Identification of Determinants for Inhibitor Binding within the RNA Active Site of Human Telomerase Using PNA Scanning†

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ABSTRACT: Telomerase is a ribonucleoprotein that participates in the maintenance of telomere length. Its activity is up-regulated in many tumor types, suggesting that it may be a novel target for chemotherapy. The RNA component of telomerase contains an active site that plays at least two roles—binding telomere ends and templating their replication [Greider, C. W., & Blackburn, E. H. (1989) *Nature 337,* 331−337]. The accessibility of RNA nucleotides for inhibitor binding cannot be assumed because of the potential for RNA secondary structure and RNA−protein interactions. Here we use high-affinity recognition by overlapping peptide nucleic acids (PNAs) [Nielsen, P. E., *et al.* (1991) *Science 254,* 1497−1500] to identify nucleotides within the RNA active site of telomerase that are determinants for inhibitor recognition. The IC_{50} for inhibition decreases from 30 μM to 10 nM as cytidines 50−52 (C50−52) at the boundary between the alignment and elongation domains are recognized by PNAs overlapping from the 5′ direction. As C50−52 are uncovered in the 3′ direction, IC_{50} increases from 10 nM to 300 nM. As cytidine 56 at the extreme 3′ end of the active site is uncovered, IC_{50} values increase from 0.5 μM to 10 μM. This analysis demonstrates that C50−C52 and C56 are important for PNA recognition and are physically accessible for inhibitor binding. We use identification of these key determinants to minimize the size of PNA inhibitors, and knowledge of these determinants should facilitate design of other small molecules capable of targeting telomerase. The striking differences in IC_{50} values for inhibition of telomerase activity by related PNAs emphasize the potential of PNAs to be sensitive probes for mapping complex nucleic acids. We also find that PNA hybridization is sensitive to nearest-neighbor interactions, and that consecutive guanine bases within a PNA strand increase binding to complementary DNA and RNA sequences.

Human telomerase is a ribonucleoprotein that adds repeating units of TTAGGG to the ends of chromosomes (Morin, 1989, 1991; Blackburn, 1992; Lingner *et al.*, 1995; Lunblad & Wright, 1996). Telomerase activity is not found in human somatic cells, with the exception of proliferative cells of renewal tissues, but is present in most established cancer cell lines and primary human tumors, leading to the suggestion that telomerase may be a target for anti-cancer chemotherapy (Kim *et al.*, 1994; Counter *et al.*, 1994; Autexier & Greider, 1996; Holt *et al.*, 1996a). This hypothesis is in dispute, as other mechanisms can regulate telomere length in yeast (Singer & Gottschling, 1994; McEachern & Blackburn, 1995), and because telomerase activity has not been detected in some human tumors (Broccoli *et al.*, 1995; Bryan *et al.*, 1995). Definitive evaluation of the role of telomerase in the development of human cancers will require a detailed understanding of functional determinants of telomerase activity and the design or discovery of highly selective inhibitors (Raymond *et al.*, 1996; Hamilton & Corey, 1996). Such inhibitors might be lead compounds for the development of chemotherapeutic agents, and, even if telomerase-independent mechanisms for telomere maintenance are present, telomerase inhibitors may be a useful component of combination therapy aimed at reduction of telomere length in rapidly dividing cells.

Studies of telomerase that examine the effect of mutations within the RNA component (Autexier & Greider, 1994, 1995; Gilley & Blackburn, 1995; Gilley *et al.*, 1996) have revealed that the RNA active site is functionally divided between an alignment domain for binding substrate and a template domain for polymerization (Figure 1). The 3′ domain of the RNA active site of telomerase binds telomere ends though base-pairing. The polymerase component then elongates the telomere using the 5′ domain as a template. A separate site within a protein component is thought to be an anchor site necessary for promoting processive elongation (Hammond *et al.*, 1997).

