mM EDTA, and 1 mM DTT, and applied to a Mono-S HR 10/10 column (developed with 0 to 1 M NaCl in the same buffer) for cation-exchange chromatography. GroES-containing fractions were identified by autoradiography as above. Following this second ion-exchange step, GroES was demonstrated to be isotopically pure by SDS-PAGE, and the concentration (54 Ci/mol) was determined by quantitative amino acid analysis.

52. Formation of the [^{35}S]GroES$_7$·ADP$_7$·GroEL$_7$–GroEL$_7$ complex was achieved by incubation of 4.3 μM GroEL$_{14}$ and 3.1 μM [^{35}S]GroES$_7$ (*45*) with 100 μM ATP for 5 min. The first round of gel-filtration chromatography (Bio-Sil Sec 400-5; 7.8 mm by 300 mm; Bio-Rad; 1 ml/min) diluted the complex ~10 times to ~430 nM. Aliquots of this complex were treated as indicated and reinjected onto the same column. The buffer throughout was 50 mM tris HCl, 10 mM $MgCl_2$, 0.50 mM DTT, 0.5 mM KCl, and 50 μM EDTA (pH 7.8).
53. [^{35}S]RuBisCO was generated as described (*44*) diluting the specific activity with unlabeled RuBisCO. The ion-exchange chromatography step was modified by developing the Mono-Q HR 5/5 column with a 0.01 to 0.5 M K-P$_i$ gradient (pH 7.0). Fractions containing RuBisCO were demonstrated to be isotopically pure, concentrated, and stored in 1 mM Hepes, 1 mM DTT, and 0.1 mM EDTA at 0°C. The [^{35}S]RuBisCO concentration was determined by assaying activity versus a known concentration of active RuBisCO; the specific activity was 5800 Ci/mol.
54. Initial complex formation was achieved by adding ATP (final concentration of 100 μM) to 180 nM GroEL$_{14}$ with or without 700 nM GroES$_{14}$ in folding buffer [100 mM Na Hepes, 5.0 mM $Mg(CH_3CO_2)_2$, 1.0 mM DTT, 0.50 mM KCH_3CO_2, 0.10 mM EDTA, and 0.010% Tween-20]. After allowing 1 min for formation of the asymmetric complex (when GroES was present), we rapidly added four aliquots (5 μl each) of ~400 nM [^{35}S]RuBisCO (acid-denatured) (a total of 66.7 nM RuBisCO added), and the quaternary complex was isolated by gel filtration in folding buffer (Bio-Sil Sec 400-5; 7.8 mm by 300 mm; Bio-Rad). We isolated ≥25% of the starting disintegrations per minute as a stable complex with GroEL (eluting at ~18 nM GroEL or asymmetric complex).
55. We achieved simultaneous addition of acid-denatured RuBisCO and other components by balancing the added components on the side of the tube until acid-denatured RuBisCO could be rapidly added, then vortexing thoroughly.
56. Because of the instability of RuBisCO-I over time, especially with aggregation constantly reducing the concentration of free RuBisCO-I (Fig. 3), complete isotopic equilibrium may occur at a relatively higher concentration of RuBisCO-I retained.
57. We thank T. R. Webb for technical assistance in the purification of the proteins used in these studies.

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Integration and Germ-Line Transmission of a Pseudotyped Retroviral Vector in Zebrafish

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The zebrafish is rapidly becoming a popular model system for the study of vertebrate development because it is ideal for both embryological studies and genetic analysis. To determine if a retroviral vector pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus could infect zebrafish embryos, and in particular, the cells destined to become the germ line, a pseudotyped virus was injected into blastula-stage zebrafish embryos. Fifty-one embryos were allowed to develop and eight transmitted proviral DNA to their progeny. Founders were mosaic, but as expected, transgenic F_1's transmitted proviral DNA in a Mendelian fashion to the F_2 progeny. Transgenic F_1 fish inherited a single integrated provirus, and a single founder could transmit more than one viral integration to its progeny. These results demonstrate that this pantropic pseudotyped vector, originally developed for human gene therapy, will make the use of retroviral vectors in zebrafish possible.

