contained additional worm sequences. The *C. elegans* sequences were present as small *Eco*RI fragments inserted directly into the *Eco*RI site of the vector. Small genomic DNA fragments could have been inserted into the *Eco*RI site of the transforming DNA during the ligation and circularization procedure. Thus, these fragments probably represent artifacts constructed during reisolation rather than junctions between genomic and foreign DNA.

In summary, a total of 11 of the 14 reisolated plasmids probably represented intact copies of the injected DNA, 2 of the 14 were direct duplications, and 1 was a deletion. Thus, the high-molecular-weight array created on injection of supercoiled molecules consists primarily of head-to-tail repeats of the injected plasmid interspersed with random duplications and deletions. The presence of these rearrangements explains the spectra of minor bands seen on hybridization to the foreign DNA (Fig. 2 and 3).

Extrachromosomal foreign DNA. As mentioned above, the high-molecular-weight arrays of foreign sequences segregated in a non-Mendelian fashion. The aberrant segregation was not due to a deleterious phenotype imparted by the foreign DNA. Furthermore, we could not detect linkage of the foreign DNA to six markers on the six linkage groups of the worm. Together, these data suggest that the foreign DNA may be maintained in the nematode as an extrachromosomal array. Extrachromosomal elements such as free duplications are detectable in *C. elegans* oocytes. Prior to fertilization, mature oocytes are arrested in prophase of the first meiotic division. Their chromosomes can be visualized at this stage as six pairs of highly condensed material by fixing and staining with the fluorogenic DNA-binding agent DAPI. Free duplications of worm chromosomes appear as diffusely staining dots independent of the bright chromosomal material (21).

To determine whether the foreign DNA in the transformants could be detected as extrachromosomal material, we picked individual hermaphrodites of a transformed strain; let them lay eggs on agar plates; and then squashed, fixed, and stained each adult with DAPI. Several of the adults contained extrachromosomal material in their oocytes (Fig. 5). To ensure that the diffuse material was not an artifact of the preparation, we grew the progeny of each adult, prepared DNA from the populations, and tested for the presence of the transforming sequences by hybridization. All the adults that contained cytologically detectable extrachromosomal material also contained the foreign DNA, as judged by hybridization analysis of their progeny.

***In situ* hybridization and mosaic transformation.** To explore the segregation of this extrachromosomal array during development of *C. elegans*, we hybridized labeled plasmid sequences to nematode tissues in situ. Embryos and bisected adults were fixed and prepared for hybridization by methods developed by Albertson (1). Preparation of ^{35}S-labeled DNA and hybridizations were performed as described above. The slides were stained with DAPI after autoradiography and were examined by epifluorescence to visualize individual DAPI-stained nuclei while the autoradiographic grains were visualized with dark-field illumination (Fig. 6). We only considered nuclei that were well separated from one another and from the cuticle or egg shell. As a control for probe sensitivity and accessibility we hybridized tissues with ^{35}S-labeled rDNA. The rDNA probe reproducibly hybridized to all distinct nuclei in the adults (Fig. 6H) and in squashed embryos (data not shown). ^{35}S-labeled pBR322 did not hybridize to uninjected worms (data not shown). However, the pBR322 sequences hybridized dramatically to the transformed *C. elegans* tissues (Fig. 6). To determine the specificity of this hybridization, we allowed individual adult hermaphrodites to lay eggs; they were then fixed, hybridized, and stained. We observed a perfect correlation between in situ hybridization to the adult gonad and the presence of foreign sequences in the progeny as assessed by standard dot blot methods.

Half of the embryos and many of the adults displayed hybridization to all nuclei (Fig. 6A). The remainder of the embryos were mosaic (Fig. 6B, C, and D). A wide spectrum of mosaicism was seen, from embryos in which only 2 of approximately 30 nuclei hybridized (Fig. 6B) to embryos in which all nuclei hybridized except two (Fig. 6C). As anticipated by the stability results, hybridization to the adult gonad was often mosaic. Figure 6E shows a patchwork pattern of hybridization to the distal arm of an adult gonad, suggesting the loss of the foreign DNA during cell proliferation of the germ line. Mosaic hybridization was also observed in intestinal nuclei of the adults (Fig. 6F). An adult with extensive hybridization to somatic intestinal nuclei but no hybridization to the gonad is shown in Fig. 6F.