Targets for telomerase inhibition include the polymerase active site, the anchor site, RNA or protein domain interfaces, specialized structures that may be formed by newly synthesized telomeric DNA (Salazar *et al.*, 1996), and the RNA template. Proteins associated with human telomerase activity have been reported (Harrington *et al.*, 1997; Nakamura *et al.*, 1997), but the rational design of inhibitors aimed at the polymerase active site or at blocking domain assembly is complicated by the current lack of detailed structural data. The primary sequences of the protein components of telomerase from *Tetrahymena* (Collins *et al.*, 1995) and *Euplotes* (Lingner *et al.*, 1997) have also been described, and the *Euplotes* protein contains motifs previously noted in reverse

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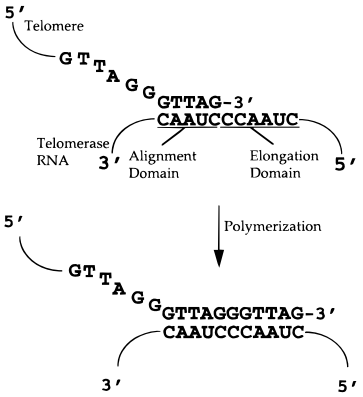

FIGURE 1: Binding of a telomere end by the RNA alignment domain of human telomerase and subsequent polymerization using the template domain to supply sequence information. Repositioning of the extended substrate to the alignment domain permits the addition of multiple repeats (Morin, 1991).

transcriptase. Existing polymerase inhibitors block activity of both ciliate (Strahl & Blackburn, 1994) and human telomerase (Strahl & Blackburn, 1996; Fletcher *et al.*, 1996; Yegorov *et al.*, 1996), and these provide a basis for the design of potent and selective inhibitors. An oligonucleotide-based strategy, that may also generate highly selective inhibitors, uses knowledge of the sequence of the RNA template (Feng *et al.*, 1995). Studies have demonstrated that addition of complementary DNA oligonucleotides (Greider & Blackburn, 1989; Shippen-Lentz *et al.*, 1990; Melek *et al.*, 1994; Feng *et al.*, 1995; Blasco *et al.*, 1995) or peptide nucleic acids (PNAs)[1] (Norton *et al.*, 1996) can inhibit ciliate and mammalian telomerase activity.

PNAs are a DNA analog in which the phosphate backbone has been replaced by (2-aminoethyl)glycine units with the nucleobases attached through methylene carbonyl linkages to the glycine amino group (Nielsen *et al.*, 1991; Hanvey et al., 1995; Hyrup & Nielsen, 1996; Nielsen, 1996; Corey, 1997). This neutral backbone avoids interstrand electrostatic repulsion, allowing complementary sequences to be recognized with high affinity and selectivity relative to hybridization of analogous DNA or RNA oligomers (Egholm, 1993). These properties allow PNAs to be used for quantification of telomere length (Landsdorp *et al.*, 1996), isolation of transcriptionally active DNA (Boffa *et al.*, 1995, 1996), antisense inhibition (Bonham *et al.*, 1995), and screening for mutations (Wang *et al.*, 1996; Carlsson *et al.*, 1996; Thiede *et al.*, 1996; Perry-O'Keefe *et al.*, 1996). Use of PNAs may also prove advantageous because they are not hydrolyzable by proteases or nucleases (Demidov *et al.*, 1994) and exhibit less propensity for nonsequence selective protein binding than phosphorothioate oligomers (Hamilton *et al.*, 1996).

Here we report that PNA-mediated inhibition of human telomerase is sensitive to single base changes in register between short PNAs and overlapping target sequences within the RNA active site. Differences in inhibition caused by the addition or loss of the potential for single base pairs provide insights into the ability of active site nucleotides to contribute to inhibitor recognition at a resolution that is now unobtainable through other methods.

MATERIALS AND METHODS

Oligomer Synthesis. PNAs were synthesized manually as described (Norton *et al.*, 1995) by solid-phase synthesis using Boc (Dueholm *et al.*, 1994) or Fmoc chemistry (Thomson *et al.*, 1995). Butyloxycarbonyl (Boc)-lysine resin [OCH_2 PAM (phenylacetamidomethyl)] was from Applied Biosystems (Foster City, CA), and the Fmoc-*N*-ϵ-*t*-Boc-L-lysine-resin was from Advanced ChemTech (Louisville, KY). PNA monomers were obtained from PerSeptive Biosystems (Framingham, MA), and the Boc-glycine was from Peptides International (Louisville, KY). All PNAs were characterized by matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry using a Voyager-DE mass spectrometry workstation (PerSeptive Biosystems). PNAs were purified by reverse-phase HPLC as described (Norton *et al.*, 1995), lyophilized, and stored in water prior to use. All PNAs contained glycine at the amino termini and lysine at the carboxy termini unless otherwise noted (Table 3). The 9-acridinecarboxylic acid used for amino-terminal modification was from Aldrich (Milwaukee, WI). DNA oligonucleotides were synthesized on a Applied Biosystems (Foster City, CA) 451 DNA synthesizer. RNA oligonucleotides were purchased from Oligos Etc. & Oligo Therapeutics (Wilsonville, OR).

