

A METHOD FOR THE DETERMINATION OF STRANDEDNESS OF DNA FRAGMENTS CLONED IN PHAGE M13

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ABSTRACT

Recombinant M13Hol phage containing Eco RI restriction endonuclease fragments B, E, and F of adenovirus type 2 (Ad2) DNA were constructed by cloning into the unique Eco RI site of the replicative form of the phage M13Hol176 DNA. Polarity of the adenovirus inserts in recombinant molecules was deduced by the following procedures: Viral DNA fragments obtained from Ad2 DNA molecules were purified, denatured, and subjected to electrophoresis. The separated DNA strands were transferred from agarose to nitrocellulose by the Southern procedure and hybridized with radioactive 3'-end labeled Hae III fragments of the recombinant phage DNAs. This procedure provided a rapid test for assaying strandedness of the cloned fragments.

INTRODUCTION

Cloning DNA fragments in single stranded phage M13 is a useful procedure because single stranded DNA is very suitable for hybridization probing and nucleotide sequence determination. Since DNA fragments are cloned in the replicative double stranded phage DNA, the chimeric phage DNA in replicative form has both strands. The phage particles, however, only have one strand (+) depending upon the orientation of DNA fragments during cloning. The (+) strand of the resulting phage recombinants therefore contain complementary sequences of foreign DNA. Various procedures have been described to identify recombinants that carry inserted sequences in opposite orientation (Sambrook *et al.* 1989). The

recombinant DNAs are screened with single-stranded probes of known polarity, but it requires the prior knowledge of the polarity of the probe. The ability of the (+) strands of recombinants to hybridize to another and then analysis of the hybrid molecule is done by agarose gel electrophoresis where double-stranded DNA migrate slower through the gel than does single-stranded DNA or by S¹ nuclease mapping. However, these procedures require screening of large numbers of recombinant clones. The polarity of the hybrid cloned DNA is also determined by sequencing. This procedure requires sequencing of large number of clones, and the prior knowledge of the sequence of the inserted DNA is prerequisite. Thus, these procedures are costly, time-consuming, and complex for routine use, particularly when multiple restriction fragments are cloned in one single transformation assay. The objective of the current study is to develop an alternative method for the determination of orientation of DNA fragments cloned in M13. A general method for determining the strandedness of cloned sequences in M13 using adenovirus type 2 (Ad2) *Eco* R1 fragments (Sharp *et al.* 1974; Goldenberg *et al.* 1981) was developed as a model system.

MATERIALS AND METHODS

Eco R1 fragments B (4.4 Kb), E (2.3 Kb) and F (1.9 Kb) of Ad2 DNA cloned in M13Hol176 phage (Goldenberg *et al.* 1981; Bhaduri *et al.* 1980; Bhaduri and Fratamico 1992) were employed in this study. Viral DNA was digested with *Eco* R1. The resulting fragments were purified by gel electrophoresis and then subjected to strand separation by a modification of the procedure of Perlman and Huberman (1977). Gel slices containing each DNA fragment were incubated for 10 min in 0.3 M NaOH at room temperature. After equilibration in 0.015 M NaOH at 0C for 1.5 h, slices were sealed with agarose (50C) into a horizontal 1.4% agarose gel and electrophoresed at 4C at 175 volts for 8 h in 0.2 × buffer (E buffer; 40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, adjusted to pH 7.2 with glacial acetic acid) to separate DNA strands. Separated strands were transferred to nitrocellulose filter paper by the Southern procedure except for omission of the alkali denaturation and neutralization steps (Sambrook *et al.* 1989).

To assay strandedness of the cloned fragments of Ad2 DNA, the recombinant single-stranded phage DNA was digested with *Hae* III for 1 h at 37C as recommended by the supplier (Bethesda Research Laboratories, Rockville, MD). The reaction mixture was heated at 66C to inactivate the *Hae* III. An aliquot was diluted to a final DNA concentration of 1.6 µg/ml in a reaction mixture containing calf thymus terminal deoxynucleotidyl transferase and [α -³²P]-dATP (purchased from Pharmacia P-L Biochemicals, Piscataway, NJ) to label the 3'-end (Roychoudhury and Wu 1980).

