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Separation of complementary strands of plasmid DNA using the biotin-avidin system and its application to heteroduplex formation and RNA/DNA hybridizations in electron microscopy

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ABSTRACT

A method for the separation of complementary strands with the help of the biotin-avidin system is described. Restriction fragments were terminally labeled at both ends with biotinylated nucleotides. The DNA was cut by a second restriction enzyme, and the fragments were bound to an avidin agarose column. The non-biotinylated strands were eluted with 0.1 M NaOH, and the biotin-labeled strands were subsequently released from the column by elution with 50% guanidine isothiocyanate/formamide. Contamination of the separated strands by complementary single strands was less than 4%. - Separated linear single strands of the vector pEMBL were prepared. On annealing with recombinant circular DNA a substitution loop is formed which provides position and orientation markers for the unambiguous electron microscopic analysis of heteroduplexes or hybrids formed with the inserted sequences. - The terminal biotin label was visualized by complex formation with a streptavidin-ferritin conjugate.

INTRODUCTION

Heteroduplex and RNA/DNA hybrid analysis are valuable tools for structural investigations of nucleic acids in the electron microsope. One of the obstacles limiting the application of these methods is the problem of homoduplex formation when the nucleic acids used for the renaturation contain a mixture of homologous complementary strands. When the sequence homologies in heteroduplexes are short, or disrupted by non-homologies, or destabilized by base-mismatches the heteroduplex formation will be much slower than the homoduplex formation. Using the right combination of separated strands homoduplex formation can be avoided, so that long incubation periods and conditions of low stringency may be applied without the depletion of the DNA pool by homoduplex formation.

Conventional means of preparing separated complementary strands from denatured DNA are density gradient centrifugation (1,2) or agarose gel electrophoresis (3,4,5). These methods depend on a favourable base distribution between the two strands. The majority of DNAs from recombinant lambda phages can be separated by agarose gel electrophoresis since the

mobility in most cases is determined by the lambda vector arms (6, and H. Delius, unpublished). But many other denatured DNAs, especially linearized plasmid DNAs, do not separate in agarose gels. A method for the separation of strands by annealing to oligo(dA)- and oligo(dT)-columns was described by Hayashi (7). It can only be used for plasmids or fragments containing a segment of poly(dA):poly(dT) about 40 to 100 nucleotides in length.

A different method which can be more generally applied for the preparation of separated strands is described below. It is based on the enzymatic incorporation of biotin-labeled nucleotides into the ends of restriction fragments and the very strong binding of such biotin labeled DNAs to avidin agarose columns (8).

MATERIALS AND METHODS

Plasmid DNAs and enzymes

E. coli strains harbouring the plasmids pEMBL8+ and pEMBL8- were obtained from R. Cortese. Plasmids containing cDNA of the H- and L-chain message of apoferritin were constructed by C. Santoro and F. Costanzo. A pBR322 clone containing the *Euglena* chloroplast EcoRI fragment I was a gift from Mario Keller, Strasbourg. The fragment was recloned into the vector pEMBL8-.

Plasmid DNA was prepared by alkaline lysis according to standard procedures (9). It was purified by CsCl-ethidium bromide equilibrium density centrifugation, extracted with isopropanol and ethanol precipitated.

Restriction enzymes were obtained from New England Biolabs, and reaction conditions were as indicated by the manufacturer.

The large fragment of *E. coli* DNA polymerase (Klenow fragment) was obtained from P. H. Stehelin & Cie A.G., Basel.

Terminal labeling with biotin

The biotin-labeling of the restriction sites was done by treatment of the restricted DNA (about 20 µg in 50 µl) with 10 units of the Klenow fragment at 37° for 15 min in a buffer containing 50 mM sodium phosphate, pH 6.5, 10 mM $MgCl_2$, 20 µM dATP, 100 µM each dGTP and dCTP, and 50 µM bio-4-dUTP or bio-11-dUTP, with biotin attached to dUTP via a short or long linker, respectively (10). Bio-4-dUTP was a gift from R. Goody and P.Vosberg (Heidelberg), and bio-11-dUTP was generously provided by Bethesda Research Labs.