Telomerase Assays. Telomerase activity was detected using the TRAPeze telomerase detection kit (Oncor, Gaithersburg, MD) essentially as described (Holt *et al.*, 1996b). The source of telomerase was human immortal primary breast epithelial cell line (HME50-5) or a cell line derived from immortal human prostrate cancer cells (DU145). Cultured cells (10^5-10^6) were lysed as recommended in the protocol supplied by Oncor. Cell lysates containing 200 cell equiv were preincubated at 25 °C for 30 min with PNA [0−100 μM, diluted in 10 mM Tris, pH 8.3, and 10 μg mL^{-1} bovine serum albumin (BSA)]. We sometimes noted aggregation of PNAs upon prolonged storage, so PNA stocks were briefly heated to 55 °C prior to addition to assay mixtures to ensure reproducible inhibition. Following preincubation, TRAPeze reaction mixture (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.01% BSA, 50 μM dNTPs, and primer mix supplied in the kit) was added directly to the mixture of PNA and cell extract. This reaction was incubated at 25 °C for 30 min to allow telomerase to elongate the TS primer. For control experiments, PNAs were added to a final concentration of 3.3 μM immediately prior to amplification as it has been established that PNAs can inhibit (Ørum *et al.*, 1993; Thiede *et al.*, 1996) or, in some cases, enhance (Demers *et al.*, 1995) the yield in PCR reactions. The tubes were transferred to a PCR thermocycler, and a two-step cycle (30 s 94 °C and 30 s 60 °C) was performed 27 times. Samples were analyzed using nondenaturing polyacrylamide gel electrophoresis.

Telomerase products and an internal PCR control were quantified by phosphorimager analysis (Molecular Dynamics). The lanes were divided into one region encompassing the telomerase products and another including the internal standard signal. The radioisotope density was integrated for each area, and the ratio of telomerase product to internal standard was determined. This value was normalized to a

[1] Abbreviations: PNA, peptide nucleic acid; BSA, bovine serum albumin; IC_{50}, concentration necessary to inhibit 50% maximal activity; T_m, melting temperature; TRAP, telomeric repeat amplification protocol; Ac, acetyl; Bz, benzoyl; Acr, acridinyl; PS, phosphorothioate; PAGE, polyacrylamide gel electrophoresis.

positive control (no PNA added) where the ratio of telomerase products to internal standard was assigned 100% activity. The extent inhibition was plotted against the final PNA concentration used in the preincubation reaction, and these graphs were used to estimate the IC_{50} values.

Melting Temperature Determination. Melting temperature studies were performed spectrophotometrically by measuring the change in absorbance at 260 nm using a 8452A UV spectrophotometer (Hewlett Packard) and an HP 89090A Peltier temperature control accessory (TEMPCO Software). Determinations were done in a volume of 0.5 mL in a 1.0 mL capped cuvette. Equimolar amounts of DNA and PNA (20 μM) were denatured at 90 °C for 5 min in 1.0 M KH_2PO_4/K_2HPO_4, pH 7.6, and reannealed upon cooling to room temperature over at least 20 min. Absorbance was recorded from 15 to 90 °C in 3, 4, or 5 °C increments using a 2 min equilibration time between each measurement. Melting temperatures were measured in duplicate and varied less than 1 °C per experiment. DNA oligomers used for measuring T_m values, and their complementary PNAs, were as follows: 5′-TTTGTCTAACCCTAACTGAGAAGG-3′ (PNAs 4−9, 22−24); 5′-CCATTTTTTGTCTAACCCTAACT-3′ (PNAs 1−3); 5′-TAACTGAGAAGGGCGT-3′ (PNAs 12−16); 5′-ACCCTAACTGAGAAG-3′ (PNAs 10−11); 5′-ACCCTAACTG-3′ (PNAs 20−21); 5′-ACCCTAACT-3′ (PNAs 18, 19, 25−28); 5′-CTAACCCTAAC-3′ (PNAs 7 and 17); 5′-CTAACTCTAAC-3′ (PNAs 7, 17); 5′-CCTAACTG-3′ (PNA 29); 5′-CTAACTGA-3′ (PNA 30); and 5′-TCTAACCC-3′ (PNA 31). RNA oligonucleotides used for measuring T_m values and their complementary PNAs were as follows: 5′-ACCCUAACUG-3′ (PNAs 18−22, 28); 5′-CCUAACUGA-3′ (PNAs 29 and 30); and 5′-UCUAACCC-3′ (PNA 31).

