Unrestricted Hybridization of Oligonucleotides to Structure-Free DNA†

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ABSTRACT: The existence of secondary structure in long single-stranded DNA and RNA is a serious obstacle to the practical use of short oligonucleotide probes (<20-mers). Here, we show that replication of a highly structured DNA in the presence of a unique set of dNTP analogues leads to synthesis of daughter DNA with a significantly reduced level of secondary structure. This replicated DNA, composed of 2-aminoadenine, 2-thiothymine, 7-deazaguanine, and cytosine bases, was readily accessible to tiled 8-mer LNA and 15-mer DNA probes, whereas an unmodified version of the same DNA was inaccessible. Importantly, while the base analogues enhanced probe−target stability, they did not significantly reduce the specificity of base pairing. The availability of structure-free DNA targets should facilitate the use of short oligonucleotide probes and promote development of generic oligonucleotide microarrays.

Long single-stranded DNA and RNA have considerable secondary structure that can prevent hybridization to complementary oligonucleotides (*1*−*8*). Accessibility is particularly problematic for very short oligonucleotides (<15-mers) because the resulting hybrids have low stability. No general solution to this problem has been described. Attempts to improve hybridization efficiency by modifying the probe to form a more stable hybrid, or by modifying the target DNA or RNA to reduce its level of secondary structure, have met with limited success (*4*, *6*, *9*, *10*). Even LNA probes (*11*), which form hybrids of unprecedented strength, are unable to address highly structured sequences. This is unfortunate because short probes are ideally suited for SNP detection and sequencing by hybridization. We have been investigating pseudocomplementary nucleic acid targets as an alternative way of improving probe accessibility (*12*, *13*). Conceptually, pseudocomplementary DNA or RNA is composed of base analogues that are unable to pair to one another but are able to pair to the standard bases of an oligonucleotide probe. Hence, pseudocomplementary nucleic acid is structure-free and should be completely accessible to short probes. Utilization of this strategy in a hybridization reaction requires amplification of the target nucleic acid by PCR and then copying of one strand into a pseudocomplementary product by asymmetric PCR or transcription. Exponential amplification of DNA by PCR cannot be carried out in the presence of pseudocomplementary dNTPs because the modified DNA is not a template for further synthesis.

In this study, we investigate the hybridization properties of DNA replicated in the presence of one or more of the following base analogues: 2-aminoadenine (nA, also known as 2,6-diaminopurine), 2-thiothymine (sT), 7-deazaguanine (cG), and hypoxanthine (hX) (see Figure 1 for structures). Each analogue has been shown to function in primer extension or PCRs (*14*−*18*), suggesting that their combined use might support DNA synthesis. The nA-sT couple was chosen as the focus of our investigation because it functions as a mismatch in chemically synthesized oligonucleotides, where hydrogen bonding between nA and sT is disrupted by steric clash between the 2-amino group of nA and the 2-thioketo group of sT. However, stable base pairing is reestablished when one of the bases is A (A-sT) or T (nA-T) (*19*). Selection of a G-C couple was not as straightforward because a pseudocomplementary version of this base pair has not been described. We utilized cG in combination with C as a way to reduce the strength of G-C pairing while maintaining the specificity. The cG analogue had the additional benefit of eliminating G-G self-pairing (*20*). Although hypoxanthine (inosine) is a more destabilizing analogue of G, it was not chosen because its degenerate pairing would have reduced specificity (*21*, *22*).

DNA composed of nA, sT, cG, and C bases should be structure-free under conditions that melt out cG-C base pairs and should therefore approximate a pseudocomplementary target. The entire sequence of such a target should be accessible to short probes that are capable of forming stable hybrids. Development of a pseudocomplementary G-C couple would permit the use of lower hybridization temperatures, thereby allowing the use of still shorter oligonucleotide probes. Here we compare the accessibility of tiled 8-mer LNA and 15-mer DNA probes to a DNA target composed of regular or modified bases. The DNA target, when made up of regular bases, had extensive secondary structure, but this same target was essentially structure-free, when made

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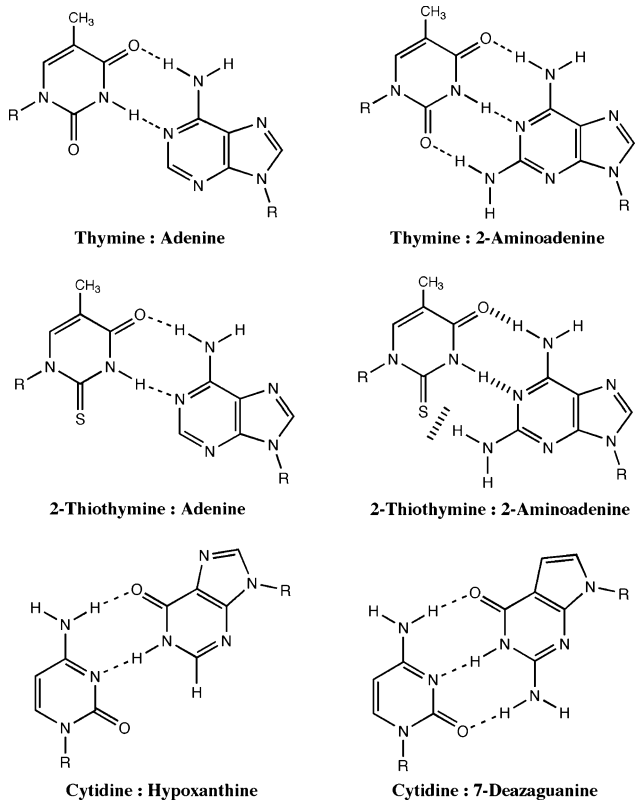

FIGURE 1: Base pairing properties of 2-aminoadenine (nA), 2-thiothymine (sT), 7-deazaguanine (cG), and hypoxanthine (hX).

