Strand Displacement Applied to Assays with Nucleic Acid Probes

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This novel method for the detection of specific nucleic acid sequences has potential applications to clinical diagnosis. During hybridization, a signal-bearing nucleic acid strand is displaced by the target nucleic acid from a partially single-stranded complementary probe strand of nucleic acid. The probe:signal strand complex is prepared by hybridizing single-stranded probe that is entirely complementary to the target nucleic acid with a shorter signal sequence that is complementary to a portion of the probe strand. The sample nucleic acid is added to this hybrid complex under hybridization conditions. The target sequence, if present in the sample, will hybridize first to the unoccupied probe sequences, and then displace the labeled strand by branch migration. By this "strand displacement" the signal strands are freed in solution, where they may be separated from those still hybridized; the quantity of label measured is directly proportional to the amount of analyte sequences in the sample. This method, demonstrated here for model and synthetic DNA, can readily be adapted for the detection of any RNA or DNA sequence and obviates the need for immobilization of sample. A wide variety of labeling techniques can be used, and the displacement can be performed in solution or with the hybrid complex attached to a solid support. This assay circumvents nonspecific binding of label to the filter matrix and the laborious washing steps inherent in other assays involving nucleic acid probes.

Additional Keyphrases: detection of RNA and DNA sequences • nucleic acid hybridization

Nucleic acid hybridization is becoming more important for detecting nucleic acid sequences, particularly those of viral genomes causing infectious diseases and human sequences related to inherited disorders. Typically, these hybridization assays are performed by Southern blots (1) or so-called "dot blots" (2, 3), in which sample DNA is denatured and immobilized on nitrocellulose, and then subjected to hybridization with labeled probe DNA homologous to the sequence of interest. After extensive washing, label remains on the filter only where homologous sample was applied. A few alternative procedures exist, such as sandwich hybridization (4, 5), in which a labeled probe is linked to an immobilized nonhomologous strand of nucleic acid only in the presence of sample strand, which can bind to each of the two strands. In another method hydroxyapatite separates probe:analyte DNA hybrids from excess single-stranded labeled probe after solution hybridization. Radioisotopically labeled probe strands are commonly used in these procedures, although other labels have been described, notably the biotin-avidin–enzyme technique of Leary et al. (6).

Each of these techniques has shortcomings. Immobilization of sample is laborious, requires partially purified DNA, and sacrifices sensitivity, owing to the inaccessibility of the immobilized DNA and the decreased rate of hybridization. Multiple steps and repeated washing are required, and nonspecific binding of label to matrix causes problems. Currently available systems thus are generally inadequate for DNA hybridization to make the transition from research facilities to clinical laboratories, where time, technical experience, and sample material are often more limiting.

In an effort to circumvent many of these limitations, we have developed a novel nucleic acid assay based on the phenomenon of branch migration within partially duplex complexes. Competition between two strands for maximal pairing with a third, complementary strand results in the displacement of the less-stable strand from the duplex.

A labeled DNA signal strand, identical to part of the target analyte sequence, is hybridized to a longer unlabeled strand (probe) that is complementary to both the signal strand and the analyte sequence. This results in the formation of a hybrid complex (Figure 1) bearing a single-stranded initial binding region capable of hybridizing with the analyte sequence. The denatured sample DNA is subjected to hybridization conditions in the presence of the hybrid complex. Analyte sequence, if present, hybridizes with the single-stranded portion of the unlabeled complex strand, resulting in a branched structure (Figure 1b). This branch then migrates, displacing the signal strand, which is subsequently found free in solution and may be separated from the hybrid complex by several means. The amount of displaced signal strand is proportional to the amount of homologous sample DNA present.

Materials and Methods

Cloning and preparation of DNA. Plasmid pBR322 DNA was cleaved with restriction endonucleases PstI and BamHI and the resulting fragments cloned into M13mp18 and -mp19 viral DNA (Figure 2), according to standard techniques (7). Clones containing the 1.1 kilobase (kb) fragment of pBR322 were selected, and the plus strand was prepared from culture supernates from Escherichia coli strains, either JM101 or JM103 (7). Concentrations were determined by absorbance at 260 nm—as only a crude approximation, given the presence of other contaminating fragments. The 27mer oligonucleotides (27-PM and 27-MM, 27-nucleotide synthetic DNA sequences, the latter containing a guanine-to-cytosine transversion nine bases from the 5' end), derived from pBR322 (nucleotides 349–375), were kindly synthesized by Dr. G. Brown (Genetics Institute). The 50mer (pBR322 nucleotides 326–375), which is comple-

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Fig. 1. The use of branch migration and subsequent strand displacement in nucleic acid assays

Complementary strands are depicted as solid and broken lines, the label by a *. The probe strand, containing an initial binding region (IBR) and a region complementary to the shorter signal strand, is hybridized with labeled signal strand, resulting in a hybrid complex (a). When the sample is added, it anneals with the IBR, forming a branched structure (b), which is then resolved by branch migration into a duplex and a released signal strand (c). If the probe strand is immobilized, the signal strand will now become separated from the solid matrix and thus will be free in solution. In solution hybridization, a separation step must be performed to distinguish bound from unbound signal strands.