RESULTS AND DISCUSSION

Inhibition of Telomerase by PNAs. We designed a series of PNAs to progressively overlap the telomerase RNA template. We incubated these PNAs with telomerase-containing cell extracts derived from a human immortal primary breast epithelial cell line (HME50-5) and a human prostate cancer-derived cell line (DU145) and assayed their influence on telomerase activity using the telomere repeat amplification protocol (TRAP) (Holt *et al.*, 1996b). In this protocol, telomerase extends an oligonucleotide primer to form elongation products. These products are then amplified by PCR to facilitate their detection. TRAP affords a sensitive and linear response over the range of telomerase activity used in these studies (Holt *et al.*, 1996b), and inclusion of an internal amplification standard in each sample (noted in Figure 2) permits reproducible quantification. The appearance of the internal amplification standard also ensures that added PNAs are not interfering with *Taq* polymerase during amplification.

As a further control, we introduced PNAs at concentrations at least 10 times greater than the measured IC_{50} value directly before initiating PCR and monitored the effect of this addition on the amplification of telomerase products. This control was necessary to ensure that PNAs were not inhibiting the appearance of elongation products by interfering with primer or template during PCR. These additions did not reduce the appearance of amplified telomerase products, confirming that the inhibition we describe below is due to sequence-selective PNA-mediated inhibition of telomerase activity.

Table 1: Effect on the Inhibition of Telomerase Activity of the Position of PNAs Relative to the 11 Nucleotide Telomerase RNA Active Site[a]

Sequence		IC_{50} (μM)	T_m°C
	70 60 55 50 45 40 3′-GAUGCGGGAAGAGU**CAAUCCCAAUC**UGUUUUUUAC-5′ (RNA Template)		
1	**TAG**ACAAAAAATG	>30	63
2	**GTTAG**ACAAAAAA	>30	63
3	**GGTTAG**ACAAAAA	0.1	66
4	**GGGTTAG**ACAA	0.01	71
5	**TAGGGTTAG**ACAA	0.001	75
6	**GTTAGGGTTAG**AC	0.01	73
7	**GTTAGGGTTAG**	0.01	64
8	**CAGTTAGGGTTAG**	0.01	72
9	CTCA**GTTAGGG**	0.01	65
10	TCTCA**GTTAGG**	0.3	58
11	TTCTCA**GTTAG**	0.3	52
12	CCCTTCTCA**GTTA**	0.3	57
13	CGCCCTTCTCA**GT**	0.3	58
14	CCCTTCTCA**GT**	0.4	48
15	GCCCTTCTCA**G**	0.5	56
16	CGCCCTTCTCA	10	53
17	**GTTAGaGTTAG**	>30	33
11/3	**TTCTCAGTTAG/GGTTAG**ACAAAAA	0.1	

[a] PNA sequences are listed from N to C termini. The RNA active site and complementary nucleotides within PNAs are in boldface. Numbering of telomerase RNA is noted (Feng *et al.*, 1995). PNA 17 contains an adenine (lower case) that is mismatched opposite C51.