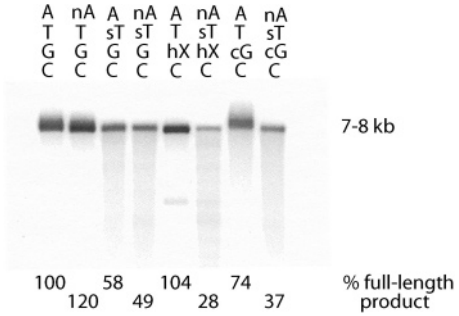

FIGURE 2: Run-on primer extension of circular single-stranded M13 DNA by Sequenase in the presence of different dNTPs.

up of modified bases. We show that both sets of probes hybridized to the modified target but not to the regular target.

RESULTS

Enzymatic Uptake of dNTP Analogues into DNA. T7 DNA polymerase that lacks 3′ → 5′ exonuclease activity (Sequenase) is a highly processive enzyme when complexed with thioredoxin (*23*). This polymerase, which can use many nonstandard dNTPs, was tested for its ability to replicate single-stranded circular M13 DNA with different combinations of regular and analogue dNTPs. Each combination of dNTPs yielded a different amount of 7−8 kb run-on product when electrophoresed in a denaturing alkaline agarose gel (Figure 2). Relative to the control reaction with standard dNTPs, substitution of dnATP for dATP or dhXTP for dGTP did not reduce the yield. Reductions of 25 and 40% were observed when dcGTP was substituted for dGTP and when dsTTP was substituted for dTTP, respectively. Premature termination of synthesis accounted for reduced yields with these triphosphates. Incorporation of two or more unnatural dNTPs further decreased the amount of run-on product. For example, DNA substituted with nA, sT, and cG was synthesized with 37% efficiency, consistent with values of 49 and 74% for uptake of nA-sT and cG into DNA, respectively.

Model Systems. Two primer extension products were used to evaluate the hybridization properties of DNA substituted with nA, sT, and/or hX/cG (Figure 3). HP25 is a 25 bp G/C-rich hairpin with a 5′ single-stranded extension that was used to evaluate the strength of intramolecular base pairing and to test the accessibility of hairpin sequences to tiled sets of short DNA and LNA oligonucleotides. SS37, which recapitulates one arm of the HP25 hairpin, is a single-stranded 37-mer that was used to investigate the stability and specificity of hybridization between modified DNA sequences and a representative DNA or LNA oligonucleotide. This DNA, which contained a 7 bp hairpin with the stem-forming region interrupted by a single G-T mismatch, provided an opportunity to test the accessibility of a less structured sequence. Each DNA was replicated from a synthetic template by Sequenase in the presence of different combinations of dNTPs. SS37 was purified on a denaturing 12% polyacrylamide gel. HP25, which was not gel purified, was released from its template via heating−cooling treatment in the presence of an extended version of the primer, which prevented reassociation of template and product hairpins by hybridizing to the 3′ overhang of the template. A 5′ ^{32}P label contributed by the primer facilitated detection and quantitation of primer extension products.

Hairpin Stability. The melting temperature (T_m) of HP25 should reflect the degree to which any given combination of bases reduces the strength of intramolecular base pairing. Because we had limited quantities of radiolabeled hairpin, the apparent T_m was determined using a gel mobility shift assay (*12*). In this method, aliquots of the hairpin were briefly incubated at different temperatures with a 25-mer DNA probe prior to quenching in an ice bath. The probe sequestered any open hairpin by engaging one arm in a hybrid. Intact hairpin was separated from hybrid in a nondenaturing polyacrylamide gel. Melting profiles were obtained by plotting percent hairpin versus temperature and taking the midpoint as the T_m of the hairpin.

Apparent T_m values of differently substituted hairpins were determined under native (5 mM $MgCl_2$) or denaturing (20% formamide) conditions (Table 1). Under native conditions, each hX-C pair (14 occurrences) or nA-sT pair (11 occurrences) reduced the T_m by 2.8 °C (39/14) or 4.4 °C (48/11), respectively, suggesting that both hX and nA-sT are highly destabilizing modifications. The relative magnitude of destabilization is consistent with hX-C being a weak base pair and nA-sT being a mismatch. Hairpin substituted with all three base analogues (nA-sT-hX) had a much reduced level of secondary structure and readily hybridized to the 25-mer probe at every temperature. A T_m of 26 °C was obtained for hairpin substituted with nA-sT-cG and is attributed to the greater stability of cG-C relative to hX-C base pairs. Unmodified hairpin or hairpin substituted with cG did not hybridize to the 25-mer probe so a T_m could not be determined by a gel mobility shift assay.

The T_m values of hairpins substituted with nA-sT or nA-sT-cG did not drop appreciably when measured under denaturing conditions (Table 1). To better understand these