That retroviruses could be used to deliver foreign DNA into the genome of an animal was first demonstrated by infecting preimplantation stage mouse embryos with the Moloney murine leukemia virus (MoMLV) and obtaining germ-line transmission of an integrated provirus (*1*). Subsequently, the ability of retroviruses to integrate exogenous DNA into the genome of infected cells has been exploited for gene therapy (*2*), for cell lineage studies (*3*), and for studies of insertional mutagenesis (*4, 5*). In addition, the use of retroviral gene traps in conjunction with mouse embryonic stem cells has proven quite effective in the search for, and mutagenesis of, genes expressed during mouse development (*5*).

In recent years the zebrafish, *Danio rerio*, has become a popular model system for vertebrate developmental studies because it offers the opportunity to combine classical genetic analysis, including large-scale mutagenesis, with an easily accessible and manipulatable embryo. Genetic studies of the zebrafish benefit from the 2- to 3-month generation time, the ability of females to routinely lay hundreds of eggs, and the small size of the adults, whereas embryological studies benefit from the large, transparent embryos, detailed fate maps, and the fact that single identified cells can be studied in living embryos (*6*). In the past, the application of retroviral vector technology to the zebrafish system was not feasible because of the limited host range of the standard vectors. However, a recent report has demonstrated that a pseudotyped retroviral vector, which can be concentrated to very high titers, can infect cultured fish cells, including those derived from zebrafish embryos (*7*). This virus contains an MoMLV-based genome surrounded by an envelope containing the glycoprotein (G protein) of the vesicular stomatitis virus (VSV), completely replacing the retroviral *env* glycoprotein. As a result of the presence of the VSV G protein, this pseudotyped virus has the broad host range characteristic of VSV, and on entry into a permissive cell will integrate retroviral sequences into the host genome.

The ability of the MoMLV(VSV) pseudotyped virus to infect cultured fish cell lines suggested that it might be possible to infect zebrafish embryos and to obtain germ-line transmission of integrated proviral DNA. However, because zebrafish embryos develop very rapidly and at 28°C whereas murine retroviruses generally require over 6 hours at 37°C to synthesize and integrate proviral DNA (*8*), it was unclear whether or not germ-line transmission could be obtained efficiently or at all in zebrafish. In these studies we describe the high-frequency infection of the zebrafish germ line with this pseudotyped virus and discuss its potential use in the study of zebrafish development.

A concentrated stock of the pseudotyped virus LZRNL(G) (Fig. 1) was generated essentially as described (*9*). This virus was titered on cultured zebrafish cells by infection of an established zebrafish cell line, PAC2 (*10*), and selection for clones in media containing G418 (*11*). LZRNL contains the neomycin phosphotransferase gene (*neo*) and thus can confer G418^r resistance to infected cells. Control mouse 3T3 cells were infected under the same conditions. The titer of the virus was 6.7×10^6 colony-forming units (CFU) per milliliter on zebrafish PAC2 cells, and 2.5×10^7 CFU/ml on mouse 3T3 cells.

To generate transgenic zebrafish, we injected LZRNL(G) virus into the blastoderm, among the cells of blastula-stage embryos, at approximately the 2000- to 4000-cell stage (*12*). On the basis of the virus titer on PAC2 cells and the volume injected, we estimate that 50 to 100 infectious units were injected into each embryo.

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To detect germ-line transmission of proviral DNA, injected embryos were raised to sexual maturity and mated, and DNA from 24-hour-old pools of their F_1 progeny were tested for the presence of LZRNL sequences by polymerase chain reaction (PCR) (*13*). In total, 8 out of 51 fish examined showed germ-line transmission of the retroviral sequences (Fig. 2A). Because only 50 to 100 F_1 embryos were collected to test for germ-line transmission, founders that transmitted proviral sequences to less than 1% of their offspring may have been overlooked.