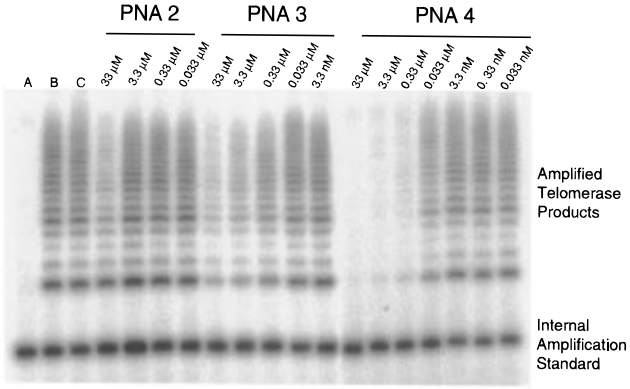

FIGURE 2: Effect on inhibition of targeting PNAs to overlapping positions on the telomerase template. Amplified telomerase products were resolved by PAGE and quantitated as described (Materials and Methods). Controls were performed lacking cell extract (A), in the absence of PNA (B), and with 3.3 μM PNA 4 added immediately before the PCR amplification (C). The sequences and IC_{50} values for PNAs 2−4 are shown in Table 1.

Effect of Recognition of Cytidines 50−52 by Overlapping PNAs. We began our analysis with PNA 1, which was complementary to the last three nucleotides at the 5′ end of the RNA template and adjacent nontemplate nucleotides. PNA 1 did not effectively inhibit telomerase activity (Table 1), a result consistent with observations from reconstitution experiments by Greider and co-workers showing that the region adjacent to the 5′ terminus of the template region of human telomerase is not essential for telomerase activity (Autexier *et al.*, 1996). PNAs 2, 3, and 4 progressively overlap three cytidines (C50−C52) within the center of the RNA active site. Binding to one of these cytidines (C50) by PNA 2 did not result in significant inhibition, while occlusion of C50−51 by PNA 3 or C50−52 by PNA 4 led to IC_{50} values of 100 nM and 10 nM, respectively (Figure 2 and Table 1). The finding that the potential for recognition

of C51 by PNA 3 and C52 by PNA 4 leads to more potent inhibition is strong evidence that both C51 and C52 are determinants for inhibition of human telomerase activity.

Scanning toward the 3′ end of the template yielded PNAs 5−9 that inhibited telomerase with IC_{50} values of 1−10 nM. Increased inhibition by PNA 5 relative to PNA 4 indicates that nucleotides U53 and A54 participate in PNA recognition, although this effect is not as dramatic as that observed with C51 and C52. Similarly, increased inhibition by PNA 5 relative to PNA 6 suggests that the potential for recognition of uridine 42 and 43 may also enhance inhibition. Scanning PNAs 10−12 began to progressively expose C50−52 from the 5′ direction and resulted in an increase in IC_{50} values from 10 to 300 nM, a result that supports C50−52 as a critical region for determining PNA-mediated inhibition. In particular, the 30-fold increase in IC_{50} for PNA 10 relative to PNA 9 implicates C50 as a determinant for PNA recognition. To further examine the importance of C50−52, we assayed PNA 17 containing a mismatched adenine opposite the central cytidine (C51). This alteration reduced inhibition by 1000-fold, emphasizing the exquisite sensitivity of PNA binding to exact complementarity at the target site, a result consistent with those observed by Nielsen and coworkers for hybridization to DNA or RNA oligonucleotides (Egholm *et al.*, 1993). Addition of adjacent PNAs in tandem (PNAs 11 and 3, Table 1) did not result in improved inhibition, suggesting that binding at the domain interface is not cooperative.

These results demonstrate that C50−52 are accessible for PNA recognition and are important determinants for PNA-meditated inhibition of human telomerase activity. Inhibition by PNAs 11−13 bases in length only approaches optimal if they possess the potential to bind to C50−52, and drops off rapidly as this potential is lost. It is interesting to note that C50−52 are at the junction of the 3′ telomerase RNA domain that binds to telomere ends and the 5′ domain that acts as template for strand elongation (Figure 1). The importance of recognition of these nucleotides for inhibition may reflect the requirement for them to be accessible during these critical events. The importance of C50−52 also suggests that they can be the target for the design of a novel class of small molecule inhibitors that can combine binding to cytidine triplets with recognition of additional telomerase-specific determinants.