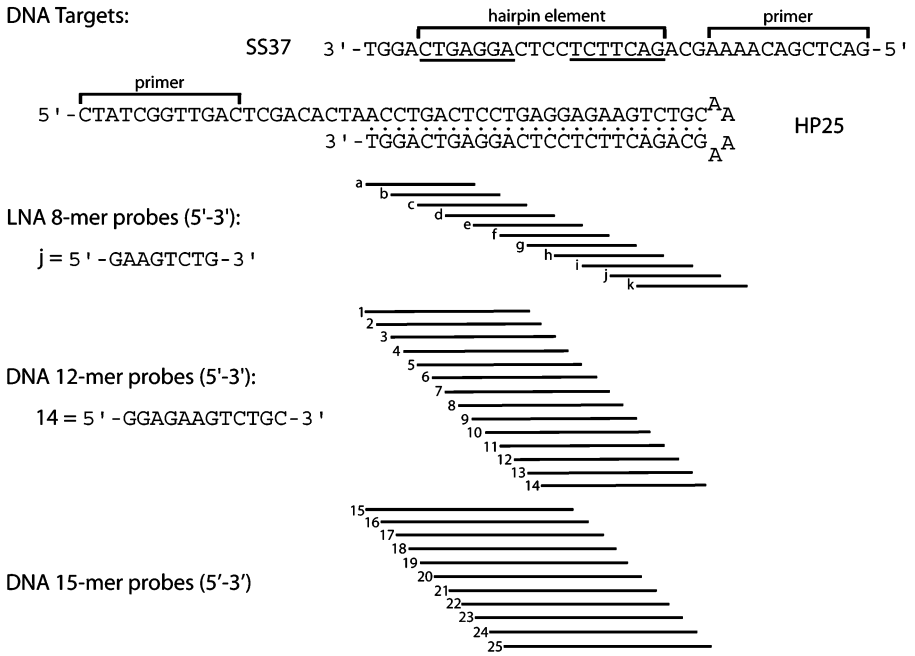

FIGURE 3: DNA targets and tiled oligonucleotide probes used in this study. Three sets of tiled probes (8-mer LNA, 12-mer DNA, and 15-mer DNA oligonucleotides) are aligned opposite complementary sequences in the SS37 and HP25 targets. The 12-mer and 15-mer DNA probes are offset by a one-base increment, while the 8-mer LNA probes are offset by two-base increments.

Table 1: Melting Temperatures of a 25 bp DNA Hairpin[a]

base composition of the DNA hairpin	T_m (°C)	
	5 mM $MgCl_2$	20% formamide
A, T, G, C	88[b]	–
A, T, hX, C	49	32
nA, sT, G, C	40	38
nA, sT, hX, C	<10	<10
nA, sT, cG, C	26	28

[a] HP25 hairpins were melted in 25 mM NaCl and 20 mM Hepes (pH 7.5) supplemented with 5 mM $MgCl_2$ or 20% formamide. [b] Predicted from Mfold.

unexpected results, we hybridized 25-mer DNA, 12-mer DNA, and 8-mer LNA probes to hairpin substituted with nA-sT-cG. The percentage of hairpin unavailable to each probe as a function of temperature was determined under both native (5 mM $MgCl_2$) and denaturing (20% formamide) conditions (Figure 4). Whereas all three probes yielded similar T_m values (25−30 °C) for the hairpin under denaturing conditions, this was not the case under native conditions. The apparent T_m of the hairpin was 26 °C when a 25-mer probe was used but >40 °C when shorter probes were used. We suggest that the 25-mer probe was able to strand invade and hybridize to the hairpin far below its true T_m. This phenomenon did not take place under denaturing conditions, nor was it observed with the shorter probes. Thus, the T_m values reported in the first column of Table 1 may be too low, which explains why some of the hairpins exhibit comparable T_m values under both native and denaturing conditions.

The melting curves depicted in Figure 4 provide additional insights into interaction of HP25 with probes. Under conditions where the hairpin was melted, the 12-mer DNA probe, and to a lesser extent the 8-mer LNA probe, did not hybridize with 100% efficiency. This result is probably artifactual since both of these hybrids, unlike the hybrid with the 25-mer probe, could be disrupted by refolding of the DNA target upon cooling. The 12 bp DNA−DNA hybrid, being the least stable, would have been more susceptible to loss by strand displacement. The level of hybrid formation also decreased when the 25-mer probe was kept at 60−70 °C in denaturing buffer. It is probable that these conditions were too stringent for base pairing. Consequently, probe−target hybridization competed with refolding of the target during the low-temperature quench step.

Thermostability of Probe−Target Hybrids. SS37 DNAs substituted with different combinations of regular and analogue bases were hybridized to an 8-mer LNA (probe **i**) and a 12-mer DNA (probe **14**) under native (5 mM $MgCl_2$) or denaturing (20% formamide) conditions. Apparent T_m values of the respective hybrids were determined by a gel mobility shift assay. In these experiments, a competitor oligonucleotide was added to aliquots of the hybrid at different temperatures. By hybridizing to the free probe, the competitor oligonucleotide fixed the ratio of unhybridized to hybridized SS37 DNA so that the ratio could be determined by electrophoretic analysis. Apparent melting temperatures were derived from the midpoint in plots of percent hybrid versus temperature.

Several conclusions can be drawn from this analysis of thermostability (Table 2). First, with only two exceptions, hybrids with an 8-mer LNA probe exhibited higher T_m values than hybrids with a 12-mer DNA probe. Second, all hybrids were less stable in a denaturing solution than in a native solution. Third, substitution of SS37 with nA-sT elevated the T_m of the hybrid, while substitution with hX or cG lowered the T_m. These changes were greatest with the LNA probe under denaturing conditions. T_m increased by 6.5 °C per nA-sT substitution and decreased by 21 or 4 °C per hX or cG substitution, respectively. Hybrids substituted with nA-sT-cG were only slightly less stable than those substituted with nA-sT.

