Use of DNA Immobilized on Plastic and Agarose Supports to Detect DNA by Sandwich Hybridization

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Cloned Salmonella DNA, which has been immobilized irreversibly on plastic and agarose solid supports, can form hybrids in both single-layer and "sandwich" hybridization protocols. In single-layer hybridization, 3 μg of immobilized DNA bound at least 30 fmol of a specific 800-base DNA sequence (equivalent to 8.5 ng, or the amount of that sequence present in 4 × 1010 organisms). In a 4-h sandwich hybridization protocol, as little as 14 amol (equivalent to 8 pg, or the amount of that sequence present in 1 × 107 organisms) of a 1600-base sequence of DNA could be detected. The methods described should be applicable to use with any set of probes—not just from Salmonella—that fulfill the criteria specified. The ability to perform DNA hybridizations on solid-phase matrices such as those used for immunoassay should bring DNA hybridization into the realm of routine clinical laboratory procedures.

Additional Keyphrases: Salmonella DNA · single-layer DNA hybridization · tubes, beads, and open-ended receptacles as solid phase for hybridization

DNA hybridizations are typically done on filters—usually nitrocellulose—and are quite cumbersome as routine procedures. Such an assay usually involves loading a filter with sample, denaturing the sample to create single-stranded DNA (which will bind to the filter), fixing the sample to the filter, and saturating the remaining filter sites with homologous DNA to prevent the single-stranded probe from sticking to the filter. All of this must be done before performing the hybridization. Moreover, nitrocellulose filters, which seem to give the best results, are brittle and difficult to manipulate and tend to become clogged when used with crude (unpurified) samples.

We have tried to develop an assay that avoids these drawbacks by using new solid-phase supports for performing "sandwich" hybridization of DNA; our aim was to bring assays involving DNA hybridization into the realm of routine laboratory procedures. Although we present data only for assays based on use of radioactive probes, the technology is equally well suited for use with non-radioactive probes.

Sandwich DNA hybridizations are not new. Dunn and Hassell (1) first used DNA sandwich hybridization to demonstrate that distal domains in the adenosine-A genome can become proximal in a mature RNA. Use of sandwich hybridization for signal amplification is summarized by Meinkoth and Wahl (2). The first diagnostic application of sandwich hybridization was by Ranki et al. (3, 4) and Virtanen et al. (5), in detecting as little as 8 amol of adenovirus DNA on filters.

Although we have not evaluated our model system with crude samples, Ranki et al. (3, 4), Virtanen et al. (5), and Palva (6) have reported that sandwich hybridization of crude samples on filters is characterized by low nonspecific binding. The solid phases we describe make the handling and the washing subsequent to the hybridization much easier than with nitrocellulose filters. In short, with the system we describe, DNA hybridization can now be done on the kinds of matrices that have been in routine use in immunoassays for many years. In fact, the competitive and sandwich methodologies used are essentially the hybridization analogs of competitive radioimmunoassays and of immunoradiometric assays, respectively.

To perform a solid-phase sandwich hybridization assay (see Figure 1), one needs a radioactive probe. The probe sequence must, however, be present in the target and proximal to the specific sequence of DNA immobilized on the solid support. In DNA sandwich hybridization, the radioactive probe will be bound to the solid phase only when the target is present to form the complementary bridge to which both the solid-phase DNA and the radioactive probe
can hybridize. It follows that, in the absence of target, no radioactivity should be bound. Therefore, in the presence of target, the amount of radioactivity bound to the solid-phase is a direct function of the concentration of target present in the system. It is advantageous that the immobilized sequence and the probe sequence be contiguous, to ensure that random breaks in the target strand will have negligible effects on sandwich hybridization. Use of nonoverlapping sequences ensures that hybridization of one sequence does not inhibit hybridization with the other. We chose the sequences used in this work specifically to fulfill these criteria.