Attachment of streptavidin-ferritin to the biotinylated ends of DNA

The biotinylated DNA (1-5 µg/ml) was mixed with the

streptavidin-ferritin conjugate (2.5 mg/ml protein; this preparation was a generous gift from Bethesda Research Labs) in 0.1 M sodium perchlorate, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 at room temperature. The conjugate was diluted 25-fold for use. After 5 min the mixture was passed through a column (4 x 50 mm) of Sepharose Cl-2B equilibrated with 4 mM magnesium acetate to remove the excess ferritin conjugate and to transfer the sample into the buffer used for the adsorption to mica.

Electron microscopy

Mica adsorption and cytochrome spreading for the electron microscopy were done as described earlier (11). Cytochrome spreading was done from 50% formamide, 0.1 M Tris-HCl, pH 8.5, 1 mM EDTA onto a hypophase of 0.005% octyl glucopyranoside (Sigma Chemical Co.). Electron micrographs were taken with a Zeiss EM 10A microscope.

Binding to avidin agarose columns and elution of single-strands

Avidin agarose (Sigma Chem. Co.) was prewashed in a column (4 x 45 mm) with 100 µl 1 M sodium perchlorate, 0.1 M NaOH, and extensively washed with 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 (T/E buffer) until the effluent was neutral. A 6 to 8 mm thick layer of the washed avidin agarose (about 80 µl) was put on top of a Sephadex G-100 column (4 x 40 mm). The column was equilibrated with 0.1 M sodium perchlorate in T/E-buffer. DNA was loaded onto the column in the same buffer adding 10 µl aliquots every 30 sec. To bind as much DNA as possible on the avidin agarose the collected fractions of the first flow-through were passed through the column a second time. A total of about 3 µg of plasmid DNA could be bound to 80 µl of avidin agarose. Non-specifically adsorbed DNA was eluted with 100 µl of 1 M sodium perchlorate, and the column then washed thoroughly with T/E buffer. The non-biotinylated strand of the double-stranded DNA was eluted with 2 x 50 µl of 0.1 M NaOH, followed by 50 µl aliquots of T/E buffer. After collecting the fractions containing the single-stranded DNA in T/E, the column was washed extensively with T/E to remove the NaOH. The biotinylated strand was eluted with 50% (w/w) guanidine isothiocyanate (Bethesda Research Labs) dissolved in formamide (to clarify this solution it was centrifuged for 10 min in an Eppendorf centrifuge before use). The single stranded DNA was collected in 100 µl T/E buffer at a concentration between 10 to 15 µg/ml.

Determination of the concentrations of eluted DNAs

Concentrations of nucleic acids were determined by fluorescence. Samples between 1 and 5 µl were loaded onto a Sepharose Cl-2B column (4 x 45 mm) equilibrated with 10 mM sodium phosphate, pH 7.0, 0.5 µg/ml ethidium

bromide. The column was eluted with the same buffer at a constant rate of about 80 µl/min, and the effluent was monitored on passage through the 70 µl flow-through cell of a Kratos FS 950 fluorometer. The concentrations were determined by comparison of the peak areas to the peaks produced by DNA standards. With an excitation wavelength of 254 nm, and a cut-off emission filter of 550 nm between 5 and 100 ng of nucleic acids were used for each determination.

RESULTS

Visualization of biotinylated nucleotides at the ends of double-stranded DNA

DNA of the plasmid vector pEMBL8 was linearized with EcoRI. Biotinylated dUTP was incorporated into the recessed ends of the restriction cut, with *E. coli* DNA polymerase I (large Klenow fragment). The reaction was monitored by determining the incorporation of radioactively labeled dATP. DNA from reactions in which the incorporation of radioactive label had reached a plateau was purified and used in experiments to visualize the biotinylated nucleotide in the electron microscope.