Because the virus was injected into blastula-stage embryos containing a large number of potential target cells, the embryos were very likely be mosaic for the presence of integrated viral sequences. To determine if the founder fish had mosaic germ lines, we analyzed individual F_1 progeny from each founder by PCR for the presence of proviral DNA (Fig. 2B). As shown in Table 1, all eight founders did indeed have mosaic germ lines and transmitted proviral DNA to less than 5% of their F_1 progeny.

We identified live transgenic F_1 fish by isolating genomic DNA from caudal fin clips and using PCR to test for the presence of the viral transgene. Two transgenic F_1's identified in this way were then mated to nontransgenic fish, and individual F_2 embryos were screened by PCR. If a transgenic F_1 fish contained an integrated provirus, that provirus should have been transmitted to 50% of the F_2 progeny. The first F_1 tested transmitted the transgene to 11 out of 25 of its F_2 progeny (44%) (Fig. 2C), and the second F_1 tested transmitted the transgene to 8 out of 17 of its F_2 progeny (47%). These frequencies are consistent with Mendelian transmission and support the notion that the proviral DNA is integrated into the zebrafish genome.

Typically, retroviral DNA integrates into a host chromosome as a single copy in a manner that maintains the linear organization of its viral genome (*14*). To confirm that the proviral sequences were integrated in the expected arrangement in the infected fish cells and transgenic animals, we performed Southern (DNA) blot analysis (*15*). Genomic DNA from two clones of cultured PAC2 cells infected with LZRNL(G), as well as from the transgenic F_1 progeny of two different founders (m4 and f13), was digested with the restriction enzyme Dra I. In addition, genomic DNA from the two infected PAC2 clones and from the transgenic F_1 progeny of all eight founders was digested with Hind III. Dra I does not

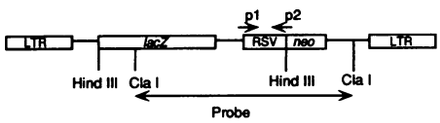

Fig. 1. Map of pLZRNL. The locations of the PCR primers used to identify transgenic fish are indicated with arrows. The Cla I fragment was used as the probe for Southern blot analysis. The construct is not drawn to scale.

Table 1. Mosaicism of germ-line transmission of proviral DNA from founders to the F_1 generation.

Founder	Transgenic F_1's	Mosaicism (%)
m4	6/306	2.0
f6	3/128	2.3
f12	2/86	2.3
f13	1/20	5.0
f36	2/110	1.8
f44	1/60	1.7
f45	2/50	4.0
m49	1/115	0.9