Fig. 2. Clones used as single-stranded DNA
M13mp19-PB1.1 and the 27-nucleotide oligomer constitute the hybrid complex, while M13mp18-PB1.1 (or a 50-mer containing the insert sequence adjacent to the BamHI site) mimics sample DNA. The thick bar fragment indicates the position on this construct of the complement to the 27mer whose sequence is shown. Because of the different polarities of the insert into M13mp18 and M13mp19, the plus strands derived from these phages are complementary for the length of the 1.1-kb insertion.

The ratio at which the quantity of free 27mer remaining after hybridization was minimized. The Gels. Nondenaturing polyacrylamide gels were prepared according to Maniatis et al. (9).

Results

We have prepared a model hybrid complex with isotopically labeled oligomer as signal strand and single-stranded M13 DNA as probe. A 2P-labeled 27-base oligomer (27-MM) was hybridized with the viral strand of M13mp19-PB1.1 (see Figure 2). The resulting hybrid complex contains a 27-nucleotide signal strand adjacent to an 1102-nucleotide initial binding region for analyte. The viral strand of M13mp19-PB1.1, which contains the complement to the pBR322-derived insert in M13mp19-PB1.1, was used to mimic sample DNA. This analyte is therefore able to hybridize to the entire 1.1-kb insert in M13mp19-1.1.

M13mp19-PB1.1 DNA was affixed to nitrocellulose filters, hybridized with 27-MM, and washed extensively. M13mp18 or M13mp18-PB1.1 was then introduced to the hybridization mixture, and samples of the supernate were monitored at various times. Dispersion of the signal strand from hybrid complex affixed to nitrocellulose is measured in Figure 3. Although some of the labeled complex is leached from the filters under conditions of hybridization, six times as much radioactivity (counts/minute) is released into the supernate in the presence of sample as in its absence. The addition of nonhomologous M13 DNA (containing no pBR322 sequences) caused no increase over background. These results indicate displacement of the signal strand specifically from the addition of target DNA, but also point out some of the shortcomings of nitrocellulose-immobilized DNA for hybridization: not all of the applied DNA is accessible to hybridization, and bound label is continuously leached (in the form of high-M, complexes, data not shown).

An alternative to hybridization of sample to immobilized hybrid complex is to perform the hybridization in solution, with subsequent separation of the bound and unbound labeled oligomers on the basis of their size difference. Hybrid complex was prepared and subdivided, sample was added under hybridization conditions, and a portion of each was analyzed by polyacrylamide gel electrophoresis and autoradiography. Results shown in Figure 4A, lane 8, demonstrate that the hybrid complex migrates as a large (83 kb) species with a slight background of unhybridized 27mer. After hybridization with homologous sample DNA, most of the labeled 27mer is displaced (e.g., lane 7). Hybridization with nonhomologous M13 DNA (lanes 9, 10, 13) does not displace the 27mer. Therefore, a simple gel electrophore-
Fig. 3. Displacement of 27-mer from immobilized hybrid complex by the target DNA in the sample
Nitrocellulose filters bearing 250-ng spots of M13mp19-PB1.1 were hybridized with 32P-labeled 27-mer 27-MM (7.2 x 10⁶ counts/min per µg). After being washed with 6 x SSC and 10 x Denhardt's solution, each spot contained about 2.3 x 10⁶ counts/min. Duplicate spots were separated and hybridized individually at 50°C in 1.25 ml of 6 x SSC plus 10 x Denhardt's solution in the presence of either no DNA (C), 2 µg of sample in M13mp18-PB1.1 (8), or nonhomologous M13mp18 (x). The radioactivity of 100-µL samples removed at various intervals was determined by liquid scintillation spectrometry.

sis suffices to determine the extent of displacement. Figure 4 also demonstrates that increasing the amounts of sample DNA results in increasing amounts of displaced signal strand. Panels A (lanes 2–7) and B show the dose-responsive displacement on hybridization with mp18-PB1.1, as do lanes 14–19 and panel C with a 50-nucleotide sample of the same polarity. The 50-mer is approximately 10 times as effective as the mp18-PB1.1 DNA in displacing the 27-mer, in part because the M13 DNA contains some contaminant that is not the nucleic acid of interest, but also probably because the large M13 circular strands place physical or topological constraints on strand displacement.