The biotinylated ends of the plasmid DNA were complexed with a streptavidin-ferritin conjugate which served as an electron dense marker for the electron microscopy. After incubation of the DNA with the streptavidin-ferritin at room temperature the sample was passed through a Sepharose column equilibrated with 4 mM magnesium acetate to remove the excess streptavidin-ferritin and at the same time transfer the sample into the buffer used for the adsorption of the DNA to mica. Figure 1a shows an electron micrograph of the complexes prepared by the mica adsorption technique. The electron dense ferritin molecules can be clearly recognized at both ends of the plasmid DNA. In a cytochrome spreading of the same preparation (Fig. 1b) the ferritin can still be recognized but the distinction between the DNA strand and the ferritin is not as good as in the mica adsorption.

The effect of the salt concentration on the binding of streptavidin-ferritin was analysed. The number of molecules with and without ferritin was counted in samples prepared at different ionic strengths. The results are given in Table 1. In low ionic strength buffers ($<$20 mM salt) the rate of complex formation is very low. At an ionic strength between 100 and 200 mM salt most of the biotinylated ends of the DNA molecules were complexed with streptavidin-ferritin after an incubation of 5 min.

Linear plasmid DNAs are frequently circularized when low concentrations

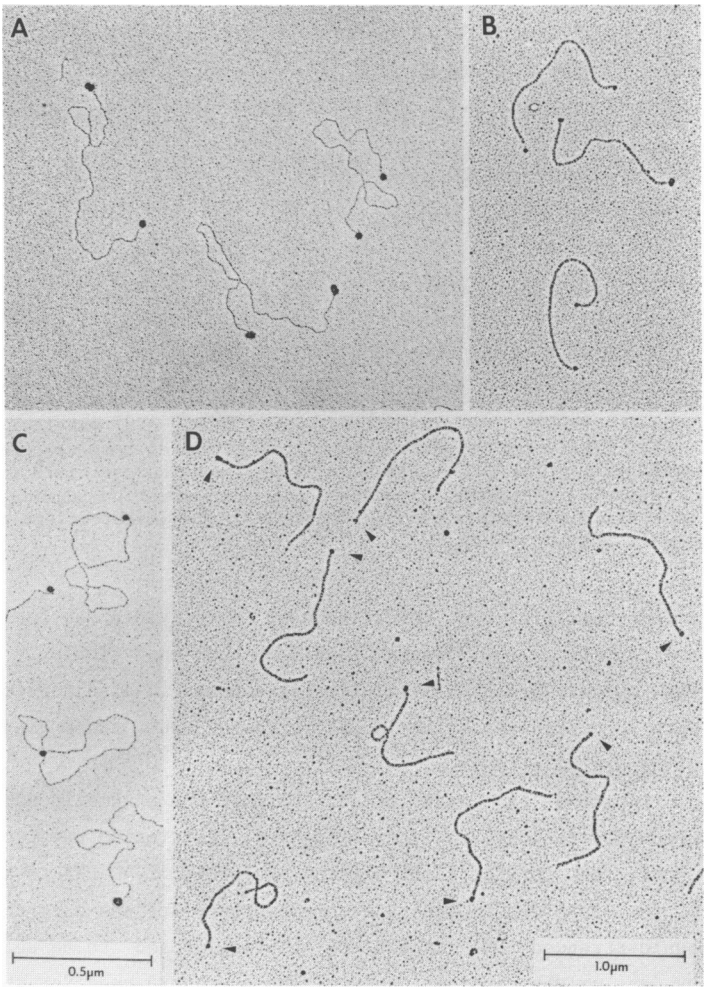


Figure 1. Electron micrographs of plasmid DNA terminally labeled with biotin and complexed with streptavidin-ferritin. (a) pEMBL8- DNA was linearized with EcoRI, and end-labeled with bio-4-dUTP using E. coli DNA polymerase (large Klenow fragment). The DNA was complexed with streptavidin-ferritin and prepared for the electron microscopy by the mica adsorption technique. (b) The same preparation as in (a) was prepared by the cytochrome spreading technique. (c) The preparation was done as in (a) but the concentration of the streptavidin-ferritin used to form the complexes with the biotin-labeled ends was 40-fold lower. (d) One of the biotin-labeled ends of a preparation as in (b) had been cut off by treatment with HindIII. The small fragment from the polylinker region of pEMBL was removed by retardation on a Sepharose Cl-2B column. After complex formation with streptavidin-ferritin and preparation by cytochrome spreading only one end of each plasmid DNA is labeled (arrow-heads). Magnification bars for (a) and (b) are given in (c) and (d), respectively.