Specificity of Hybridization. Specificity was investigated by comparing the hybridization of SS37 to a perfect-match

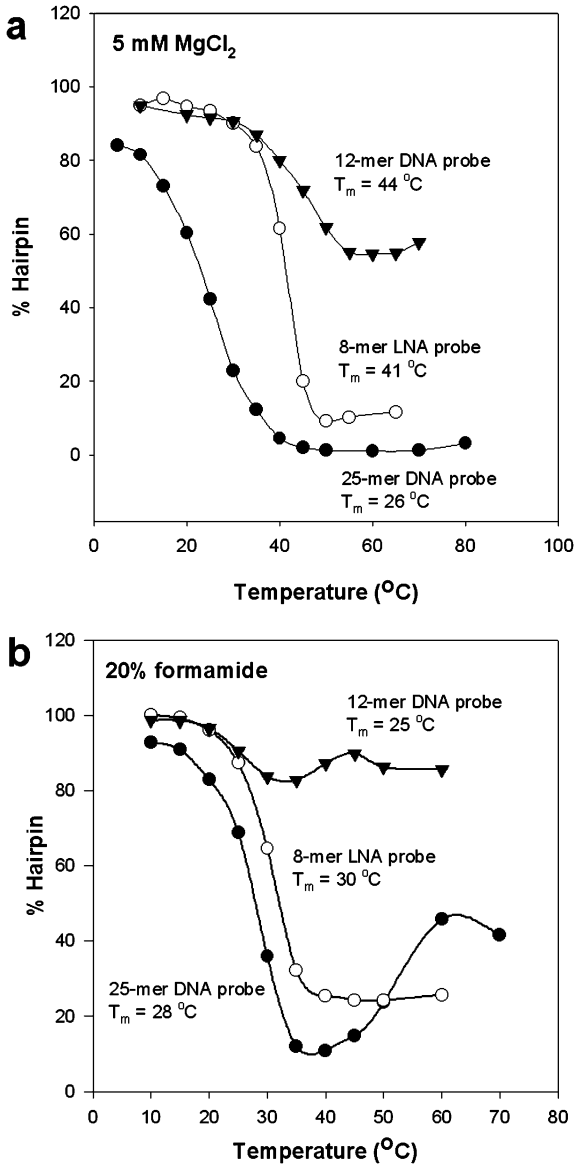

FIGURE 4: Apparent melting profiles of HP25 in (a) 5 mM $MgCl_2$, 25 mM NaCl, and 20 mM Hepes (pH 7.5) or (b) 25 mM NaCl, 20 mM Hepes (pH 7.5), and 20% formamide. Profiles represent the percentage of hairpin (substituted with nA, sT, and cG) unavailable to oligonucleotide probes. The 25-mer DNA probe was complementary to one arm of the HP25 hairpin. The 12-mer DNA (probe **14**) and 8-mer LNA (probe **i**) were complementary to the 5′ end of the same hairpin arm.

Table 2: Melting Temperatures of Short DNA−DNA and DNA−LNA Hybrids[a]

	T_m (°C)			
	8-mer LNA probe		12-mer DNA probe	
base composition of DNA target	5 mM $MgCl_2$	20% formamide	5 mM $MgCl_2$	20% formamide
A, T, G, C	57	37	46	23
A, T, hX, C	42	16	43	17
A, T, cG, C	55	33	46	22
nA, sT, G, C	66	63	55	34
nA, sT, hX, C	66	48	49	23
nA, sT, cG, C	66	60	53	32

[a] Hybrids between SS37 DNA targets and LNA (5′-GAAGTCTG) or DNA (5′-GGAGAAGTCTGC) probes were melted in 25 mM NaCl and 20 mM Hepes (pH 7.5) supplemented with 5 mM $MgCl_2$ or 20% formamide.

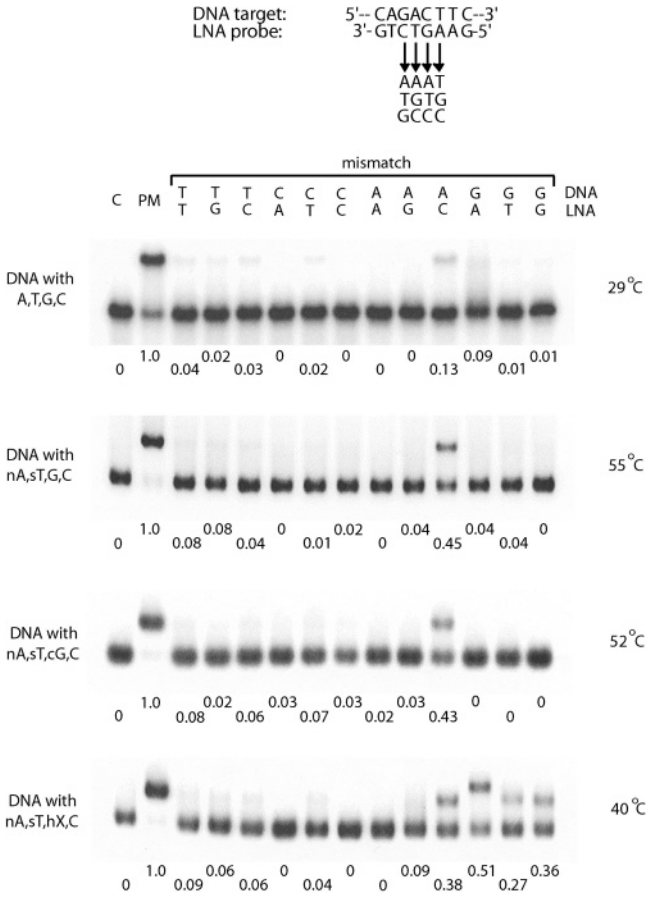

FIGURE 5: Specificity of hybrid formation between 8-mer LNA probes and SS37 DNA targets. Radiolabeled SS37 DNAs composed of different bases were hybridized to a perfect-match (PM) LNA probe (5′-GAAGTCTG, 1 μM) and to a dozen mismatched versions of the same probe under stringent conditions [8 °C below the T_m of the perfect-match hybrid in 25 mM NaCl, 20 mM Hepes (pH 7.5), and 20% formamide for >10 min]. After addition of an LNA competitor (5′-CAGACTTC, 10 μM) to sequester free probe, the reaction mixtures were quickly cooled and analyzed by gel mobility shift analysis. The extent of hybrid formation in each reaction is listed below the gels.