Materials and Methods

DNA Reagents

Plasmid DNAs were prepared from 2- to 10-L cultures by the method of Birnboim and Doly (7) as modified by Ish-Horowicz and Burke (8). The characterization of RF321 (plasmid 321, a 4900-base-pair (bp) sequence of Salmonella DNA contained within an 11 000-bp plasmid vector and isolated from a genomic library of Salmonella typhimurium) has been described by Fitts et al. (9). The Salmonella portion of RF321 can be digested by the restriction endonucleases BamHI, HindIII, and EcoRI into three sequential fragments: B2, an 800-bp BamHI/HindIII fragment; A10, a 1600-bp HindIII/EcoRI fragment; and A9, a 2500-bp EcoRI/BamHI fragment. We used only B2 and A10 in these experiments. Plasmid B2, a subclone of RF321, consists of the BamHI/HindIII B2 fragment of RF321 in the BamHI/HindIII site of pBR322. A10 phase and B2 phase were prepared by separately subcloning the B2 and A10 fragments by standard means (10) into a recombinant filamentous phage fd (8500 bp). Hybridization showed that B2 and A10 phase were from the same strand of RF321. Plasmid B2 was digested with EcoRI under conditions recommended by the manufacturer of the enzyme (New England Biolabs, Beverly, MA).

We labeled EcoRI-digested plasmid B2 with $^{32}$P by using the large fragment of DNA polymerase I "Klenow fragment" (New England Biolabs), as described by Maniatis et al. (10), to a specific activity of $2.2 \times 10^{8}$ dpm/μg. Plasmid B2 was radiolabeled in vivo, essentially as described by Croes and Falkow (11), to a specific activity of $5.2 \times 10^{9}$ dpm/μg by using $^{32}$P-thymidine (New England Nuclear, N. Billerica, MA). We prepared the radioactive probes from A10 phase and B2 phase as described by Hu and Mesang (12), but using the sequencing primer rather than the probe primer. In both cases, this produced probes (gamma A10 and gamma B2) that were the complements of the respective cloned sequences. After synthesis, we released the radiolabeled product from the template by denaturation and subsequently purified it by gel filtration. Specific activities of gamma probes were approximately $3.5 \times 10^{6}$ dpm/μg. $^{32}$PdATP was purchased from Amersham, Arlington Heights, IL.

Solid Supports

CNBr-activated Sepharose-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ), 12 x 75 mm polypropylene test tubes, and polypropylene solid-phase receptacles (SPRs, described below) were coated with protein–DNA complexes by well-known methods (13, 14) 8

The DNA used for immobilization was digested plasmid B2 (4600 bp). After coating, all solid-phase matrices were washed repeatedly at high temperature. Tubes and SPRs were then dried and stored at room temperature until used. CNBr beads were stored wet at 4 °C until used. SPRs were regenerated by boiling for 15 min in twofold concentrated SSC buffer.

SPRs, which resemble pipet tips that are closed on the top, were designed to function in the KinetiCount 48™ immunoassay system (15) (Medical & Scientific Designs, Inc., Rockland, MA), which cyclically increases and decreases the pressure around them. SPRs, used as solid-phase matrices, are coated on the lower inner surfaces with the material to be immobilized. For the purposes of this study, we coated each SPR with 10 μg of digested B2 plasmid, of which 3 μg remained attached irreversibly. Incubations in SPRs were done in sequential pressure cycles—2 min in which surrounding air pressure forced 0.5 mL of reaction mixture into the SPR, followed by a 5-s release of pressure to allow the liquid to drain from the SPR into the tube below— for various numbers of cycles. These hybridizations were performed at 65 °C, far above the recommended operating temperature of the current model of the KinetiCount 48. Thus, we used a specially adapted temperature-controlled pumping chamber.

Hybridization

In the single-layer experiments examining the capacity of the solid phase for B2 sequence and in competitive assays, we used the $^{32}$P-labeled complement of the B2 sequence (gamma B2) as the probe. When used, targets were either plasmid 321 or the single-stranded B2 phase.