Table 1

Salt dependence of the binding of streptavidin-ferritin.

Salt	Ferritin bound: none	one end	both ends	circles	Total number counted	percentage of complexed ends
a) 100 µg/ml streptavidin-ferritin						
none	45%	34%	12%	9%	285	36%
20 mM	10%	40%	38%	12%	252	63%
100 mM	4%	24%	62%	10%	223	83%
200 mM	1%	15%	71%	13%	204	91%
b) 2.5 µg/ml streptavidin-ferritin						
100 mM	3%	7%	20%	70%	266	84%

1 µg/ml of pEMBL8- DNA linearized with EcoRI and labeled with bio-4-dUTP using Klenow enzyme was incubated for 15 min at room temperature with streptavidin-ferritin, and then passed through a Sepharose Cl-2B column equilibrated with 4 mM magnesium acetate for the adsorption to mica. The number of streptavidin-ferritin conjugates bound to the linear molecules or circularizing the DNA were counted.

of streptavidin-ferritin are used, because each avidin has four binding sites which can bind the biotin labels at both ends (Table 1; Fig.1c). Although the amount of streptavidin-ferritin bound is also reduced at low ionic strength the circularization is not increased under these conditions.

Isolation of separated strands

It was reported by Langer et al. (8) that the enzymatic addition of biotin-labeled nucleotides to the staggered restriction cut produced by EcoRI is sufficient for the tight binding of labeled SV40 DNA to an avidin agarose column. In order to separate complementary strands only one strand in the double-stranded DNA should be biotin-labeled to be selectively retained by an avidin agarose column. The procedure used for the strand separation is schematically outlined in Fig. 2.

A restriction fragment is labeled at both ends with biotin and then cut with another restriction enzyme. This results in two fragments which are only labeled on one strand. The fragments are bound to an avidin agarose column. The non-biotinylated strands are eluted by denaturing the DNA with NaOH and the biotinylated strands are subsequently eluted with guanidinium isothiocyanate/formamide.

Following this scheme separated strands from plasmids containing cDNA inserts of mRNA for the L-chain and for the H-chain of apoferritin were prepared. The plasmids were linearized with EcoRI, and labeled with bio-4-dUTP using Klenow DNA polymerase. After a second cut with SalI the