There are several explanations for the lack of inhibition with PNAs 1 and 2 and relative lack of inhibition by PNAs 3 and 10−16. They may bind to the telomerase RNA, but not to residues that are essential for telomerase function. Alternatively, all or part of the RNA target sequence may not be accessible for PNA binding. The latter phenomenon may weaken any binding that does occur sufficiently that the PNAs can be displaced upon polymerization. We do not distinguish between these possibilities, but do demonstrate that optimal PNA inhibition is dependent upon association with sequence-specific determinants in the active site. The fact that addition of some PNAs yields efficient inhibition is positive evidence that the RNA that they are directed to is accessible and available for Watson−Crick base pairing, and that binding to the targeted residues will interfere with strand elongation.

Effect of Recognition of the 3′ Region of the RNA Template by PNAs. We extended scanning toward the 3′ end of the telomerase RNA by synthesizing PNAs 12−16. These PNAs continued to inhibit telomerase with IC_{50} values of approximately 300−500 nM. Between 9 and 11 bases of these PNAs are complementary to RNA past the 3′ termini of the active site (C56−G63), and inhibition suggests that some of these nucleotides must be accessible for hybridization. This conclusion is supported by earlier observations that the analogous 3′ region within the *Tetrahymena* RNA component is accessible to *in vivo* methylation footprinting (Zaug & Cech, 1995). As PNA's 15 and 16 reached the extreme 3′ end of the alignment domain, IC_{50} values increased to 0.5 μM and 10 μM, respectively. The jump in IC_{50} from 0.5 μM to 10 μM upon losing the ability to recognize C56 indicates that this cytidine is accessible and important for oligomer recognition. Accessibility of C56 is consistent with the assumption that it is available to recognize and align substrate DNA. It is important to note that PNA 5, which possesses the lowest IC_{50} value, 1 nM, and PNA 4, which possesses an IC_{50} value of 10 nM, have the potential to bind determinants C50−C52, but not C56. Therefore, while C56 appears to be a useful determinant for inhibitor binding, its recognition is not essential for inhibition of telomerase by PNAs 11−13 bases in length as they are long enough to bind through interactions with multiple lesser determinants.

Measurement of Melting Temperatures of PNAs to Complementary Sequences. We measured melting temperatures (T_m) of duplexes between PNAs and their DNA complements to ensure that the PNAs used in these studies could form duplexes with complementary sequences at the assay temperature of 25 °C. DNA oligonucleotides were used for most assays because employment of DNA rather than RNA facilitated analysis of the large number of PNAs that required assay. Comparison to RNA−PNA melting temperatures is justified because T_m's for PNA−RNA hybrids are at least as high as those for PNA−DNA hybrids (Egholm *et al.*, 1993) (Table 3). We observed that PNAs 2, 3, and 4 varied greater than 3000-fold in IC_{50} values and had similar melting temperatures of 63, 66, and 71 °C, respectively (Table 1), demonstrating that their differing potentials for binding complementary sequences were not responsible for the large differences in inhibition by these PNAs. Similarly, PNAs 10−16, which inhibit less, all have melting temperatures that significantly exceed the assay temperature (Table 1). Thus, while we cannot determine whether suboptimal inhibition is caused by a failure to bind to telomerase, a failure to block a critical nucleotide, or the likelihood that a PNA inhibitor will be displaced, we can conclude that a lack of inhibition is not due to an inability of any of the PNAs tested to bind to complementary sequences at the assay temperature. It is likely, therefore, that the level of inhibition of telomerase is at least partially governed by structural and functional determinants that are unique to telomerase.

Interestingly, PNAs containing three consecutive guanines exhibit substantially higher melting temperatures than similar PNAs that contain three cytosines (Table 1 and Figure 3A). For example, the 11 base PNA 14 of sequence CCCTTCT-CAGT possessed a T_m of 48 °C, while the 11 base PNA 4 of sequence GGGTTAGACAA exhibited a T_m of 71 °C, despite the fact that the ratio of guanine to cytosine within PNA 4 (5/11) is lower than that of PNA 14 (6/11). These results suggest that consecutive guanines within a PNA permit tight binding to target sequences, and the suprisingly large difference in T_m between PNAs 4 and 14 supports the need to determine melting temperature values for PNA