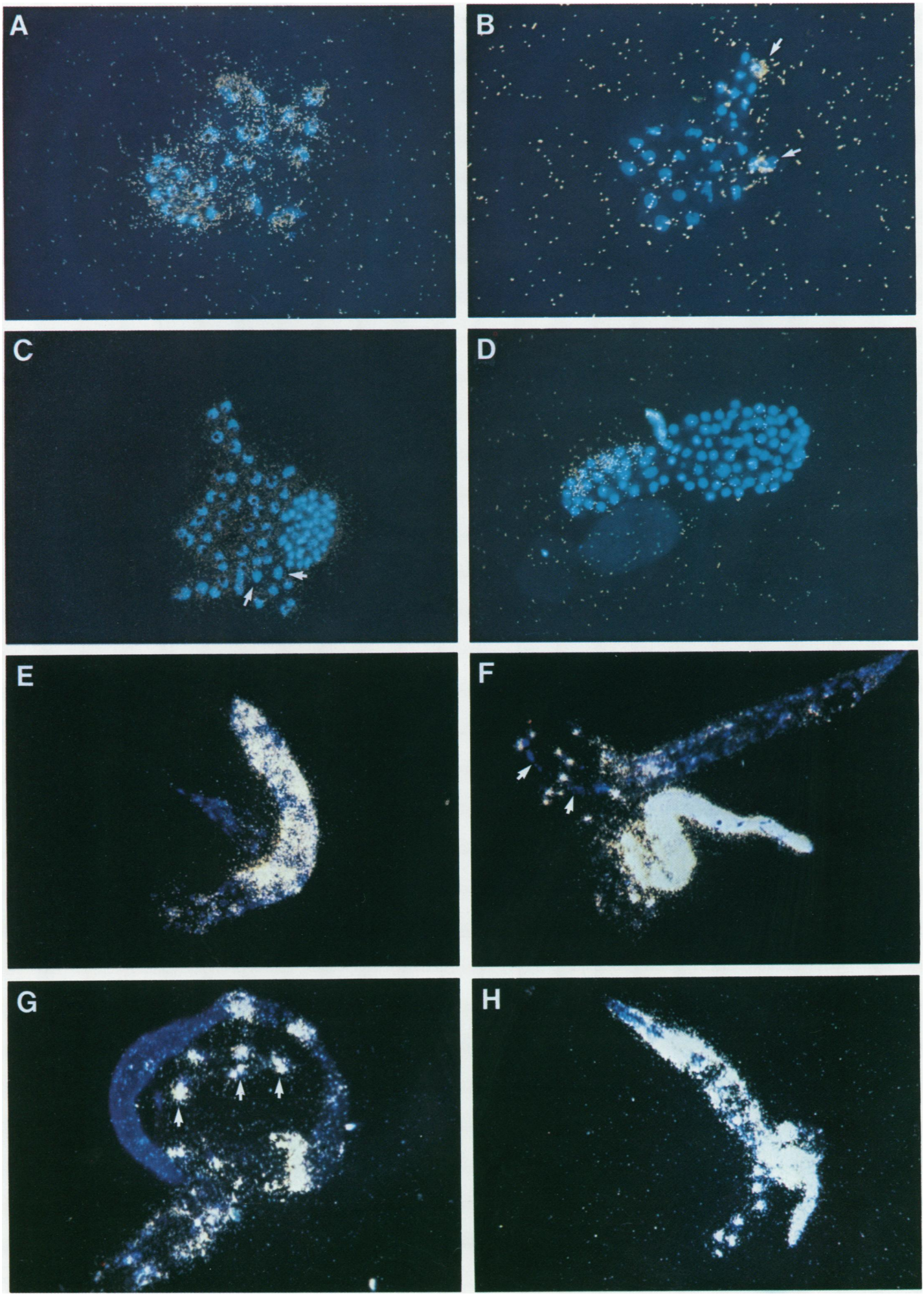
These in situ hybridization experiments demonstrate that a population grown from an isolated transformant contains nonmosaic animals with foreign DNA sequences in all cells, mosaic transformants with exogenous sequences in some somatic or germ line cells, and nematodes with no foreign sequences. Presumably, loss of the foreign DNA may occur at random times during development, giving rise to the various patterns of mosaicism we observed.