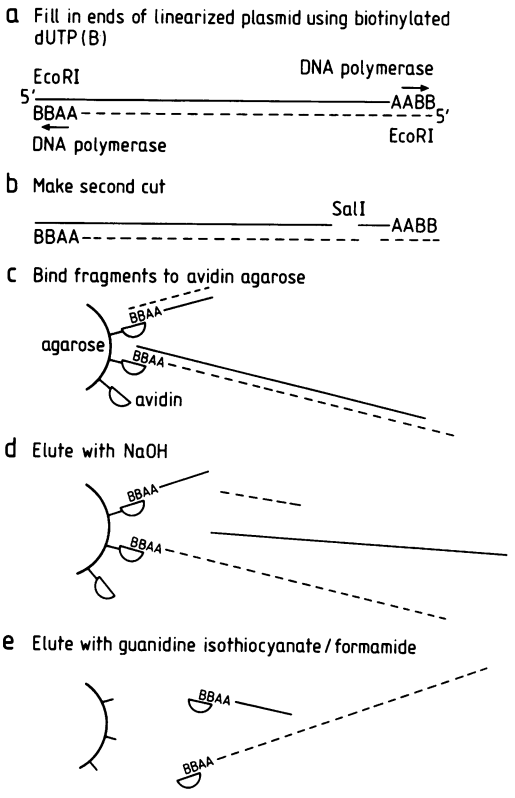


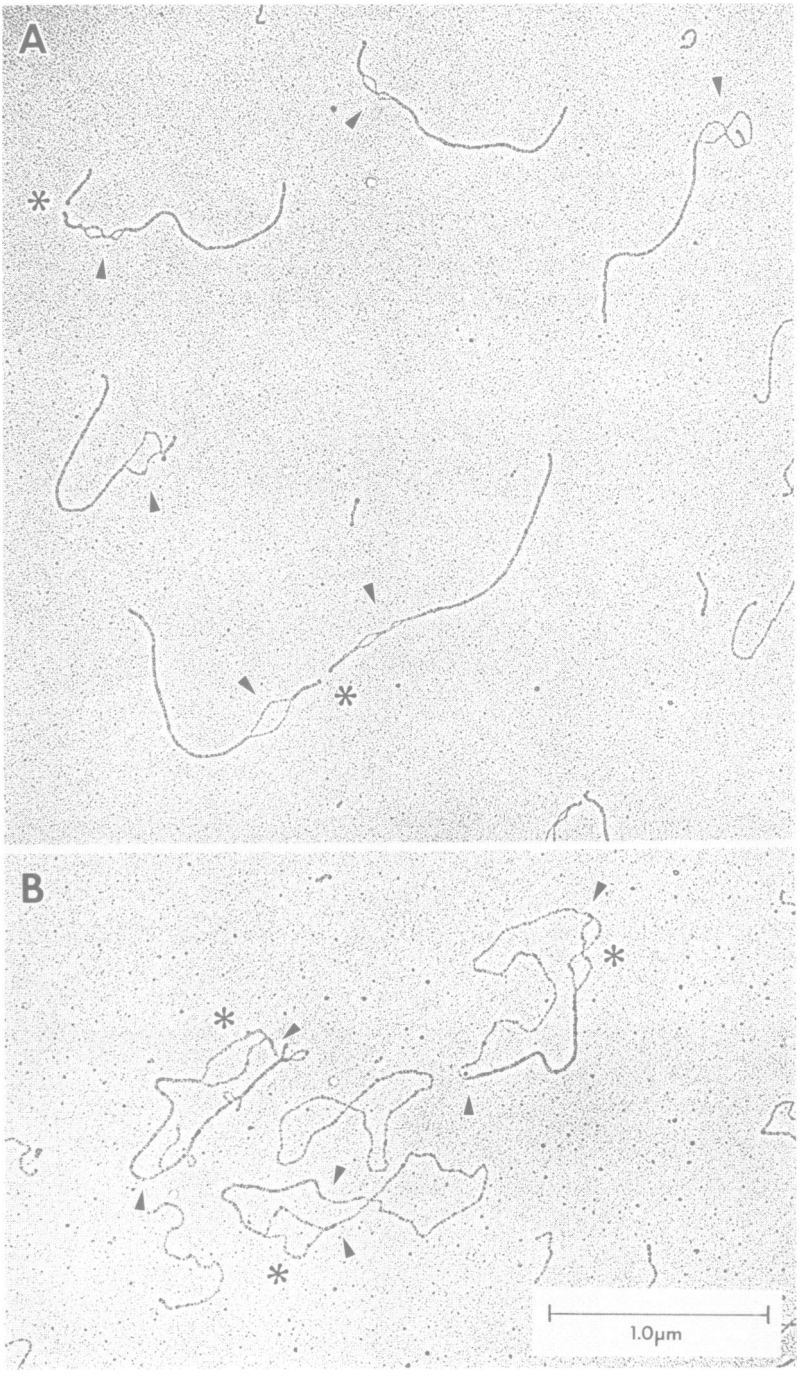
Figure 2. Scheme for the preparation of separated strands from double-stranded linearized plasmid DNAs or restriction fragments.

Table 2

Determination of contaminating complementary strands in preparations of separated strands.

	single strands	double strands		content of complementary strands
		partial	complete	
a) cDNA plasmid of L-chain of apoferritin				
NaOH eluted	200	6	2	4%
G-Iso eluted	206	3	3	3%
b) cDNA plasmid of H-chain of apoferritin				
NaOH eluted	304	3	4	2%
G-Iso eluted	250	3	3	2%

The preparations of separated strands were incubated under reannealing conditions for 16 hours, prepared for electron microscopy by cytochrome spreading, and single strands, fully double stranded molecules, and partially double stranded molecules were counted.