8-mer LNA (probe **i**) versus 12 versions of the same probe with a single mismatch. The SS37 target contained different combinations of regular and analogue bases. Hybridizations were conducted in native buffer at a temperature 8 °C below the T_m of the perfect-match hybrid so that a centrally placed mismatch in the hybrid should prevent annealing if the reaction is specific. Each reaction was terminated by adding a competitor 8-mer LNA that hybridized to the probe, followed by placement of the reaction mixture in an ice bath. The gel mobility shift assay allowed us to measure the extent of hybridization obtained with each probe−target combination. By utilizing 12 mismatched probes, the effect of each mismatch on hybrid stability could be determined.

The results (Figure 5) indicate that DNA substituted with nA, sT, and cG was nearly as specific as DNA when hybridized to the 8-mer LNA probes. No loss of specificity was observed when T or G in the target was replaced with sT or cG, respectively. However, substitution of hX for G was accompanied by degenerate pairing of this base to A, T, and G, consistent with previous studies (*21*, *22*). Substitution of nA for A was accompanied by an increased tolerance of the nA-C mismatch relative to the A-C mismatch. This

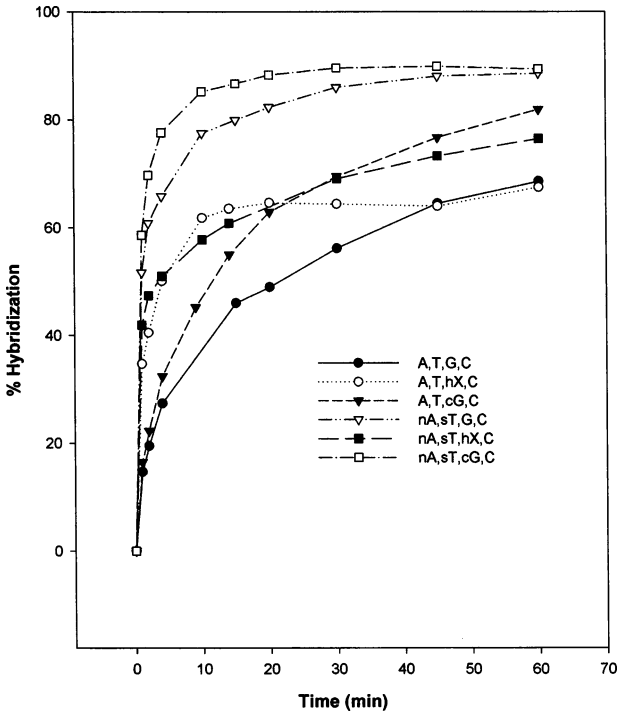

FIGURE 6: Time course for hybridization of an 8-mer LNA probe to SS37 DNA targets substituted with different bases. Reactions were carried out with radiolabeled target and LNA probe (5′-GAAGTCTG, 1 μM) at 10 °C in 25 mM NaCl, 20 mM Hepes (pH 7.5), and 20% formamide. Aliquots were quenched with an LNA competitor (5′-CAGACTTC, 10 μM) and analyzed for hybrid formation by gel mobility shift analysis.

has been observed previously for duplexes which contain an LNA backbone (*24*, *25*) and may reflect wobble pairing between nA and C. The average mismatch frequency in duplexes which contained nA-sT-cG was approximately twice that observed for unmodified duplexes (6.4% vs 2.9%) and was primarily attributable to the nA-C mismatch. This was not the case for hybrids formed with a DNA 12-mer (probe **14**), where the nA-C mismatch was not tolerated under stringent conditions (data not shown). A thermodynamic study of base pairing by nA supports the specificity of this base analogue in DNA (*26*).

Hybridization to a Short DNA Hairpin. The SS37 DNA contained a 7 bp hairpin (see Figure 3) which was stable at 10 °C in denaturing (20% formamide) buffer. Hybridization of an 8-mer LNA (probe **i**) to one arm of this hairpin (Figure 6) was much slower than would be expected for a single-stranded target sequence. Substitution of SS37 DNA with nA-sT, nA-sT-cG, or nA-sT-hX was expected to destabilize the hairpin and enhance the rate of hybridization. This prediction was tested experimentally, and the results are presented in Figure 6. Hairpin substituted with nA-sT-cG was readily accessible to the LNA probe. Hybridization was nearly complete within 10 min and established a 9:1 equilibrium of hybrid to hairpin. Hairpin substituted with nA-sT hybridized nearly as well, underscoring the effectiveness of this pseudocomplementary couple in promoting hybrid formation. Replacement of G with cG or hX resulted in intermediate rates of hybridization. Unlike nA-sT, these base analogues did not contribute added stability to the probe−target hybrid. Target DNA substituted with nA-sT-hX probably had minimal secondary structure but nonetheless hybridized slowly to probe for unknown reasons.

Hybridization of Tiled Probes to SS37 and HP25 Targets. As a more rigorous test of the accessibility of short probes to DNA substituted with nA-sT-cG, we evaluated hybridization of tiled sets of 8-mer LNA, 12-mer DNA, and 15-mer DNA probes (Figure 3) to both the SS37 and HP25 DNA targets composed of regular (A, T, G, and C) or modified (nA, sT, cG, and C) bases. Reactions were conducted at 45 °C in native (5 mM $MgCl_2$) buffer. Under these conditions, the modified SS37 and HP25 targets should have been completely single-stranded (Figure 4) and readily accessible to every probe. By contrast, the unmodified SS37 and HP25 targets had hairpin elements with predicted T_m values of 49 and 88 °C, respectively. The presence of secondary structure in these targets was expected to interfere with or block the hybridization of short probes. This was indeed the case for the unmodified HP25 target, which was inaccessible to all three probe sets (data not shown).