DISCUSSION

We transformed the genome of *C. elegans* by microinjecting DNA into the cytoplasm of the syncytial gonad. Injection of DNA into the cytoplasm of mammalian cells leads to rapid degradation (8). DNA injected into the meiotic cytoplasm of the syncytial gonad of the nematode was not extensively degraded. In approximately 10% of the injections, intact foreign DNA sequences were detected in the progeny.

The exogenous DNA was propagated in the form of high-molecular-weight arrays. Such arrays could be kinetically favored on introduction of exogenous DNA, or the concatamers could be a prerequisite structure for subsequent replication or segregation. Surprisingly, the arrays were extrachromosomal; no evidence for integration into the genome has been obtained to date. Although we know that the copy number of foreign sequences is high in the transformed nematodes (80 to 300 per haploid genome), we know little about the molecular architecture of the extrachromosomal arrays. We cannot rule out the possibility that worm sequences were incorporated by the high-molecular-weight array, nor do we know the topology (circular or linear) or the accurate size of the concatamers. From its electrophoretic mobility, we estimate that there are greater than 10 copies of the injected DNA in a given array.

FIG. 6. Mosaic transformants. In situ hybridizations were performed using simple modifications (see the text) of methods developed by Albertson (1). The figure shows embryonic and adult tissue as viewed with dark-field and epifluorescent illumination. The exposed autoradiographic grains appear as bright gold dots; the nuclei fluoresce light blue. (A) An embryo with 24 nuclei: all hybridized with the ^{35}S-labeled pBR322. (B through D) Mosaic embryos. In panel B, two of approximately 30 nuclei hybridized; in panel C, all but two of the squashed nuclei hybridized; in panel D, hybridization was clustered over 4 to 8 of approximately 100 nuclei. (E through H) In situ hybridization to adult tissues. In panel E, a gonad from a transformed worm shows a patchy pattern of hybridization which is indicative of loss of the transformed sequences in some of the germ line nuclei. In panel F, the gonad of a transformed nematode (lower portion of the frame) shows strong hybridization; several intestinal nuclei that failed to hybridize are indicated by the arrows. Panel G shows a transformant in which the gonad (staining a faint blue in the left portion of the picture) fails to hybridize, whereas intestinal nuclei (in the center of the frame) and other somatic cells still carry the foreign DNA. Panel H shows a wild-type worm hybridized with ^{35}S-labeled rDNA sequences; all somatic and germ line nuclei reproducibly hybridized.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In many DNA transformation systems, the introduction of linear DNA has important consequences. In yeast cells, the ends of linear molecules are highly recombinogenic (36); in mammalian systems, linear DNA increases the frequency of transformation (33); in the transformation of the sea urchin, linear DNA is absolutely required (16). In *C. elegans*, the only observed effect of linear DNA on injection was to alter the mechanism of formation of the high-molecular-weight array. Injected supercoiled DNA formed predominantly tandem (head-to-tail) arrays. The linear DNA showed a facility for end-to-end ligation resulting in an array that was a mixture of head-to-head, tail-to-tail, and head-to-tail repeats. Such ligation of linear DNA in eucaryotes has been a longstanding observation (31). In addition, the ligation of linear molecules accounted for the rearrangements of foreign sequences present in all of the arrays. If the DNA is sheared or otherwise cleaved during injection, the ligation of linear molecules containing random endpoints would create random duplications and deletions. These duplications and deletions of injected sequences were produced within the first three or four generations after injection. Thereafter the arrays did not undergo extensive rearrangement; all the progeny derived from a single transformed worm showed similar patterns of hybridization. The metabolism and uptake of injected DNA in *C. elegans* gonads warrants further investigation.