Competitive assays were performed sequentially. We added a small but constant amount of radioactive probe (30 or 100 ng) to the target DNA to be measured, and incubated under conditions that allowed the target and probe to hybridize maximally in the absence of solid phase. We then added the mixture to the solid-phase DNA, which was used essentially as a specific adsorbent for unbound probe (the probe that has not hybridized to the target in solution is free to bind to the solid phase). In the presence of competitor, the amount of radioactivity bound is inversely proportional to the amount of competitor present.

Sandwich hybridization was performed as described in the introduction, with $^{32}$P-labeled gamma A10 probe in solution. For sandwich assays, we added target and probe to the solid-phase DNA simultaneously. For competitive assays, target and probe were pre-incubated together for 30 min, unless otherwise specified, before addition to the solid-phase DNA.

DNA used for immobilization was denatured by boiling for 15 min in distilled water, then quickly chilled and maintained at 0 °C until used. Hybridization was always preceded by a 30-min prehybridization at 65 °C in fivefold concentrated SSC, fivefold concentrated Denhardt's solution (unconcentrated Denhardt's contains, per liter, 200 mg each of bovine serum albumin, polyvinylpyrrolidone, and ficoll) (16), and sodium dodecyl sulfate (SDS), 1 g/L. For the experiments with SPRs, we also included 500 mg of heparin per liter (17). Hybridizations were performed in fivefold concentrated SSC, Denhardt's solution, and SDS, 1 g/L, in either the presence or absence of dextran sulfate (100 g/L). Incubation volumes were 0.4 mL for beads, 0.5 mL for SPRs, and 1.0 mL for tubes. After incubation, we washed the solid phases with three 10-min washes at 65 °C. The first two washes were in twofold concentrated SSC plus SDS, 1 g/L, and the last wash in 10-fold diluted SSC plus SDS. To quantify our results, we used either a scintillation counter, to measure light emitted by either scintillation fluid or by Cerenkov radiation (18), or the KinetiCount 48.
gamma counter (validation of this method to be described in a future communication). Comparisons of the various solid supports have been drawn from various separate experiments originally designed to look only at capacity to support hybridization.

Results

We coated SPRs, tubes, and beads with plasmid B2 DNA to compare the capacities of the various solid phases. Figure 2 shows that all three solid-phase media can irreversibly bind similar proportions of the added DNA. Using very high DNA concentrations to coat tubes indicated that above 17 μg of DNA per tube the amount that binds to the solid phase decreases drastically.

The hybridization capacity of such solid phases is illustrated in Figure 3. SPR-immobilized plasmid B2 was annealed with various concentrations of labeled gamma B2 (described in Materials and Methods) for increasing times. In addition, we evaluated the effect of dextran sulfate, a reported hybridization rate enhancer (4, 19), on the hybridization of these novel DNA supports. Figure 3 shows a linear relationship between the initial concentration of gamma B2 probe and the amount hybridized. Moreover, the capacity of the SPR to support hybridization was not exceeded even at the highest concentration of target, 63 fmol (16.5 ng). This amount of target is equivalent to the amount of specific sequence that would be found in 4 × 10^10 organisms, far in excess of the amount of DNA to which a test such as this would ever be exposed. In the absence of dextran sulfate, we believe the reaction is almost complete by 20 h, there being little difference between the extent of hybridization observed for the 20- and 40-h incubations. In the presence of dextran sulfate, hybridization was more than double that which would have occurred in 40 h in its absence.