A
B
1.0μm

biotinylated DNA was bound to an avidin column in 100 mM salt. After a wash with 1 M salt to remove non-specifically bound DNA the two strands were eluted. Of 3.5 µg of double-stranded DNA bound to 80 µl of avidin agarose between 1.4 and 1.7 µg were recovered in each single strand preparation.

The purity of the preparations was checked in the electron microscope. After extensive self-annealing of the eluted strands the percentage of homoduplexes was determined. The results are given in Table 2. The contamination of the separated single strands with complementary strands was between 2 and 4%.

Heteroduplexes were prepared by annealing a mixture of the NaOH-eluted strand of one plasmid and the guanidine isothiocyanate-eluted strand of the other plasmid (Fig.3a). Heteroduplexes can be recognized by substitution loops formed in the region of the insert sequences. Nearly all the annealed DNA molecules showed heteroduplex loops confirming that there is very little contamination with homologous complementary strands.

It is not known how the biotinylated DNA strand is eluted from the avidin agarose by guanidin isothiocyanate. This strand was reannealed with the non-biotinylated strand eluted with NaOH. To test for the presence of biotin at the end of the resultant homoduplex the preparation was incubated with streptavidin-ferritin. No complexes were formed. But without added streptavidin-ferritin the majority of the molecules showed small knobs at one end when prepared by the mica adsorption method (not shown). It is possible that avidin and perhaps material from the support matrix might still be attached to the eluted biotinylated strands. This is supported by an effect noticed in the preparation of heteroduplexes shown in Fig. 3a. At

Figure 3. Electron micrographs of heteroduplexes and hybrids using purified preparations of separated strands. (a) Heteroduplexes prepared from the complementary separated strands of two plasmids carrying cDNA inserts of the L-chain and H-chain genes of apoferritin. The substitution loops indicating the regions of non-homology are marked by arrow-heads. Some of the molecules were sticking together before they were pulled apart in the cytochrome spreading (stars). (b) Heteroduplexes between the circular DNA of a recombinant plasmid containing the Euglena chloroplast DNA EcoRI fragment I of 5 kb cloned in the vector pEMBL8- and the strand complementary to the vector sequences from pEMBL8+. The substitution loops in the F1 region of the (+)- and (-)-pEMBL vectors are marked by stars. The ends of the vector duplexes are marked by arrow-heads. The insert sequences of the heteroduplex to the left have formed hybrids with spliced mRNA from Euglena chloroplasts. The 5'-end of the gene on the left side is not contained in the cloned fragment, therefore this end of the mRNA is seen as an unhybridized single-stranded tail. Two of the small intron loops were not opened to a circular form by the cytochrome spreading.

the positions marked by a star the ends of heteroduplexes had apparently been sticking together before they were separated by the spreading forces during the cytochrome preparation. This is most likely due to avidin molecules attached to one of the single strands at the end of the heteroduplexes which is close to the substitution loops.

Application to the analysis of DNA/RNA hybrids using the pEMBL vector system

Plasmid pEMBL- and other M13-derived recombinant phage DNAs are a good source of single strands which can be hybridized with RNA or DNA under conditions of low stringency. However, the circular structure of the recombinant phage DNAs makes it difficult to localize and orientate the ends of the cloned fragments in the electron microscope. To overcome this difficulty we used a marker heteroduplex with the vector part of the recombinant circular DNA. The pEMBL vectors were constructed in two versions (12). The (+)- and the (-)-series differ in the orientation of the F1(IG) fragment but are otherwise identical. If a single strand of a vector from the (+)-series is annealed with the complementary strand of the (-)-series a substitution loop at the position of the 1.2 kb inverted F1(IG) fragment is formed.