The extrachromosomal high-molecular-weight arrays are surprisingly stable to propagation in *C. elegans*. The foreign DNA appears to segregate to at least half of the progeny in a single generation. An adult hermaphrodite contains approximately 1,000 germ line nuclei (23), generated from two embryonic precursors (35) in approximately 14 mitotic cell divisions (5 to generate the two embryonic precursors and another 9 if one assumes simple proliferative division during growth of the gonad). Therefore, a stability of 50% per worm generation corresponds to a stability of 95% per cell division $[(0.95)^{14} = 0.50]$ or a rate of loss of 5% per cell division. In mammalian cells, unintegrated arrays are lost at a rate of 3% per cell division (8, 41). Extrachromosomal concatamers have also been observed in *Schizosaccharomyces pombe*; they are lost at a rate of 5 to 13% per division (40). Clarke and Carbon (9) have deleted the centromere of chromosome III of *Saccharomyces cerevisiae*; their data indicate that the acentric chromosome is lost at a rate of 10 to 15% per cell division.

In *C. elegans*, chromosomal fragments known as free duplications show stabilities of approximately 50% per worm generation (21). The formation of high-molecular-weight arrays with similar stabilities does not require the injection of any specific worm sequences. Indeed, the plasmids that successfully transformed *C. elegans* shared only 2,297 bp of pBR322 sequences. Any sequences that were required in the injected DNA for successful transformation and propagation might be contained within this 2,297 bp. *C. elegans* chromosomes do not have discrete kinetochores or centromeres (2, 21). Perhaps there is no strict sequence requirement for chromosomal segregation in *C. elegans*. Alternatively, the extrachromosomal arrays might contain *C. elegans* sequences that were not present in the injected DNA. We are currently exploring the factors that may contribute to the stability of the extrachromosomal concatamers.

Preliminary results demonstrate that the foreign sequences in these arrays can be expressed (R. Jefferson, M. Klass, and D. Hirsh, unpublished data). If such expression is under proper spatial and temporal regulation, we can contemplate the use of this mechanism of DNA transformation to study genes of developmental importance. The extrachromosomal location of the foreign DNA precludes concerns of position effects that could be observed upon integration into random genomic locations. However, the presence of rearranged plasmid sequences in the high-molecular-weight arrays may be a drawback to studies of sequences that regulate developmental expression. It would be difficult to establish that expression is due to the original injected sequence rather than a fortuitous duplication or deletion. Perhaps properly packaged DNA (condensed with histones) or suitably engineered vectors (such as linear molecules capped with functional telomeres [51]) would not undergo these rearrangements.

The occasional loss of the injected sequences is not likely to prevent the detection of proper gene expression. We have shown that half the progeny of a transformant still carry some exogenous sequences, and approximately half of these transformed animals contain foreign DNA in every cell. Thus, we would expect to be able to observe the expression of genes that are required in specific cells or tissues with a penetrance of approximately 25%.

While a reasonable frequency of transformed worms carry DNA in all their cells, many are mosiac for the exogenous sequences. Mosaic animals can be used to identify the anatomical location of gene action. For instance, it may be possible to determine which cells need to carry a particular transformed gene to complement a mutation. Herman (20) has analyzed mosaic animals generated by the low mitotic stability of genetically defined free duplications. Generating mosaics by DNA transformation would augment such studies. Thus, this mechanism of DNA transformation may offer dual benefits. Complementation by transformation might be feasible, and once a gene is identified, mosaic transformants may be used to define its cellular site of action.

Other mechanisms of transformation, particularly those by which DNA is stably integrated into the genome by recombination or transposition, may be feasible. For instance, injection of hybrid molecules with large regions of homology might permit recombination; a screen or selection for stable transformation might reveal such events. Injection of *C. elegans* transposable elements (13, 27) or their derivatives may permit mobilization and insertion of foreign DNA into the genome. Thus, the results of the microinjection experiments we have described suggest avenues by which alternative mechanisms can be explored.

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