Examining the capacities of SPRs onto which different amounts of single-stranded DNA were immobilized as well as of regenerated and unpressurized (static) SPRs leads us to several conclusions (see Figure 4). First, the more DNA immobilized on the SPR, the larger the percentage of probe bound. Also, with one-third (1.3 μg) of the usual concentration of DNA on SPRs, the percentage of probe bound begins to decrease at the highest concentration of probe added, suggesting that the capacity of the SPR is being approached. After regeneration, the SPRs lost about 30% of their original capacity. Given that the SPRs used in this experiment actually had been through two regeneration cycles before this experiment, a 30% loss in capacity is a worst-case estimate for a single regeneration cycle. The resistance of the immobilized DNA to denaturing conditions demonstrates the stability of the linkage of the DNA to the SPR. The effect of pressurized pumping of sample into the SPR was to increase hybridization by approximately 30%. Al-

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**Fig. 2.** Comparison of capacities of beads (●), tubes (X), and SPRs (Δ) for immobilized DNA

Polyethylene tubes, SPRs, and CNBr-activated Sepharose 4B beads were exposed to the amounts of DNA indicated on the abscissa. After four washes at 65°C, the amounts of DNA bound to each were measured (ordinate). Tubes and SPRs were labeled with tritiated plasmid B2 added during the coating to follow the percentage of input immobilized. 32P-labeled B2 plasmid was used to trace the amount of DNA immobilized on beads. Volumes and surface areas were: 10 μL/μL of packed beads (external surface area = 0.65 cm², internal surface area known but unknown); 1 mL per test tube (4 cm²); 0.5 mL per SPR (4.7 cm²). The amount of radioactive plasmid B2 added in the various experiments are as follows: test tubes, experiment 1 (X), all plasmid added as 3H-labeled plasmid B2; experiment 2 (C), 0.18 μg of 3H-labeled plasmid B2 added per tube, 0.27 μg of 3H-labeled plasmid B2 per SPR (Δ), and 0.16 μg of 32P-labeled plasmid B2 per 50 μL of packed beads (●)

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**Fig. 3.** Comparison of the effect of dextran sulfate on binding of 32P-labeled gamma B2 probe with increased time of incubation

The incubations in the presence and the absence of 100 μg/mL dextran sulfate were performed in two separate experiments: 3 h (C), 20 h (X), and 40 h (○). In the absence of dextran sulfate; 4 h (●) in the presence of dextran sulfate

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**Fig. 4.** Results for 4-h incubations in SPRs in the presence of 100 μg/mL dextran sulfate and the following amounts of plasmid B2 DNA: (●), 3 μg; (○), 1.3 μg; (□), 3 μg after two regenerations of SPR; (X), 3 μg after two regenerations of SPR, without pressurized pumping cycle
though the data presented here are only for static (unpumped) incubation of regenerated SPRs, similar experiments indicate the same extent of improvement for fresh SPRs in both single-layer and sandwich hybridizations.

Table 1 summarizes the results of a sandwich hybridization performed with SPRs onto which 3 μg of DNA had been immobilized. Hybridization was in either the presence or the absence of 150 ng of heterologous (calf thymus) DNA per SPR. (This amount was chosen as being equivalent in mass to the highest concentration of target used in the hybridization.) As shown, our prototype sandwich assay can detect adequate signal from as little as of 14 amol of target (equivalent to DNA from 1 x 10⁷ organisms) either in the presence or the absence of heterologous DNA. Although the presence of heterologous DNA does dampen the signal, it does not change the sensitivity of the assay. Sandwich hybridization of regenerated SPRs in the presence of calf thymus DNA gave approximately 25% less hybrid formation than did fresh SPRs. However, as shown in Table 1, the target is detected at least quantitatively at low concentrations (see Discussion), so the sensitivity of the assay is really determined by the amount of nonspecific binding. Separate observations (unpublished) indicate that nonspecific binding is a direct function of the quality of the labeled probe used.

The results of two competitive assays are presented in Figure 5. For these assays both a double-stranded (321 plasmid) and a single-stranded (B2 phage) target were used. With single-stranded or double-stranded target DNAs, the sensitivity of the competitive assay was only 10% or 1%, respectively, of that of the sandwich assay.