Despite the single-stranded character of the hairpin target substituted with nA-sT-cG, the 12-mer probes encountered difficulty in forming stable hybrids (Figure 3). This probe set hybridized with only 13% efficiency to the modified HP25 target versus 75% efficiency to the two SS37 targets (Figure 7a). Considering that the 12-mer probes were expected to form hybrids with T_m values greater than 50 °C, the level of hybridization to these targets was less than expected. We propose that probe−target hybrids were indeed formed at 45 °C but that most were displaced by refolding of the target when the reaction mixtures were placed in an ice bath. Refolding would have been more extensive with the modified HP25 target than the two SS37 targets, accounting for the small amount of hybrid formed with the hairpin.

Hybrid formation was much improved under the same conditions when the DNA probes were increased in length from 12- to 15-mers (Figure 7b). With the tiled 15-mer probes, robust hybridization was observed with all three targets. The extent of hybrid formation was greater than 90% for the single-stranded targets and 75% for the modified hairpin target. When the temperature of the hybridization reaction mixture was changed in either direction by 10 °C, individual probes responded differently, but in each case, the average occupancy of the probe set decreased to ~60% with the modified HP25 target (data not shown). Hybrids formed by these probes were relatively resistant to dissociation by strand displacement, caused by refolding of the target during the low-temperature quench. Despite some probe-to-probe variation in hybridization efficiency with the modified HP25 target, we predict that most sequences in any DNA target substituted with nA, sT, and cG should form stable hybrids with 15-mer DNA probes.

Hybridization of the tiled 8-mer LNA probe set to SS37 and HP25 DNA targets was also carried out at 45 °C in native buffer (Figure 7c). This probe set hybridized with 83% efficiency to unmodified SS37, 94% efficiency to nA-, sT-, and cG-substituted SS37, and 76% efficiency to nA-, sT-, and cG-substituted HP25. The near equivalence of 8-mer LNA and 15-mer DNA probes with respect to hybrid formation reflects the enhanced stability of LNA−DNA hybrids relative to DNA−DNA hybrids and suggests that most 8-mer LNA probes should also form stable hybrids with DNAs which contain nA-sT-cG bases. However, some hybrids, like those formed by probes **e** and **f**, may be

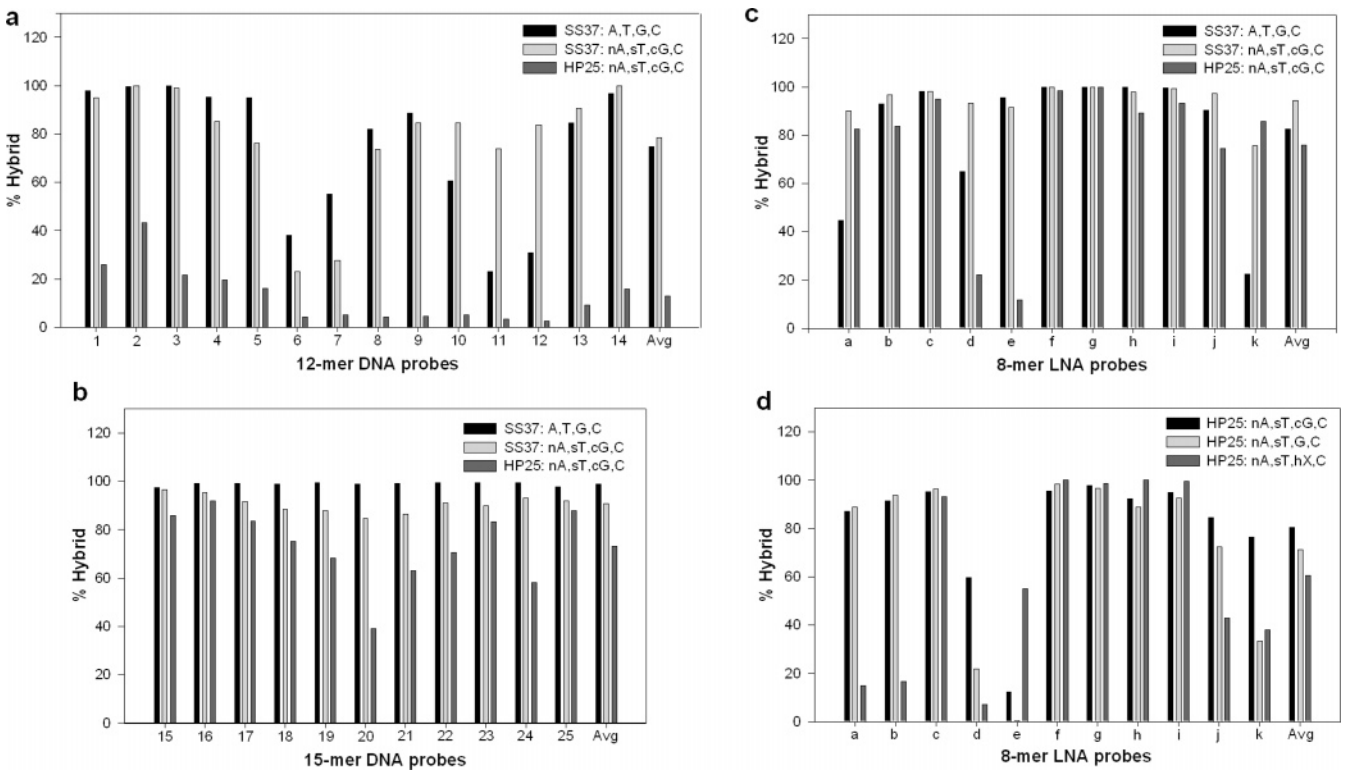

FIGURE 7: Hybridization of tiled oligonucleotide probes to SS37 and HP25 DNA targets. The profiles in panels a−c were obtained after incubation for 20 min at 45 °C in 5 mM $MgCl_2$, 25 mM NaCl, and 20 mM Hepes (pH 7.5). The profiles in panel d were obtained after incubation for 10 min at 40 °C in 25 mM NaCl, 20 mM Hepes (pH 7.5), and 20% formamide. Reactions, which included 5 μM probe, were quenched in an ice bath and analyzed by gel mobility shift. Longer incubation times did not increase the extent of hybridization.

underrepresented due to low T_m values or susceptibility to strand displacement.

