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DNA exhibits multi-stranded binding recognition on glass microarrays

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ABSTRACT

In the course of exploring the hybridization properties of glass DNA microarrays, multi-stranded DNA structures were observed that could not be accounted for by classical Watson–Crick base pairing. Non-denatured double-stranded DNA array elements were shown to hybridize to single-stranded (ss)DNA probes. Similarly, ssDNA array elements were shown to bind duplex DNA probes. This led to a series of experiments demonstrating the formation of multi-stranded DNA structures on the surface of microarrays. These structures were observed with a number of heterogeneous sequences, including both purine and pyrimidine bases, with shared sequence identity between the ssDNA and one of the duplex strands. Furthermore, we observed a strong binding preference near the ends of duplexes containing a 3′-homologous strand. We suggest that such binding interactions on cationic solid surfaces could serve as a model for a number of biological processes mediated through multi-stranded DNA.

INTRODUCTION

Glass cDNA microarrays are widely used to profile thousands of expressed gene sequences simultaneously by hybridization. In a typical production process, cDNA clones are amplified by PCR and then spotted onto chemically modified glass slides (1,2). In early applications, efforts were made to covalently attach the (+) strand and remove the (–) strand from the surface by washing under denaturing conditions (3). More recently, we have concluded that such denaturation steps are unnecessary, since spotted double-stranded (ds)DNA microarrays exhibit good sensitivity and reproducibility without denaturation (4). Nevertheless, it is generally assumed that hybridization to cDNA microarrays occurs by Watson–Crick base recognition. The underlying assumption is that the spotted dsDNA duplexes become partially denatured upon binding to the surface, thereby promoting base recognition with single-stranded hybridization probes. Another possibility (although heretofore unrecognized) is multi-stranded base recognition, as is known to occur in solution during the process of homologous recombination. Such binding interactions would potentially require only partial unwinding of the arrayed duplexes rather than complete denaturation.

Most biochemical models of homologous recombination involve single-stranded (ss)DNA and linear dsDNA in the presence of ATP and DNA-binding proteins. In the presence of ATP-γ-S, a non-hydrolyzable analog of ATP, RecA protein associates with and lengthens dsDNA by a factor of ~1.5 (5). In many protein–DNA complexes, the double helix is unwound and distorted from the classical Watson–Crick B-form (6,7). Despite the views provided from classical molecular models, DNA itself is a highly elastic molecule (8). The double helix under external tension exhibits a transition to a stretched state that is 1.7 times longer than its 'relaxed' B-DNA form (8,9). These and similar studies suggest that DNA is capable of forming multi-stranded structures when subjected to an external force, such as binding to a solid surface.

Here we present a series of hybridization experiments demonstrating the formation of multi-stranded DNA structures on chemically modified glass surfaces. These structures show a strong preference for single strands associating with homologous 3′-strands near the ends of arrayed dsDNA elements and *vice versa*. We propose that such binding interactions could serve as a model for a variety of biological processes involving multi-stranded DNA, including homologous recombination.

MATERIALS AND METHODS

Glass substrates

Aminopropylsilylated glass slides were produced at the Incyte Genomics Microarray Facility (Fremont, CA). These slides were reacted with cyanuric chloride to promote attachment of both synthetic polynucleotides and longer PCR-amplified dsDNA (P.L.Lee *et al*., manuscript in preparation). Briefly, the aminopropylsilanated glass slides were immersed in a stirred slurry of cyanuric chloride (12.7 g) (Aldrich, Milwaukee, WI) and sodium carbonate (25 g) in *n*-hexane (1 l) at 4°C for 1 h. The slides were then rinsed with *n*-hexane in an ultrasonic bath and air-dried. FTIR was performed on a Nexus Model 470 instrument to confirm coating compositions (Nicolet Instrument Corp.).

Synthetic oligonucleotides

Synthetic oligonucleotides were modified with either a 5′-alkylamino linker for microarraying or 5′-fluorescent dyes (cyanine-3 and cyanine-5) for use as hybridization probes (Operon Technologies, Alameda, CA). They were each HPLC purified and ranged from 30 to 99 bases in length (see Fig. 1).