Discussion

Both single-layer and sandwich hybridizations can be performed with use of DNA immobilized onto plastic surfaces. Using SPRs to perform 4-h sandwich hybridizations in dextran sulfate, we have detected as little as 14 amol (4 pg of sequence B2 or 8 pg of sequence A10) of target.

The upper limit (i.e., 17 μg per tube according to Figure 2) to the amount of DNA that can be immobilized on a surface is probably determined by the fact that, at high concentrations, DNA forms networks so that only a small proportion of the added DNA is sterically available for immobilization. Single-layer hybridization experiments with agarose beads show that the more densely a surface is coated with DNA, the lower the percentage of the DNA that is hybridizable, probably because of both crowding and self-hybridization (data not shown). Fortunately, as little as 3 μg of DNA on a SPR has the capacity to hybridize with more of a specific sequence than is present in 4 x 10⁹ organisms.

Although the capacities of test tubes and beads for DNA are equal to or better than that of SPRs, our preliminary experiments show that SPRs present several advantages when used for hybridization assays. For example, the surface-to-volume ratio for SPRs is more than twice that for tubes (4.7 cm² for a 0.5-mL SPR vs 2.0 cm² for a 0.5-mL tube); the DNA immobilized onto SPRs does not seem to continue to bleed off during repeated washes (as it can from beads, data not shown); the pressurized pumping action for which SPRs were designed accelerates the rate of hybridization; and the SPR design facilitates washing.

The use of dextran sulfate in our system increases the rate of both single-layer and sandwich hybridizations by seven- to 10-fold in 3- to 4-h incubations, and facilitates hybrid formation to quantities not attainable in even 40 h in its

Table 1. Sandwich Hybridization

<table>
<thead>
<tr>
<th>Target added, amol</th>
<th>Heterologous DNA a</th>
<th>SPR pretreatment b</th>
<th>Probe c bound</th>
<th>% of maximum possible binding</th>
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<td>-</td>
<td>N</td>
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<td>31</td>
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<tr>
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<td>-</td>
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<td>N</td>
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<td>14</td>
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<td>N</td>
<td>0.51</td>
<td>19</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>N</td>
<td>0.31 d</td>
<td>136</td>
</tr>
<tr>
<td>14000</td>
<td>+</td>
<td>R</td>
<td>2.0</td>
<td>184</td>
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<tr>
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<td>+</td>
<td>R</td>
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<td>R</td>
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<td>0</td>
<td>+</td>
<td>R</td>
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<td>100</td>
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</table>

a 150 ng of calf thymus DNA present (+) or absent (−). b N, no pretreatment (fresh SPRs); R, regenerated SPRs. c 9450 amol (5 ng) of 32P-labeled γ-A10 probe added. d Nonspecific binding.
absence. In contrast, Ranki et al. (3) reported no enhancement in hybrid formation by dextran sulfate on 17-h sandwich incubations on filters. Because Ranki et al. presumably looked at end-point rather than rate experiments, and because dextran sulfate affects the rate, not the extent, of reaction, both results are probably correct. Our results are similar to those reported by Wahl et al. (19) for two-phase hybridizations on DNA paper strips by using nick-translation probes. The major effect of dextran sulfate is the exclusion of the nucleic acid from the hydration volume of the polymer, effectively increasing the nucleic acid concentration (20). Generally, in the presence of dextran sulfate, the rate of hybridization of a probe with membrane-bound DNA is increased three- to fourfold. In addition, for double-stranded probes, dextran sulfate increases the hybridization signal more than fourfold.