One of the features of clinical nucleic acid samples is that they contain nucleic acids other than those of the target organism, e.g., other organisms and patient genomic DNA. To ascertain whether excess nontarget DNAs would either cause spurious displacement or interfere with specific displacement, we examined the effect of simple and complex nonhomologous DNAs on displacement. Neither M13 nor denatured calf thymus DNA displaced the labeled oligomer (Figure 5, lanes 4 and 6), and their presence in excess did not inhibit the displacement from the targeted sample (compare lanes 2, 7 and 9). Hybridization assays that depend on hybridization of labeled probe to the sample DNA (i.e., all previously described assays) are subject to "false positives" from cross hybridization with partly homologous sequences—especially between oligomeric probes and complex DNA, or with sample DNA containing sequences related to probe vectors. Because the mechanism of strand displacement is fundamentally different from these approaches, requiring two steps of recognition (initial binding and branch migration), adventitious binding of the initial binding region to a related sample sequence need not lead to strand displacement and generation of a positive signal.

The use of oligomeric DNA as a hybrid constituent in strand displacement (although oligomers are not required, see Discussion) imposes constraints upon the hybridization conditions used for incubation. The rate of annealing of long sequences is greatest at about 25°C, below the melting temperature of the duplex, and decreases rapidly at lower temperatures (9). However, as the temperature used to anneal approaches the melting temperature of an oligomeric sequence, more of the oligomer spontaneously dissociates from the hybrid. The melting temperature of the 27-PM from M13mp19-PB1.1 in 2 x SSC solution was determined to be approximately 68–70°C (data not shown); when the M13mp19-PB1.1 had been immobilized on nitrocellulose, the oligomer melted off at lower temperatures and over a broader range. This difference may imply that all immobilized sequences are not equally accessible to hybridization.

In view of these temperature constraints, we assessed the rate and extent of displacement at different temperatures. Figure 6 shows that displacement proceeded to completion more rapidly at 50°C than at 40°C, and that the hybrid was still stable at the higher temperature (lane 9). The rate of annealing and displacement needs to be maximized to be...
useful for rapid diagnosis. We have found that enhancers of solution hybridization (10, 11) such as polyethylene glycol do accelerate displacement, presumably by accelerating annealing of sample and initial binding region (data not shown). In addition, recA protein from E. coli (12) accelerates displacement at 37 °C (manuscript in preparation and B. K. Davis, personal communication), which may be especially useful with enzymatic labels that do not tolerate high temperatures.

Discussion

As we have shown, branch migration and strand displacement can be utilized to determine the presence of targeted nucleic acid sequences. The amount of signal strand released in the unbound form is proportional to the amount of target nucleic acid in the sample. Because introduction of nontarget DNA does not displace the signal strand, displacement is thus specific for the sequence of interest, and is not inhibited by the presence of unrelated nucleic acids.

Results presented here were obtained by using a model system of M13-borne plasmid and synthetic sequences and radioisotopic labeling. However, the procedures are also applicable to nucleic acid sequences found in naturally occurring samples (manuscript in preparation), and the signal strand can carry various kinds of labels (unpublished results; see also 13).

The unique nature of nucleic acid sequences provides a
potentially highly discriminatory diagnostic tool for infectious agents and genetic disorders. At present, nucleic acid probes for species-specific sequences are widely used in clinical research laboratories and in a few commercial kits. Current technologies, however, are limited for use as a routine diagnostic procedure in the clinical laboratory by the level of technical skill required to perform the tests and their labor-intensive methodology and by the high background signals from nonspecific adsorption of label to solid matrix or from nonspecific hybridization between probe sequences and similar sequences present in the sample. The model system we describe eliminates, or significantly reduces, these deficiencies and is also amenable to various simple formats involving nonisotopic labels (13). It is applicable to both DNA and RNA analytes (data not shown) and can be adapted to detect any nucleic acid sequence by judicious design of the probe complex.

These features make strand displacement an attractive candidate for use in clinical laboratories. It is unnecessary to immobilize sample DNA, and less extensive purification may be required. Because the hybrid complex can be prepared in advance, only incubation with sample under hybridization conditions and the separation step are necessary to complete the assay, obviating several labor-intensive steps in a clinical setting. In addition, such assays are adaptable to automation. Figures 4–6 illustrate electrophoretic gel separation of free signal strand from the hybridized strands, but we have also accomplished such separation with small, rapid gel-filtration columns (data not shown).