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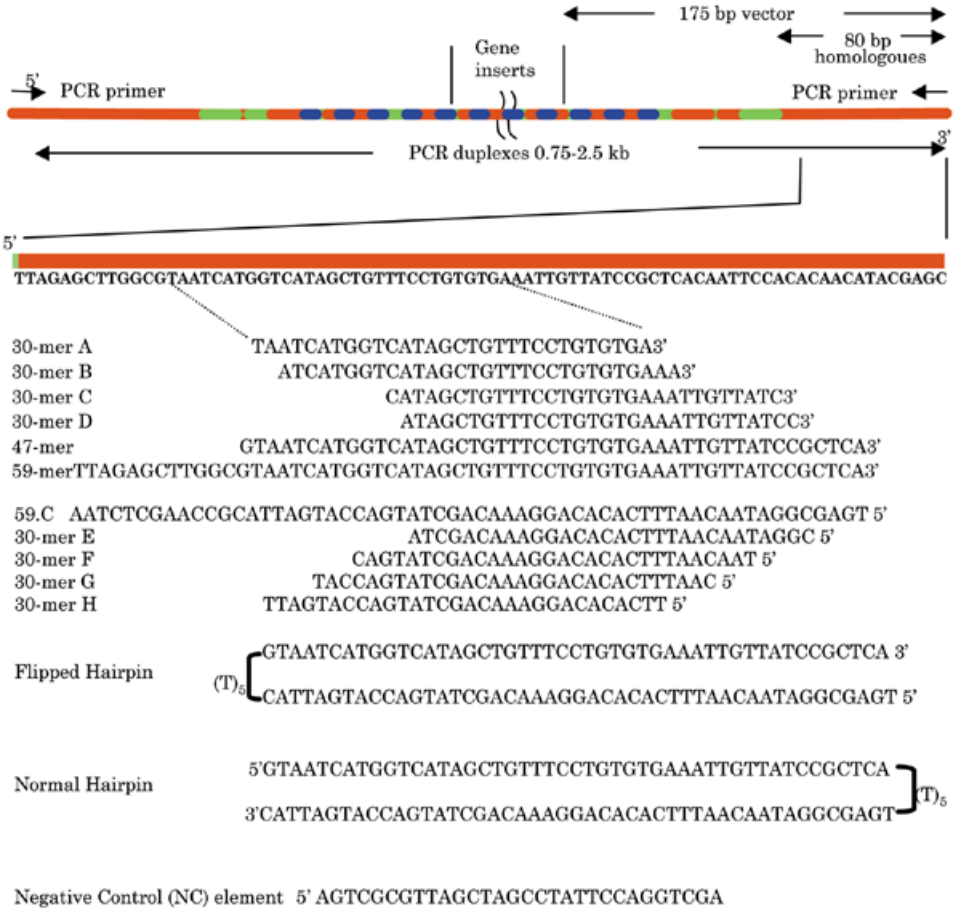

Figure 1. Alignments of the vector sequence of 3′-end PCR fragments relative to the array elements and synthetic probes.

Stock solutions for microarraying were prepared in 2.5× SSC buffer at a final concentration of 25 µM.

PCR-amplified dsDNA

Human cDNA clones containing ~750 bp–2.5 kb expressed gene inserts (Incyte Genomics) were PCR amplified as described previously (9). This was done using unmodified vector primers SK536 (5′-GCGAAAGGGGGATGTGCTG-3′) and SK865 (5′-GCTCGTATGTTGTGTGGAA-3′) (Operon Technologies, Alameda, CA). The resulting purified PCR products were suspended in 2.5× SSC buffer for microarraying at a final concentration of 100 ng/µl.