Wahl et al. (19) hypothesize that the hypermolar hybridization results from network formation by double-stranded probe in solution. However, we are using single-stranded probes, which should not be able to form networks as effectively, but the rate increase is substantially greater than three- to fourfold. Perhaps the probe we used was contaminated with fragments of the phage from which it was prepared (and to which it is complementary) by primer extension. Consistent with this possibility is the finding (unpublished observations) that heat denaturation of the probe before hybridization increases the hybridization signal—which is unexpected for single-stranded probes. The presence of such complementary pieces could account for network formation and could, in fact, account for the greater-than-theoretical amount of hybridization we see at low concentrations of target in sandwich assays. Nick-translation probes have been used in a similar manner for signal amplification in the past (2). The implications of this hypothesis are that addition of linearized or damaged phage B2 should increase signal (possibly resulting in a more sensitive assay), whereas the use of "cleaner" probe should decrease signal in the presence of dextran sulfate. Nonspecific binding has varied from 0.02% to 0.3% as a function of probe preparation (unpublished observations). Conceivably, high nonspecific binding may also be a function of networking and the purity of the probe. The significantly lower signal in the presence of calf thymus DNA may result from the interference of heterologous DNA with the networking process, by acting as a competitive inhibitor of hybrid formation (21).

The prototype sandwich assay that we have described is quantitative over the range of target concentrations tested. The fact that signal is decreased in the presence of heterologous DNA could affect the limit of sensitivity of the assay for quantitative detection of signal, should the extent of signal depression be a function of the concentration of heterologous DNA added. We expect, however, that this is a threshold phenomenon. If so, once a certain concentration of heterologous DNA is reached, the signal generated in its presence will become independent of its presence. The results of Ranki et al. (3, 4), investigating sandwich hybridization on filters, strongly suggest that assays of this sort are quantitative in the presence of excess heterologous DNA and crude sample extracts. In fact, they used 500-fold more heterologous DNA in their quantitative assays than we added to our system, and their specific binding, like ours, was decreased by 30% in the presence of the heterologous DNA.

The attachment of the DNA to the solid phase is quite stable, as demonstrated by the fact that the DNA retains its ability to hybridize, even after repeated regeneration cycles. These results do not imply that the solid phase is reusable—our best results are obtained with fresh SPR—but indicate the firmness of the binding of the DNA to the solid phase, which affects the sensitivity of the sandwich assay. The loss of even a very small percentage of the bound DNA would lead to a solution-phase competition with the solid phase DNA, with a concomitant decrease in the sensitivity of the assay.

Sandwich assays are typically more sensitive than competitive assays, both because all components except target are present in excess to drive the reaction and because signal is detected as an increase over a small amount of nonspecific binding, rather than as a decrease in specific binding. However, we wanted to determine the limit of detection (sensitivity) of a competitive assay with our system. Our preliminary studies indicate that competitive DNA assays are significantly less sensitive than sandwich assays, especially for double-stranded DNA targets. The limit of detection of single-stranded target was approximately \(7 \times 10^3\) organisms (30 pg or 100 amol) when 30 pg of radioactive probe was used. Detection of double-stranded target, however, resulted in both a low-dose "hook" phenomenon and a sensitivity of only \(7 \times 10^3\) organisms. These phenomena, which may be related, may result from the networking of the target with immobilized plasmid strands of both senses. As the target concentration increases at low concentrations, its availability for formation of networks increases, thereby increasing its ability to bind the probe. Because overall target concentration is still low, however, it is very likely to be bound to the solid phase as a network. As target concentration increases even further, the networks become larger and more plentiful, but less densely labeled. Thus, the amount of probe bound to the solid phase finally begins to decrease. If correct, this hypothesis suggests that competitive assays will not provide a sensitive means for measurement of double-stranded DNA.

In conclusion, we have demonstrated "sandwich" DNA hybridization on novel solid-phase supports in a model system. The assay format described should be adaptable for routine use in clinical laboratories, because it readily lends itself to automation and has adequate sensitivity to detect \(1 \times 10^6\) organisms in less than 5 h. The methodology should be applicable for use with any appropriate set of DNA probes, as well as with isotopic and nonisotopic signal detection.

References