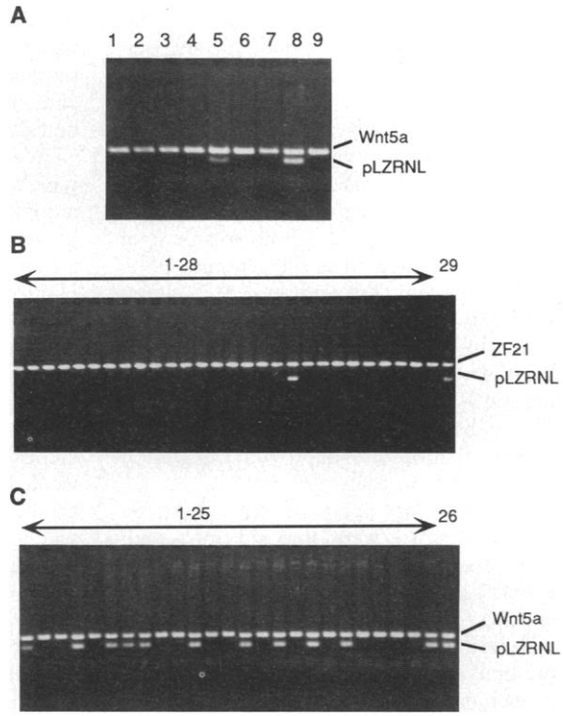

Fig. 2. Polymerase chain reaction (PCR) analysis to detect germ-line transmission of proviral DNA (**A**) PCR analysis with DNA extracted from pools of the F_1 progeny of individual injected, potential founder fish. Two pairs of primers were used for PCR analysis of zebrafish genomic DNA. The first pair of primers is specific to the injected pseudotype proviral DNA (as shown in Fig. 1) and generates a 290-bp PCR product. The second pair of primers is specific to the zebrafish Wnt5A gene and generates a 387-bp PCR product (*20*). Lanes 1 to 7 show the PCR products generated with DNA from pools of embryonic F_1 progeny. Lane 8 shows the PCR products generated with DNA from a zebrafish cell line that contains a proviral insertion. Lane 9 shows the PCR product generated with DNA from an uninjected fish. (**B**) PCR analysis with DNA extracted from individual F_1 progeny of a positive founder fish. In this case, the second pair of primers is specific to the zebrafish homeobox gene, ZF21 (*21*), and generates a 475-bp PCR product. Lanes 1 to 28 show the PCR products generated with DNA from individual F_1 fish. Lane 29 shows the PCR products generated with DNA from a zebrafish cell line that contains a proviral insertion. (**C**) DNA was extracted from the individual F_2 embryos of a transgenic F_1 fish and was analyzed by PCR. Lanes 1 to 25 are DNA from individual F_2 fish. Lane 26 shows the PCR products generated with DNA from a zebrafish cell line that contains a proviral insertion.

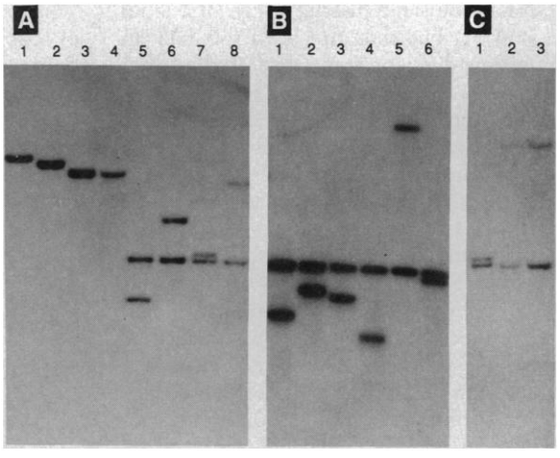

Fig. 3. Confirmation of proviral DNA integration by Southern blot analysis. (**A**) Southern blot analysis of genomic DNA from two clones of zebrafish PAC2 cells generated by virus infection (lanes 1, 2, 5, and 6) and from two transgenic F_1 fish from founders m4 (lanes 3 and 7) and f13 (lanes 4 and 8). Genomic DNA samples were digested with Dra I (lanes 1 to 4) and Hind III (lanes 5 to 8) and were probed with the Cla I fragment of pLZRNL (shown in Fig. 1). (**B**) Southern blot analysis of Hind III digests of genomic DNA from the transgenic F_1 progeny of founders f6, f12, f36, f44, f45, m49 (lanes 1 to 6, respectively). (**C**) Genomic DNA from three transgenic F_1 progeny of a single founder fish was digested with Hind III and probed with the Cla I fragment of pLZRNL. The hybridization pattern in lane 1 is different from those in lanes 2 and 3, indicating that the genomic sites of integration are different.

cleave within the LZRNL sequence and should yield one or more fragments, depending on the number of integrations, with sizes larger than the proviral genome (7.2 kb). Hind III cleaves twice within the LZRNL sequence (Fig. 1) and is expected to yield a 3.7-kb internal fragment for all insertions, and two junction fragments with sizes dependent on the presence of Hind III sites in the surrounding genomic sequences. The Cla I fragment of pLZRNL, containing lacZ, RSV, and neo sequences, was used as the hybridization probe (Fig. 1) and was expected to hybridize to both the internal 3.7-kb Hind III fragment and to the 3′ junction fragment.