Microarray fabrication

Synthetic polynucleotides and PCR-amplified dsDNA reagents were spotted onto chemically modified glass slides (see above) at 175 µm spacing (center to center) using a micro-capillary tip robotic arrayer (Incyte Genomics, Fremont, CA). The microarray slides were rinsed in 0.2% SDS for 2 min followed by three 1 min rinses in deionized water. Next, they were treated with 0.2% I-Block reagent (Tropix, Bedford, MA) in 1× Dulbecco's phosphate-buffered saline (Life Technologies, Gaithersburg, MD) at 60°C for 30 min. After treatment with I-Block reagent, the slides were washed again at room temperature with 0.2% SDS and water as described above.

Fluorescent labeling of PCR-amplified dsDNA for hybridization experiments

Nick translated (NT) hybridization probes were generated from a pool of 576 individual PCR products (each containing common 3′- and 5′-vector sequences; see Fig. 1). A nick translation kit (Promega, Madison, WI) was used according to the manufacturer's protocol except that Cy-3-labeled dUTP (Amersham Pharmacia Biotech) was included in the labeling reaction (1:1 molar ratio with unlabeled dUTP) and reaction times were varied from 1 to 72 h (see Results). All NT probes were purified by ethanol precipitation. The resulting labeled NT probes were compared against the original pool of PCR products by agarose gel electrophoresis using ethidium bromide staining. A 1 h nick translation reaction showed no measurable difference in size distribution, whereas longer reaction times showed evidence of shorter fragments.

Hybridization reactions

Fluorescently labeled oligonucleotides (1–10 ng/reaction) and NT probes (50–100 ng/reaction) were each suspended in 20 µl of hybridization buffer (5× SSC, 0.2% SDS, 1 mM DTT) and applied to microarrays under 22 × 22 mm raised coverslips (Incyte Genomics). The microarrays were then placed in a sealed chamber to prevent evaporation and incubated at 60°C

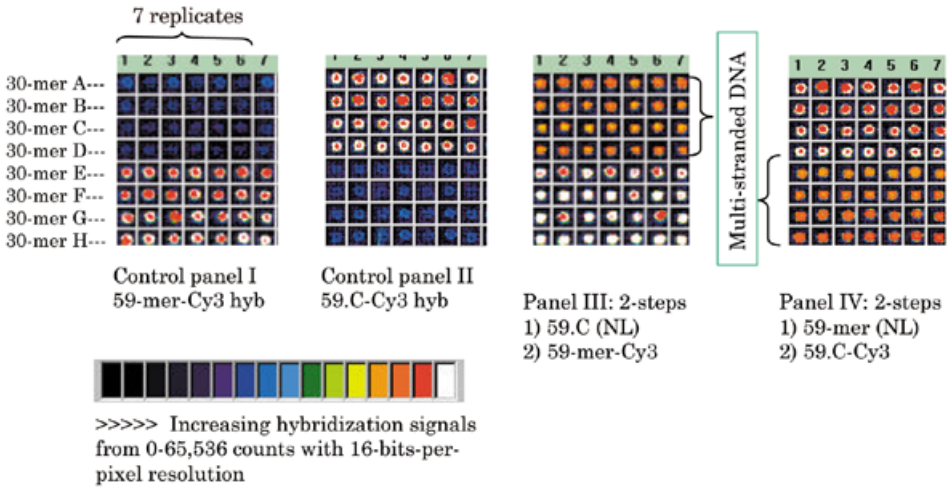

Figure 2. Demonstration of the stepwise formation of multi-stranded structures on the surface of the DNA microarrays. (**I**) Conventional Watson–Crick hybridization of a Cy3-labeled 59mer probe to elements E–H. (**II**) A Cy3-labeled 59.C probe hybridizes only to elements A–D. (**III**) Results of two sequential hybridizations. First, a non-labeled (NL) 59.C probe (10 ng) was hybridized under the same conditions as those used in (I) and after washing and drying the arrays, they were hybridized with a Cy3-labeled 59mer (10 ng). (**IV**) As (III) except that the probes were reversed, with a non-labeled (NL) 59mer being used in the first step and a Cy3-labeled 59.C probe being used in the second step.

for 6 h. After hybridization, the arrays were washed in 1× SSC, 0.1% SDS, 1 mM DTT at 45°C for 10 min, then in 0.1× SSC, 0.2% SDS, 1 mM DTT at 25°C for 5 min.