A critical concept in this design is the process of branch migration, through which a branched tripartite structure is resolved into a duplex and a displaced single strand. Branch migration of forked structures has been known since Lee et al. (14) observed a disparity in the distribution of forked branches in renatured, terminally redundant, circularly permuted DNA. The phenomenon has been studied extensively in both linear (15) and circular structures (16), and plays a role in recombination and the behavior of replication forks in vivo as well as in various in vitro phenomena (see 17 for review).

To design optimal signal strands for use in diagnostic assays, one must know the rate-limiting factors of hybridization and branch migration. Studies of the rate of migration of branch points during the release of short single-stranded DNAs from nicked double-stranded circles (16) indicate that the time required to exchange one nucleoside for another is 12 μs. Large linear-branched structures were designed to investigate branch migration when initiated by reassociation of denatured complementary strands (15). The displaced strand (1.6 kb) had a lifetime in the branched structure of less than 10 s, and the displacement followed second-order kinetics, limited by the rate of renaturation of the two separated strands. This is consistent with our observation that the length of the signal strand does not seem to affect its displacement. We have examined displacement of oligomers, oligomers tailed with extensive tracts of nonhomologous homopolymer at the 3' end, and single-stranded DNA several hundred nucleotides long (unpublished data) and found no effect of length. Although increasing the temperature towards the hybridization optimum does accelerate displacement of the signal strand (see Figure 6), this is most likely due to more rapid initial pairing. Therefore, it is probably advantageous to use somewhat longer signal strands, which are not as subject to melting at optimal hybridization temperatures.

We also compared the displacement of oligomers that are perfectly matched to their complementary sequence with that of oligomers containing a single mismatch (i.e., at nine bases from the 5' terminus). There appears to be no difference in the rate of displacement by sample, although the mismatched oligomer is less stable and thus is more susceptible to melting. This is consistent with the expectation that displacement in our system is not limited by the rate of branch migration, but by association within the initial binding region.

The branched tripartite structure formed when the sample DNA "recognizes" the single-stranded initial binding region is resolved by branch migration to release a labeled single-stranded signal molecule. As discussed above, the release of the signal strand requires not only sufficient base-pair homology to cause hybridization of sample DNA to the initial binding region, but also the presence of adjacent sequences homologous to the signal strand to resolve the branched structure. Therefore, background caused by hybridization of related sequences (which may be significant in currently used methods) will be minimized.

We envision strand displacement as being useful for nucleic acid assays in two formats. The first involves an immobilized hybrid complex. Sample nucleic acids are introduced to this complex and, if the appropriate target sequences are present, they hybridize with the single-stranded initial binding region as depicted in Figure 1. After resolution by branch migration, the smaller signal strand is displaced into the surrounding solution. In this case, removal of the solution from the immobilizing matrix (e.g., nitrocellulose) effectively separates the bound signal strands from those that have been displaced, and assay of the solution allows quantification of the target sequences in the sample. Under some conditions it may be advantageous to perform the sample hybridization in solution; the attendant increase in speed would result from diffusion of both hybridizing species and possibly a more rapid stereochemical readjustment of polymer chains. The latter format requires a quick separation step after the hybridization and strand displacement. We have demonstrated the utility of size separation, using both gel electrophoresis and gel filtration, but affinity chromatography could also be used for rapid post-displacement separation of appropriately modified hybrid complexes from displaced signal strands. Affinity matrices are particularly advantageous for use with large nonisotopic labels, which might minimize the size difference used in preparing Figures 4–6, or when the size of the released strand is not greatly different from that of the hybrid complex. We have used avidin-agarose and poly(dG)-cellulose columns for this (data not shown).

Affinity chromatography may also be used to immobilize the hybrid complex itself, as an alternative to traditional means of immobilizing DNA (13). With immobilized hybrid complexes (e.g., Figure 3), nonspecific adsorption of the labeled probe strand (or of the label itself) to the solid matrix does not contribute to background signal, because results are read from the solution phase. Dot or Southern blots, however, do adsorb some enzymatic labels onto the filter surface, which interferes with measuring the label that is specifically bound.

Strand displacement provides a novel type of clinical diagnostic assay with potentially great flexibility in format and utilization. It is applicable to many types of analytes, and can easily be adapted to accommodate nonisotopic readout.

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References