Samples digested with Dra I revealed only single bands of variable size, indicating the presence of single copies of integrated provirus (Fig. 3A, lanes 1 to 4). In the case of Hind III digests, a 3.7-kb fragment of LZRNL was detected in the genomic DNA of the transgenic fish and the PAC2 clones (Fig. 3A, lanes 5 to 8, and Fig. 3B, lanes 1 to 6). In addition, each lane has a second band of variable size, presumably representing the 3′ junction fragment. The variation in size between these junction fragments suggests that the sites of proviral integration were distinct in each case.

The fact that approximately 50 to 100 infectious units were injected into each embryo suggested that multiple integration events could readily have occurred in each embryo. To determine whether or not a founder fish transmitted more than one proviral integration through its germ line, we compared the insertions of three different transgenic F_1's from a single founder (m4) by means of Southern blot analysis. Genomic DNA was digested with Hind III and probed as described earlier. As expected, all three F_1's had the internal 3.7-kb band as well as a 3′ junction fragment. A comparison of the putative junction fragments, however, indicated that two of the fish had the same insertion (Fig. 3C, lanes 2 and 3), but that the third fish had an insertion different from that of the other two (Fig. 3C, lane 1). This result demonstrates that for this founder, at least two insertions had been transmitted independently through the germ line.

The viral construct used in this study contains two reporter genes—*lacZ*, driven by the Moloney LTR, and *neo*, driven by an internal Rous sarcoma virus (RSV) LTR (Fig. 1) (*16*). Previous transfection and microinjection experiments have indicated that the Moloney LTR does not drive lacZ expression in zebrafish cells at levels detectable by staining with the chromogenic substrate, X-Gal (*17*). Therefore, we were not surprised to find that neither PAC2 clones infected with LZRNL(G) nor embryos containing the LZRNL transgene stained blue with X-Gal in our present studies. Efforts are currently under way to determine if the *neo* gene is expressed from the RSV promoter in fish containing the viral transgene as it is in the PAC2 fish cell line.

Our results demonstrate that the LZRNL(G) pseudotyped virus can be used to infect zebrafish embryos and to generate transgenic zebrafish. Additional technological advances promise to make the infection of zebrafish embryos with this class of viral vectors an extremely powerful tool for the study of vertebrate development. Such advances should include the identification of suitable promoter and promoter-enhancer combinations for gene expression in zebrafish cells.

We are interested in using this class of pseudotyped vector to generate insertional mutants in zebrafish. Mutagenesis in zebrafish has typically been performed with gamma rays or chemical mutagens such as *N*-ethyl-*N*-nitrosourea (ENU) (*18*). However, a drawback of these mutagenesis methods is that the genes identified cannot be cloned readily because of the current absence of high-resolution genetic and physical maps of the zebrafish genome. Genes mutated by insertional mutagenesis would be more amenable to cloning because the integrated exogenous DNA would provide a "tag" for the insertion site.

One possible strategy for studies of insertional mutagenesis would be to generate as many transgenic lines as possible, breed them to homozygosity, and screen them for mutant phenotypes. Although this approach is currently possible, it would be made more feasible by an increase in transgenic frequencies. Such an increase might be made possible by injection of a higher titer virus stock earlier in embryonic development. The stock of virus used in this study was more than 10-fold lower in titer than stocks that have been obtained with other vectors (*7*).

These results suggest that the advantages of retroviral vector technology, which have been well documented in the mouse and chicken, will now be applicable to the zebrafish. This advance should accelerate the study of zebrafish development and consequently help to elucidate the general mechanisms controlling vertebrate development.