Data acquisition and analysis

The microarrays were scanned at 532 (cyanine-3) and 635 nm (cyanine-5) using a dual laser fluorescence scanner (Axon Instruments, Foster City, CA) at 10 µm resolution. The fluorescent signal was collected at 16 bits/pixel resolution. The images were analyzed with GEMTools image analysis software (Incyte Genomics) to provide corrected signal intensities, including local background subtraction. Positive signals were reported above a threshold of 2.5 times local background and were all within the linear dynamic range of the scanner. Tab delineated text files were exported from GEMTools for data analysis using Microsoft Excel software.

RESULTS

Figure 1 summarizes the sequences of synthetic oligonucleotides used in the experiments described below. As illustrated in the figure, these oligonucleotides were aligned 22–39 nt from the 3′-terminus of PCR-amplified cDNA clones and correspond to a common vector region outside the cloned gene inserts. A complementary sequence to the 59mer (59.C) permitted investigation of various arraying and hybridization possibilities with either strand fluorescently labeled, including sequential hybridizations (see below). Additionally, two complementary 47mer sequences were synthesized as single strands for arraying as well as hairpin constructs with T_5 loops at either end to provide stabilized duplexes for hybridization. The 30mers A–D are complementary to 30mers E–H, respectively, but do not overlap completely. Finally, a non-complementary negative control oligonucleotide sequence (NC) was included on each microarray to investigate non-specific hybridization.

In general, the hybridization experiments described below did not show evidence of non-specific hybridization. The hybridization signals from NC array elements were within 20% of local background signals in all cases. Furthermore, complex probe mixtures generated from human placenta mRNA did not show significant cross-hybridization to the arrayed oligonucleotide sequences (data not shown).

Sequential hybridization of complementary single-stranded oligonucleotides with arrayed oligonucleotides

The 30mer oligonucleotide sequences A–H were arrayed individually as seven replicate spots per microarray. Fluorescently labeled or unlabeled versions of the 59mer and 59.C sequences were then hybridized to this array in various combinations. As shown in Figure 2I and II, separate hybridization reactions with either Cy3-labeled 59mer or Cy3-labeled 59.C produced positive signals only at their corresponding complementary 30mer array elements. However, when the microarrays were first hybridized with unlabeled 59.C and then hybridized a second time with Cy3-labeled 59mer (Fig. 2III), positive signals were observed across all the array elements, including 30mers that were the ‘same sense’ as the Cy3-labeled 59mer. The sequential hybridization experiment was then repeated in reverse order, using unlabeled 59mer and Cy3-labeled 59.C, yielding a similar result (Fig. 2IV). Thus, both of the sequential hybridization experiments provided evidence for multi-stranded binding recognition to same sense array elements mediated by a previous hybridization with complementary unlabeled oligonucleotide. This experiment demonstrated that pre-formed duplexes on an array surface could further hybridize a third strand of oligonucleotide.

Hybridization of hairpin duplexes with arrayed single-stranded oligonucleotides

The hairpin duplexes illustrated in Figure 1 (each containing a Cy3-label at their 5′-ends) were hybridized to microarrays containing single-stranded oligonucleotide elements (30mer

Table 1. Hybridization signal intensities and strand preference of various labeled duplexes hybridized to arrayed oligonucleotide elements

Cy3-labeled probe	Hybridization signal intensity on elements				Strand preference	
	59mer	59.C	30mers A–D	30mers E–H	59mer/59.C	(A–D)/(E–H)
N hairpin	1590	61 300	545	27 900	1/39	1/51
F hairpin	39 300	2040	1930	546	19/1	4/1
NT 1 h	9890	202	1770	55	49/1	32/1
NT 72 h	56 200	11 400	12 800	2960	5/1	4/1
NT 72 h (O/N)	50 890	5760	14 800	963	9/1	15/1
59 bp duplex	12 700	14 600	20 100	16 700	1/1	1/1