The LNA probe set was also hybridized under stringent conditions to three HP25 targets substituted with different combinations of bases (Figure 7d). Here, the hybridizations were conducted at 40 °C in denaturing (20% formamide) buffer. The results confirmed our expectation that secondary structure was primarily disrupted by the presence of nA and sT bases in the target. As long as the hairpin contained these pseudocomplementary bases, the accessibility of the targeted sequences was similar regardless of whether the hairpin contained G, cG, or hX. Clearly, introduction of a pseudocomplementary G-C pair into the target along with the nA-sT couple would have a greater effect on lowering the temperature required for disruption of intramolecular pairing.

DISCUSSION

It is well-known that hybridization of oligonucleotides to long single-stranded RNA or DNA targets has variable efficiency and can be problematic. Different probes to the same target can hybridize with efficiencies that differ by orders of magnitude (*3*, *4*). Most of these difficulties arise from target sequences participating in intramolecular base pairing (*5*). Unlike proteins, single-stranded nucleic acids can exist in multiple alternative conformations (*27*), with each presenting a different set of probe-accessible sequences. Accessibility becomes a growing concern as probe length is decreased. Shorter probes require lower temperatures of hybridization which in turn stabilize secondary structure. Sequence and base composition of DNA can also modulate hybridization to a particular probe by influencing the T_m of the hybrid (*5*). Here again, these parameters alter the T_m to a greater extent as the length of the probe is decreased.

The variability in the efficiency and specificity of hybridization prevents the practical use of very short probes and impairs the performance of longer probes (<30-mers). Elimination of secondary structure in nucleic acid targets as proposed here would ideally equalize the hybridization efficiency of all probes to a more consistent level. This in turn would allow the use of shorter probes with increased specificity, a distinct advantage in detection of single-nucleotide polymorphisms by hybridization. The availability of pseudocomplementary targets should permit the use of DNA probes as short as 8−10-mers, thus allowing development of inexpensive universal oligonucleotide microarrays for applications such as genomic profiling or sequencing by hybridization.

In principle, pseudocomplementary nucleic acid is free of secondary structure yet able to hybridize to regular oligonucleotide probes. In this study, we show that dNTPs of nA, sT, cG, and C could be incorporated into DNA by Sequenase and that the resulting primer extension product, as exemplified by a 25 bp hairpin, had a significantly reduced level of secondary structure. The modified hairpin melted under conditions which were permissive for hybridization with short LNA and DNA probes. All three base analogues exhibited specific pairing, with the exception of wobble hydrogen bonding between nA and C when using an LNA probe. The base analogues also helped to equalize the strength of base pairing in probe−target hybrids, with nA-T and A-sT being more stable than A-T and cG-C being somewhat less stable than G-C.

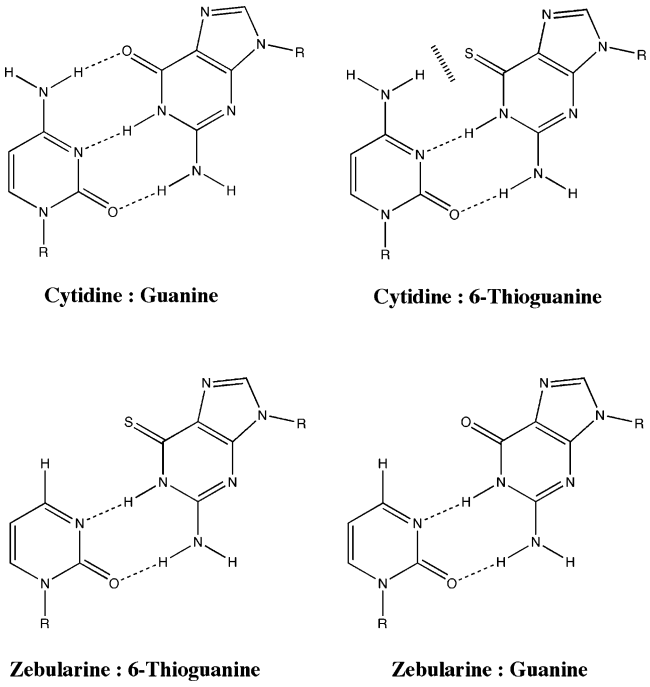

FIGURE 8: Base pairing properties of 6-thioguanine (sG) and zebularine (Z).

Our results suggest most DNAs composed of nA, sT, cG, and C should be denatured and hence "structure-free" under conditions that permit stable hybridization by 8-mer LNA or 15-mer DNA probes. A generic LNA microarray, with the length of each probe adjusted to equalize hybrid stability, should perform well with such targets. We envision that genes of interest could be amplified by PCR and then converted to a structure-free form by asymmetric PCR. This modified DNA could be conveniently labeled using a dCTP analogue conjugated to a fluorophore, a biotin, or an alkylamine group. A recently described mutant of Taq DNA polymerase with an expanded tolerance for modified dNTPs such as dcGTP would be a good choice for catalyzing the second amplification reaction as long as the fidelity of synthesis is not compromised (*28*).