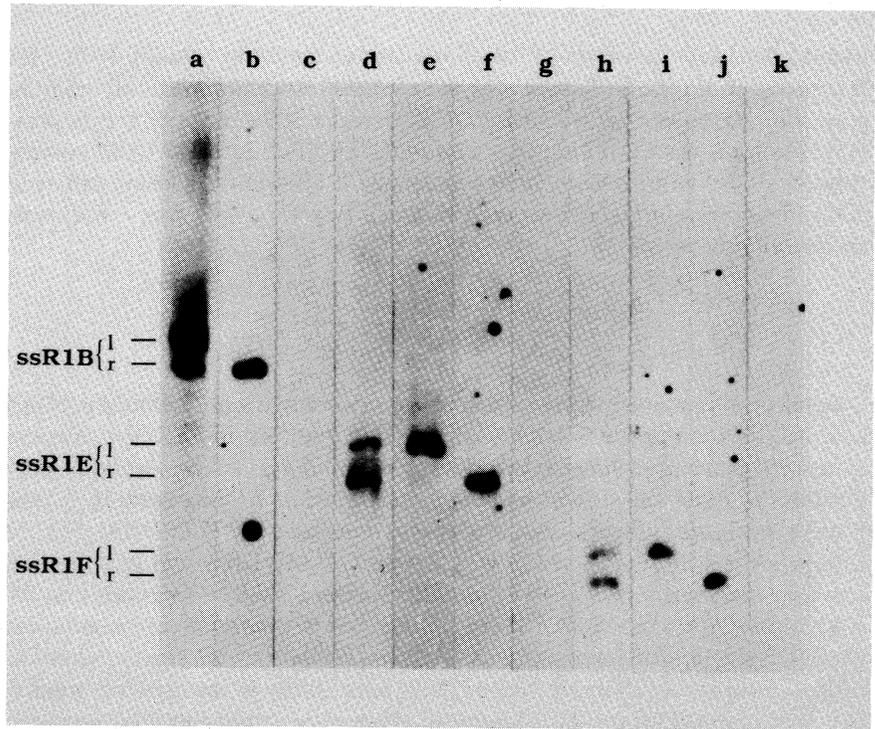


FIG. 1. HYBRIDIZATION ANALYSIS OF THE POLARITY OF CLONED ADENOVIRUS SEQUENCES

Southern blots of the separated strands of AD2 *Eco* R1 restriction fragments B, E, and F were hybridized with radioactive, end-labeled *Hae* III restriction fragments of recombinant phage DNA preparations. Poly dA tails were synthesized employing [α - 32 P]-dATP and terminal transferase. Strands were separated by alkaline denaturation of restriction fragments in agarose gels followed by horizontal agarose gel electrophoresis at 4C in low ionic strength buffer (modified from Perlman and Huberman 1977).

Strips in lanes a-c, d-g, and h-k are Southern blots containing the separated strands of *Eco* R1 fragments B, E, and F, respectively. Each strip contains approximately 6 μ g equivalents of DNA fragment. 3'-End labeled and nick-translated radioactive probes were as follows: lanes a, d, h: nick-translated Ad2 lanes c, g, k: M13Hol176 phage DNA; lane b: M13 R1 B (1) phage DNA (clone B-70); lane e: M13 R1 E (r) phage DNA (clone E-110); lane f: M13 R1 E (1) phage DNA (clone E-144); lane i: M13 R1 F (r) phage DNA (clone F-75); lane j: M13 R1 F (1) phage DNA (clone F-7).

The separated DNA strands on nitrocellulose strips were then hybridized with 3'-end labeled *Hae* III fragments of the recombinant phage DNAs as described by Sambrook *et al.* (1989). Nick-translated 32 P-labeled viral DNA (specific activity 1.7×10^7 cpm/ μ g) probe was prepared according to manufacturer's specification (Radiochemical Center, Amersham, Arlington Heights, IL) and was

used to determine positions of faster and slower migrating strands of *Eco* R1 fragments, B, E, and F. Both probes were used at concentration of 10^6 cpm/ml of hybridization mix. Single-stranded DNAs were not denatured prior to hybridization. Stringent washes were performed in $0.1 \times$ SSC, (SSC: 0.15 M sodium chloride, 0.015 M trisodium citrate) containing 0.1% sodium dodecyl sulfate at 50C. Filters were dried and exposed to X-ray film for 1 to 2 h at $-80C$ with an intensifying screen.

RESULTS

Results from a representative experiment are shown in Fig. 1. Previously, Sharp *et al.* (1974) showed that *Eco* R1 fragments B, E and F from Ad2 DNA migrate as doublet upon electrophoresis depending on which strand the fragment originated. Positions of faster and slower migrating strands of *Eco* R1 fragments B, E, and F were localized by hybridization with nick-translated ^{32}P -Ad2 DNA (Fig. 1; lanes a, d, and h). To determine which strand of Ad2 DNA was incorporated into the recombinant phage DNA, parallel Southern blots of separated *Eco* R1 strands were hybridized with ^{32}P end-labeled *Hae* III fragments of recombinant phage DNAs. Recombinant phage DNAs from 20 separate R1 B were screened (Fig. 1, lane b). No chimeric phage DNAs with inserts in the opposite orientation were detected for the R1 B fragment, although recombinants were prepared from three separate cloning experiments. Since the faster migrating strand of R1 B is from rightward viral strand (Sharp *et al.* 1974), all of these recombinant DNA contain viral DNA from the leftward strand. Seven recombinant phage DNAs from R1 E were tested for polarity. Four of them hybridized with the leftward strand of R1 E (Fig. 1, lane e). The remaining three R1 E clones hybridized with the rightward strand (Fig. 1, lane f). Of five R1 F clones tested, three recombinant phage DNA were hybridized to the slower migrating strand of R1 F (Fig. 1, lane i), while phage DNA from two other clones hybridized to the faster migrating strand (Fig. 1, lane j). As expected, no significant hybridization was observed when preparations of ^{32}P end-labeled, *Hae* III fragments of M13Holl76 phage DNA were annealed with the separated strands of *Eco* R1 fragments B, E, or F (Fig. 1, lanes c, g, and k, respectively).

DISCUSSION

This method for determining the strandedness of inserts is simple and reliable. The method is based on the observation that the slower migrating strand of AD2 *Eco* R1 fragments B, E, and F originate from the leftward (1) viral DNA strand

(Sharp *et al.* 1974). The polarities of adenovirus inserts in recombinant phage DNAs were assigned as follows: Chimeric phage that hybridized with the faster migrating strand must contain Ad2 sequences from the leftward (l) strand; conversely, recombinant phage that hybridized with the slower migrating strand must contain Ad2 sequences from the rightward (r) strand. Southern blots of separated DNA strands were hybridized to 3'-end-labeled *Hae* III fragments of single stranded recombinant phage DNAs. This method can be used for rapid characterization of clones in single-stranded phage vectors and permits the assignment of strandedness without knowledge of any restriction site in the cloned sequence. Further, it may prove especially useful for applications where determination of the polarity of the inserted sequence is not a prerequisite to the use of the clone.

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