Strand preferences are reported as the ratios of average hybridization intensities from replicate oligonucleotide elements. Data for the 59 bp duplex is extrapolated from the two-step hybridization experiment with single-stranded oligonucleotides (see Results). O/N, overnight preannealing of the NT 72 h hybridization probe prior to microarray hybridization.

sequences A–H, 59mer and 59.C). As described in the previous example, each of these oligonucleotides was arrayed as seven duplicate spots for signal averaging. As summarized in Table 1, these two hairpin duplexes showed opposite binding preferences for the two sets of complementary oligonucleotides (30mers A–D and 59mer versus 30mers E–H and 59.C). In each case, binding preference was observed at arrayed oligonucleotide elements sharing sequence identity with the free 3′-strand of the hairpin probes. For example, the normal hairpin probe (Fig. 1) showed binding preference for arrayed 59mer and 30mer sequences of 39:1 and 51:1, respectively. The flipped hairpin probe (Fig. 1) showed lower but opposite binding preferences. This experiment demonstrated that constrained duplex probes could bind arrayed single-stranded target oligonucleotides sharing sequence identity with one of the duplex strands. Furthermore, our results show that there is a preference for sequences sharing identity with the 3′-strand of the hairpin.

Hybridization of PCR-amplified dsDNA with arrayed single-stranded oligonucleotides

Table 1 lists the data for NT PCR duplexes along with the hairpins. Hybridization probes generated from longer PCR-amplified dsDNA (containing ~750 bp–2.5 kb expressed gene inserts) were fluorescently labeled by nick translation for hybridization onto microarrays containing single-stranded oligonucleotide elements (see previous example). Based on agarose gel electrophoresis (see Materials and Methods), the product from a 1 h nick translation labeling reaction was indistinguishable from the starting material, indicating that this probe population is largely double-stranded and representative of full-length PCR amplicons. However, the product generated from a 72 h nick translation reaction showed less duplex character (based on ethidium bromide staining) and a shorter size distribution compared to the starting material (~100–200 bp size range). As summarized in Table 1, the hybridization probe generated from a 1 h nick translation reaction showed a strong binding preference for the arrayed 59mer and 30mers A–D (averaged signal ratios of 49:1 and 32:1, respectively, compared to the corresponding complementary oligonucleotide elements). As in the previous experiment with synthetic hairpin probes, these array oligonucleotides share sequence identities with the 3′-strand of the PCR duplexes. The hybridization probe generated from a 72 h nick translation reaction showed a reduced strand preference compared to the 1 h NT probe (Table 1), although the hybridization signals were higher (as expected, based on increased label incorporation). However, pre-annealing the 72 h NT probe overnight prior to hybridization resulted in increased signal ratios between 59mer and 59.C oligonucleotides, indicating that duplex integrity is an essential feature of the observed strand preference.

Unlike the constrained duplexes (hairpins and long PCR amplicons), the control 59 bp duplexes showed no strand discrimination. Therefore similar hybridization intensities were observed for the elements in both orientations, as shown in Table 1, which was demonstrated by pre-formation of duplex from ssDNA (59mer and 59.C) or the sequential array hybridizations, as illustrated in Figure 2.

Hybridization of single-stranded oligonucleotides with arrayed PCR-amplified dsDNA

In this experiment, the PCR-amplified dsDNA products described above were arrayed individually in the presence of a non-denaturing buffer (2.5× SSC) or a denaturing buffer (150 mM NaOH, 20 mM EDTA). Fluorescently labeled oligonucleotides containing the 59mer or 59.C sequence (Fig. 1) were then hybridized separately on this microarray. As shown in Table 2, dsDNA elements that were arrayed in a non-denaturing buffer showed binding preferences for the 59mer versus 59.C hybridization probes, with averaged signal ratios ranging from ~7:1 to ~68:1 (average ~28:1). As in the previous example, this strand preference corresponds to the oligonucleotide sharing sequence identity with the 3′-strands of the duplex DNAs. However, this strand preference was not observed when the dsDNA elements were arrayed in a denaturing buffer, where hybridization signal ratios averaged close to unity (Table 2). Therefore, these results support the formation of multi-stranded structures in the former case and indicate that the observed strand preferences are an intrinsic property of duplex DNA and not an artifact of asymmetrical PCR.