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9. A 293-derived cell line stably expressing the MoMLV *gag* and *pol* genes as well as the LZRNL genome was transiently transfected with a plasmid encoding the VSV-G gene driven by the human cytomegalovirus promoter. Virus containing supernatant was collected 48 to 72 hours later and concentrated as previously described (*7*).
10. The PAC2 cell line was derived from 24-hour-old embryos and is maintained in Lebowitz-15 media supplemented with 15% fetal bovine serum and 5% zebrafish embryo extract (*19*).
11. Concentrated stocks of LZRNL(G) were diluted and used to infect NIH 3T3 cells, and PAC2 cells for 3 hours in the presence of polybrene (8 μg/ml). Approximately 4 hours after completion of the infection the infected cells were trypsinized, serially diluted into both selective media and nonselective media, and plated. The plates were stained with crystal violet 10 days later and the number of colonies was counted.
12. Concentrated pseudotyped virus derived from the vector pLZRNL was resuspended in TNE [50 mM tris-HCl (pH 7.8), 130 mM NaCl, 1 mM EDTA] containing polybrene (8 μg/ml; Sigma). For injection, dechorionated eggs were incubated in Holtfreter's solution at 25 to 27°C for about 4 to 5 hours, and the late blastula–stage embryos were injected with a total of 10 to 20 nl of virus into multiple locations in each embryo with use of a glass needle and a dissecting microscope.
13. DNA was extracted from pools of 50 to 100 F_1 embryos at 24 hours of development, or from individual fish by incubation for 4 to 12 hours at 55°C in a lysis buffer [10 mM tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.4% SDS, proteinase K (200 μg/ml)]. DNA was precipitated by ethanol and dissolved in TE (pH 8.0). Approximately 10 ng of DNA was used for PCR with AmpliTaq Polymerase (Perkin Elmer, Cetus). The reaction was carried out at 94°C for 30 min, 60°C for 45 min, and 72°C for 60 min for 32 cycles with an initial 2-min denaturation step at 94°C. The two primers used to detect the presence of LZRNL DNA sequence yield a 300-bp PCR product. The 5′ primer (P1) is 5′-GGGAATGTAGTCTTATGCAATAC-3′. The 3′ primer (P3) is 5′-GCACACCAATGTGGTGAATGGTC-3′. A pair of internal control primers homologous to the zebrafish Wnt5A gene (*20*) or to the ZF21 gene (*21*) was included in each reaction.
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Preferential Nucleosome Assembly at DNA Triplet Repeats from the Myotonic Dystrophy Gene

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The expansion of CTG repeats in DNA occurs in or near genes involved in several human diseases, including myotonic dystrophy and Huntington's disease. Nucleosomes, the basic structural element of chromosomes, consist of 146 base pairs of DNA coiled about an octamer of histone proteins and mediate general transcriptional repression. Electron microscopy was used to examine in vitro the nucleosome assembly of DNA containing repeating CTG triplets. The efficiency of nucleosome formation increased with expanded triplet blocks, suggesting that such blocks may repress transcription through the creation of stable nucleosomes.

Several human genetic diseases have been traced to the expansion of blocks of repeating nucleotide triplets. The expansion of a CGG repeat was first observed in the 5′-untranslated region of the *FMR-1* gene, responsible for fragile X syndrome (*1*). Subsequently, five more disorders were linked to expanded triplet blocks: a CTG (or CAG) repeat was found in the 3′-untranslated region of the myotonic dystrophy gene (*2*) and in the protein-coding regions of the genes for Kennedy's disease (*3*), Huntington's disease (*4*), spinocerebellar ataxia type 1 (*5*), and dentatorubralpallidoluysian atrophy (*6*). Although the repeat blocks occur at different locations relative to the coding sequences, a correlation between the length of the triplet block, the severity of the diseases, and the age of onset exists in all six disorders. Furthermore, a phenomenon called genetic anticipation has been observed in which the severity of the disease increases with succeeding generations (*4–7*). When the expression of a human myotonic dystrophy protein kinase gene containing an expanded CTG triplet block was examined in the absence of the normal allele, no mRNA from the kinase gene was detected (*8*). Furthermore, the threshold for the onset of the phenotype of the triplet diseases frequently approximates the amount of DNA (146 base pairs) in a nucleosome. These observations suggest that DNA that contains long triplet repeats might form stable nucleosomes that repress transcription.