Further extension of this approach to smaller DNA probes will require a pseudocomplementary G-C couple to be used in conjunction with nA-sT. Its development will probably involve a strategy different from that used to develop the nA-sT couple. In this respect, 6-thioguanine (sG) and C are attractive candidates (Figure 8). The dNTP of sG is incorporated into DNA (*29*−*31*) where it forms a weak base pair with C due to steric clash between the 6-thioketo group of sG and the 4-amino group of C (*32*−*35*). The sG-C couple in a DNA target could be considered pseudocomplementary if the corresponding oligonucleotide probes contained a C analogue which strongly pairs to sG. Zebularine (Z) is a candidate because sG-Z is reported to be as stable as A-T (*29*, *32*). An additional advantage of Z is that it does not pair well to G, thus reducing the likelihood of secondary structure in probes.

Alternatively, the level of G-C pairing in the DNA target could be reduced by utilizing analogues of cG that pair less strongly to C. For instance, substitution of the C7 position of cG with alkyl groups of increasing size progressively weakens base pairing when the corresponding dNTPs are enzymatically incorporated into DNA (*36*). Hybridization to cG-containing DNA could be concomitantly strengthened by using probes in which C is replaced with an analogue with greater binding affinity (*37*−*40*). Efforts along these lines are underway with the aim of developing a more efficient and reliable strategy for utilizing oligonucleotide microarrays.

METHODS

Materials. dNTPs of A, T, G, C, and cG were purchased from GE Healthcare Biosciences (Little Chalfont, U.K.), and dNTPs of nA, sT, and hX were provided by TriLink Biotechnologies (San Diego, CA). Sequenase was obtained from USB Corp. (Cleveland, OH). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Proligo (Paris, France) contributed the LNA oligomers.

Run-On Primer Extension. A ^{32}P end-labeled universal primer was hybridized to a 2-fold molar excess of M13 mp18 single-stranded circular DNA and extended by Sequenase in the presence of different combinations of dNTPs. Reaction mixtures were incubated for 60 min at 37 °C in the presence of each dNTP (100 μM). Aliquots were directly loaded onto a small 1% alkaline agarose gel and electrophoresed at 35 V for 6 h in the presence of molecular weight markers. The gel was equilibrated in a neutral buffer, dried under vacuum onto DEAE paper, and visualized by phosphorimaging.

Preparation of SS37 and HP25 Target DNAs. A radiolabeled DNA primer (containing standard bases) was annealed to a chemically synthesized DNA template by rapid heating and cooling. Run-off primer extension was carried out by Sequenase in the presence of 100 μM dNTPs at 37 °C for 30 min. The HP25 product was recovered along with its template after centrifugation through a gel filtration column (Centri-Spin 20; Princeton Separations, Adelphia, NJ) previously equilibrated in 10 mM Hepes (pH 7.5). A 21-mer oligonucleotide (7 μM) was added to the flow through, which was heated at 90 °C for 1 min and placed in an ice bath. This heating−cooling treatment disrupted the hybrid between template and product hairpins, allowing both to reanneal as separate molecules. By hybridizing to the overhang of the template hairpin, the 21-mer blocked reassociation of template and product hairpins through their single-stranded tails. Labeled hairpin was stored at −20 °C for up to 1 month.

The primer used for SS37 synthesis hybridized a few bases downstream from the 3′ end of the DNA template, thus ensuring that the product of primer extension would be resolved from the template strand in a denaturing polyacrylamide gel. Workup consisted of stopping the reaction with 20 mM EDTA, extracting the mixture with an equal volume of a phenol/chloroform/isoamyl alcohol mixture, and precipitating the DNA with ethanol in the presence of a glycogen carrier. Recovered nucleic acid was electrophoresed through a 7 M urea−12% polyacrylamide gel. The labeled SS37 band was excised, extracted overnight into TE, clarified through a 0.45 μm filter, concentrated by ethanol precipitation, and dissolved in 10 mM Hepes (pH 7.5).

Gel Mobility Shift Assay for Hybridization. This assay has been described previously (*12*). For the determination of T_m values of intermolecular hybrids, the radiolabeled SS37 target was annealed to 1 μM probe. Aliquots of this solution were then incubated for at least 3 min at different temperatures, supplemented with 10 μM competitor oligonucleotide, and

placed in either an ice or a dry ice bath. The competitor, which was identical in backbone and complementary in sequence to the probe, sequestered free probe and fixed the equilibrium between free and hybridized SS37. Electrophoresis of samples in a 12% polyacrylamide gel with 5 mM $MgCl_2$ in a cold room resolved the two states of SS37. An apparent T_m could be determined from the midpoint in a plot of percent hybrid versus temperature.

Permutations of the protocol described above were used to monitor hairpin melting, assess the specificity of hybridization, and gauge the performance of tiled probe sets. For determining the T_m of HP25, a 25-mer DNA probe (5 μM) complementary to one arm of the hairpin was incubated together with radiolabeled HP25 at different temperatures for at least 3 min. Rapid cooling in an ice bath fixed the ratio of free to bound hairpin. An apparent T_m for the hairpin was determined from a plot of percent hairpin versus temperature. In the specificity experiments, both matched and mismatched probes (1 μM) were heated and cooled with an SS37 target and then kept for at least 10 min at a stringent temperature. Reaction mixtures were fixed by adding competitor oligonucleotide (10 μM) followed by quick cooling in ice. The competitor was complementary to the perfect-match probe and hence mismatched with the other probes. Reactions were analyzed for hybrid formation by gel shift analysis. Hybridization of tiled LNA or DNA probes (5 μM) to SS37 or HP25 targets was straightforward since no competitor oligonucleotide was involved. Following hybridization, the reactions were quenched in an ice bath and the mixtures analyzed by gel electrophoresis.

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