DISCUSSION

The results described above provide four separate lines of evidence for the formation of multi-stranded DNA structures

Table 2. Hybridization signal intensity and strand preference of labeled 59 mer/59.C probes onto cDNA elements arrayed under native and denaturing conditions

Arrayed elements	Native array buffer (control, 2.5× SSC)			Denaturing array buffer (NaOH, EDTA)		
	Hybridization intensity		Preference	Hybridization intensity		Preference
	59mer	59.C	59mer/59.C	59mer	59.C	59mer/59.C
cDNA 1	6620	521	12.7	9170	12 800	0.7
cDNA 2	13 300	559	23.8	25 700	22 600	1.1
cDNA 3	2690	392	6.9	17 400	10 100	1.7
cDNA 4	5710	561	10.2	10 100	21 100	0.5
cDNA 5	19 700	468	42.1	9080	17 100	0.5
cDNA 6	8010	351	22.8	27 100	15 600	1.7
cDNA 7	3610	416	8.7	10 800	17 500	0.6
cDNA 8	22 800	543	42.1	26 500	19 400	1.4
cDNA 9	16 400	733	22.4	22 400	32 900	0.7
cDNA 10	45 000	658	68.4	25 400	28 900	0.9
cDNA 11	28 300	648	43.6	37 400	18 900	2
Average	15 600	532	27.6	20 100	19 700	1.1

on glass microarrays. First, hybridization of a complementary oligonucleotide to arrayed oligonucleotides enabled a third strand of 'same sense' oligonucleotide to hybridize in a subsequent round of hybridization. Secondly, synthetic hairpin duplexes were shown to hybridize arrayed oligonucleotides. Thirdly, PCR-amplified duplex DNA was shown to hybridize arrayed oligonucleotides. And fourthly, oligonucleotide probes were shown to hybridize arrayed PCR-amplified dsDNA elements ranging in length from ~750 bp to 2.5 kb. In the last case, strand preference was only observed when the dsDNA elements were arrayed in non-denaturing buffer; arraying in a denaturing buffer resulted in no strand preference (i.e. hybridization more reflective of traditional Watson–Crick base pairing).

Taken together, our results support the formation of multi-stranded structures on glass microarrays wherein two homologous strands bind a third complementary strand. These results support the formation of triple-stranded structures, although alternative structures such as D-loops and cruciforms could also explain the data. Zurkin *et. al* (10) and Kiran and Bansal (11) proposed an attractive model for the triple strand possibility. According to this model, duplex stretching results in a widened major groove that supports binding a third strand that is homologous and parallel to one of the Watson–Crick strands. The resulting triple-stranded complex is predicted to form sequence-specifically through the following base recognitions: A:T-T*, T:A-A*, G:C-C* and C:G-G* (wherein the third strand is represented by the asterisk).

These strand preferences are summarized and illustrated in Figure 3. In all cases, strand preference was observed corresponding to oligonucleotides sharing sequence homology with free 3′-strands of dsDNA, which was indicated by the results from the normal hairpin and flipped hairpin. Similarly, long PCR duplexes showed consistent strand preferences, independent of the configuration of probes and elements. The control duplex, which has both ends free, showed no strand discrimination, as expected.