Using the strand separation technique described above linear single strands were prepared from the pEMBL vectors which are complementary to the vector sequence in the circular recombinant phage DNA. When a linear separated strand of a (-)-vector is annealed with the vector sequence of a recombinant phage DNA of the (+)-series (or vice versa) a substitution loop is formed at the position of the F1(IG) fragment while the insert sequences remain single-stranded, as indicated schematically in Fig. 4. Heteroduplexes of this type can be obtained in high yield. As an example heteroduplexes between a circular single strand from a pEMBL8- recombinant and a complementary vector strand from pEMBL8+ are shown in Fig. 3b. The ends of the insert sequences are marked by the ends of the homologous vector duplexes (Fig 3b, arrow-heads). The orientation of the insert can be deduced from the position of the asymmetrically located F1(IG) substitution loops in the heteroduplexes (Fig. 3b, stars).

Such heteroduplexes have been used for the analysis of RNA/DNA hybrids from wheat and Euglena chloroplasts (13; Koller, Clarke and Delius, in preparation). A chloroplast DNA fragment was cloned into the vector pEMBL8-. The circular recombinant phage DNA was annealed with the linear complementary strand of the vector pEMBL8+ and with chloroplast mRNA. The genomic chloroplast DNA formed several intron loops in the hybrid. The

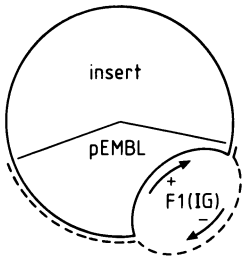


Figure 4. Schematic representation of the heteroduplex between a recombinant single-stranded DNA circle from the pEMBL(+)-series with a complementary single strand derived from a pEMBL(–) vector.

substitution loop in the vector provides an unambiguous reference for the 5'-3' orientation of the hybrid (Fig. 3b).

DISCUSSION

The end-labeling of DNA with biotin and the complexing with streptavidin-ferritin can be done with high efficiency. A high concentration of ferritin conjugate has to be used to saturate all biotin-labeled ends. Otherwise several biotinylated ends may be bound to the same streptavidin-ferritin complex.

At low ionic strength the complex formation is slowed down. Single-stranded biotinylated DNA could not be complexed efficiently (unpublished observations). At low ionic strength the effect may be the same as for double-stranded DNA. At high ionic strength the strong secondary structure probably lowers the accessibility of the biotinylated ends. Therefore, double-stranded rather than single-stranded DNA was bound to the avidin agarose columns. Alkaline denaturation of the DNA after the binding did not dissociate the complex and released only the non-biotinylated strand.

When avidin-agarose was used without the preceding wash procedure very little DNA was eluted from the columns. A control experiment (data not shown) in which free avidin was loaded onto a Sephadex column ahead of a DNA sample showed that the DNA was stuck on the column. It is therefore important that traces of free avidin should be washed out from the avidin agarose before preparing the affinity columns.

The terminal labeling of the restriction fragments was done either with bio-4-dUTP or bio-11-dUTP (10). The length of the linker had no obvious effect on the binding of the labeled DNA to the avidin agarose columns or on the complex formation with the streptavidin-ferritin conjugate. But it is possible that for internally labeled DNA the length of the linker is of

importance as was observed for in situ and for filter hybridizations (10,14).

Only DNA with protruding 5'-ends should be labeled by the Klenow enzyme. Therefore, it should theoretically be possible to prepare fragments for single-end labeling by double-digestion with two restriction enzymes: One producing 5'-protruding ends, and a second one producing blunt ends or 3'-protruding ends. But when double-digested SalI (5'-protruding) - PvuII (blunt end) fragments were labeled and used for strand separation the preparations had a much higher contamination with complementary strands.

Isolated single strands of fragments cloned into M13 or pEMBL vectors can easily be obtained in large quantities. But the circular structure of these recombinant DNAs presents two problems for their use in electron microscopy. First, they lack a reference marker for the orientation and position of the insert. This problem can be overcome by the method described here with the introduction of a substitution loop in the vector duplex. Second, the annealing of two circular structures is severely inhibited for topological reasons. This may prevent the annealing of the circular recombinant DNAs with other covalently closed circles or with sequences which are flanked by inverted repeats. In these cases the use of isolated linear single strands may improve the yield of heteroduplex structures substantially.

The method described can be universally used as long as two convenient restriction enzyme sites are available, one suited for the terminal labeling and the second for the production of subfragments.

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