To examine this hypothesis, we carried out in vitro nucleosome reconstitution experiments, starting from high salt buffer. This method has been used by researchers to examine DNA sequence–directed nucleosome positioning in vitro (*9–11*). We used electron microscopy (EM) in our analysis to create a map showing the relative affinity for nucleosome formation along plasmid DNAs containing CTG triplet blocks.

Numerous DNA structural transitions are facilitated by negative supercoiling or influenced by heating in the presence of Mg. A Bluescript-based plasmid, pSH2, isolated from *Escherichia coli*, with a negative superhelical density of −0.06 and containing 130 CTG repeats derived from an individual with myotonic dystrophy (*12*) was first treated with Mg and heat and then reconstituted with a limiting amount of histones. We then prepared the DNA for EM by cutting it with restriction enzymes and labeling one end with streptavidin (Fig. 1). This placed the CTG triplets in pSH2 between 32 and 43 map units along the DNA (*12*).

Analysis of the location of 100 single nucleosomes (Fig. 2A) revealed that 48% of all the nucleosomes were present in the region between 30 and 45 map units. In contrast, the data for the vector alone (Fig. 2B) showed a uniform distribution of nucleosomes over the length of the DNA with only 10% of the nucleosomes present in the region between 30 and 45 map units. When linear (Fig. 2C) or supercoiled (Fig. 2D) pSH2 DNA that had not first been treated with heat or Mg was reconstituted, in both cases 29% of all the nucleosomes were localized to the triplet repeat. The data suggest that primary DNA structure is the major factor favoring nucleosome positioning over the triplets and that supercoiling or exposure to heat and Mg is less effective.

In all triplet repeat–related diseases, the severity of the disease increases with the length of the triplet block. To explore the relation between the length of the triplet block and the efficiency of nucleosome assembly, we used six pUC19-based plasmids (treated with heat and Mg) containing 26 to 250 contiguous CTG triplet repeats [$(CTG)_{26}$ to $(CTG)_{250}$] (*12*) as templates. Nucleosome assembly was found to be two- to fivefold more likely over the triplet blocks than over the adjacent flanking regions (Fig. 3).

When the values were normalized (Fig. 4), we observed that as the number of the CTG repeats increased, the efficiency of nucleosome formation at the repeats also increased (Fig. 4), up to a repeat size of 180. For the DNAs with a $(CTG)_{250}$ repeat, two side-by-side nucleosomes were occasionally observed (Fig. 1C). Plasmids pSH2 and pRW1981 (*12*) contain the same $(CTG)_{130}$ repeat cloned into different vectors. Nonetheless, they showed similar efficiencies of nucleosome formation (Figs. 2 and 3), which suggests that the efficiency of nucleosome assembly is independent of the nature of the flanking sequences.

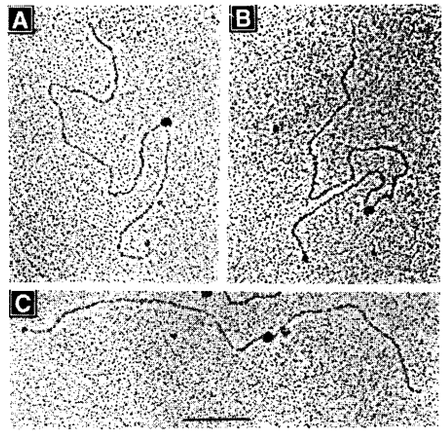

Fig. 1. Visualization of nucleosomes assembled on streptavadin-labeled pSH2 DNA containing a $(CTG)_{130}$ repeat (**A** and **B**) and pRW3222 containing a $(CTG)_{250}$ repeat (**C**), derived from individuals with myotonic dystrophy. The DNAs (curved filaments) contain streptavadin protein bound to one end [small particles at bottom in (A) and (B), and at left in (C)] and one or two nucleosomes assembled near the center of each DNA. In the absence of added histone, no nucleosome-like objects were observed. Reconstitution of the DNA with histones was as described (*16*) and preparation for EM, including rotary shadowcast with tungsten, was also as described (*17*). Bar, 100 nm.

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