The multi-stranded structures and strand preferences reported here have also been reported for *in vitro* studies of homologous recombination mediated by RecA protein. For example, RecA protein was shown to promote the formation of a triple-stranded DNA complex between a 33 bp synthetic duplex and a circular plus strand of M13 DNA (12). A number of studies have demonstrated that recombinant hybridization orients the single strand in the same direction as the 3′-homologous '+' strand of the duplex (10,13) (with the possible exception of long DNA molecules; 14). It has also been observed that 3′-homologous ends were essential for stable joint molecule formation between linear ssDNA and supercoiled DNA (i.e. 3′-ends were 50–60 times more reactive than 5′-ends) (15). Linear ssDNAs with homology at the 5′-end were considerably less reactive (16). The preferential reactivity of 3′-homologous ends was thought to be attributed to the 3′-ends of ssDNA being more heavily coated with RecA proteins (4,17). Our studies show that similar strand recognition occurs in the formation of multi-stranded DNA structures on glass microarrays in the absence of a protein catalyst.

As stated in the Introduction, duplex DNA is an elastic molecule that is capable of elongating up to 1.7-fold when subjected to an external force (7,8). Similarly, the binding of RecA protein has been shown to elongate duplex DNA by a factor of ~1.5 (18). We suggest that such deformations could also explain the formation of multi-stranded DNA structures on glass microarrays, particularly the cationic microarray surfaces investigated here. Furthermore, the strand preferences that we observed are similar to those described from solution studies of homologous recombination catalyzed by RecA protein. Thus, the formation of multi-stranded structures on glass microarray surfaces appears to reflect an intrinsic property of DNA that may be important in a variety of biological processes.

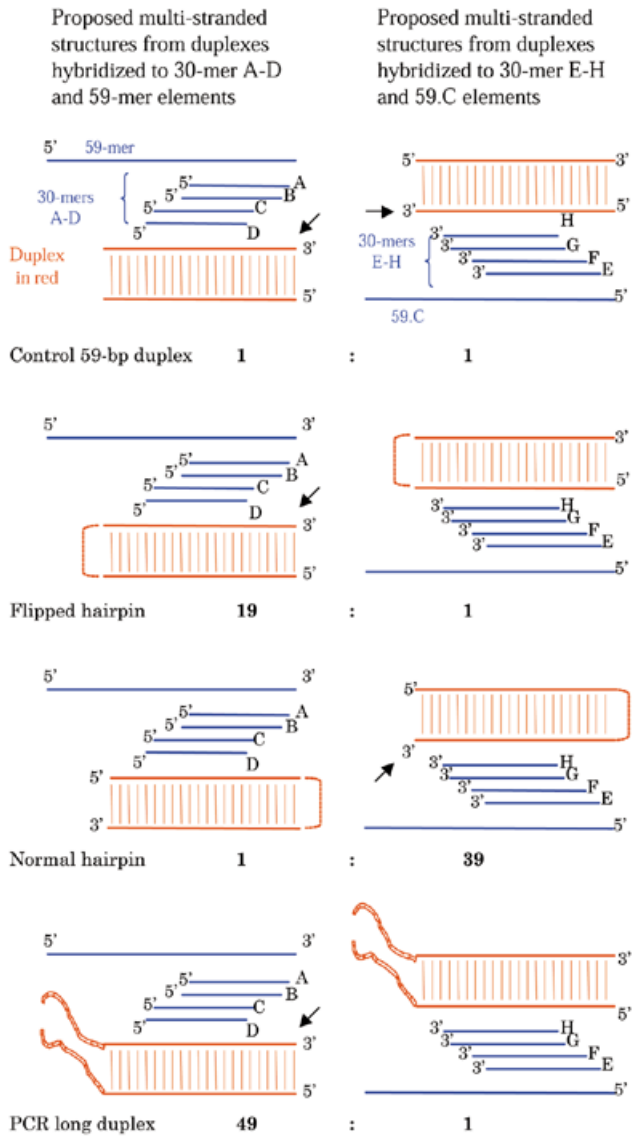

Figure 3. Proposed structures of various duplex probes with single-stranded elements. The single-stranded elements that share sequence identity with the 3′-strand of the duplex (indicated by an arrow) hybridized preferentially. The elements are shown in blue and probes in red.

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