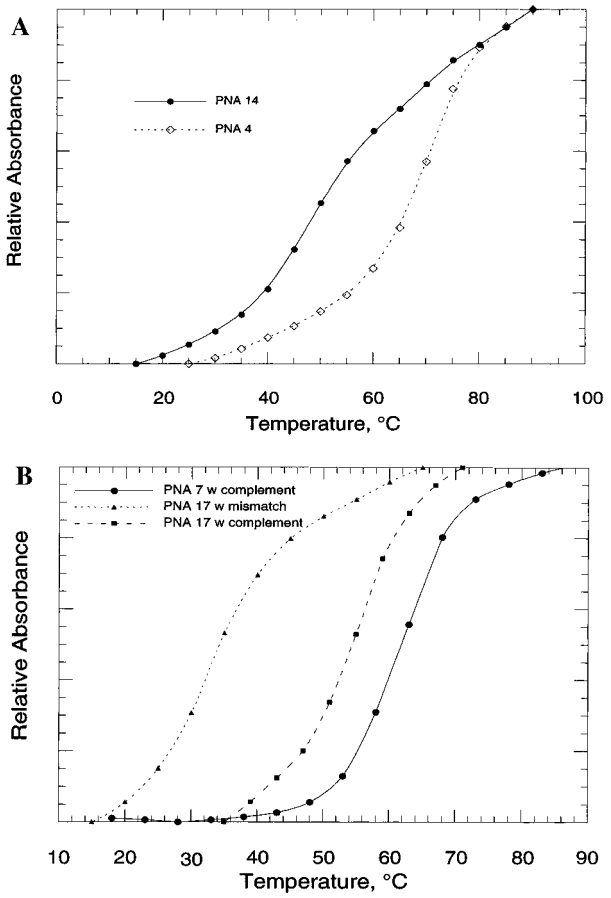

FIGURE 3: (A) Relative melting temperatures of PNAs 4 and 14 with complementary DNA oligonucleotides. (B) Relative melting temperatures of PNA 7 with its complementary DNA oligonucleotide and PNA 17 with a complementary DNA oligonucleotide or a DNA oligonucleotide containing a cytidine opposite the central adenine. Values for the *Y* axis are normalized to account for differing initial absorbances. Sequences and IC_{50} values for PNAs 4, 7, 14, and 17 are shown in Table 1.

recognition of complementary sequences in addition to IC_{50} values. The importance of recognition of the three guanines for high affinity is further supported by analysis of PNA 17, in which recognition of consecutive cytidines is disrupted by insertion of adenine in place of the central guanine. This PNA exhibited a reduction of T_m relative to PNA 7 from 65 to 56.8 °C for hybridization to a DNA oligonucleotide with exact complementarity, and 33 °C to a DNA oligonucleotide containing a mismatch (Table 1 and Figure 3B).

Nearest-Neighbor Effects on PNA Hybridization. To systematically address the influence of consecutive guanines within the PNA strand on melting temperatures of PNA/DNA and PNA/RNA duplexes, we made a series of PNAs based on PNA 7, an 11 base PNA with 3 consecutive guanines in its middle positions (Table 1). We compared the melting temperatures of the duplexes formed by these PNAs with analogous DNA/DNA, DNA/RNA, and DNA/phosphorothioate (PS) duplexes to determine if the melting temperatures of PNA-containing duplexes are more dependent on nearest-neighbor interactions. The importance of nearest-neighbor interactions for duplex formation by DNA has been extensively analyzed (Ornstein & Fresco, 1983; Breslauer *et al.*, 1986; Delcourt *et al.*, 1991), and recent work has indicated that DNA duplex stability follows the trend GC > CG > GG > GA ∼ GT ∼ CA > CT > AA > AT > TA (SantaLucia *et al.*, 1996).

We first replaced the three consecutive guanines with cytosines to examine whether this would alter the melting temperature of PNA-containing duplexes even though the flanking bases and the overall number of cytosine/guanine base pairs remain the same. As we observed for PNAs 4 and 14 (Table 1 and Figure 3A), this substitution had a substantial influence on melting temperature. PNA/DNA duplex A (GGG^{PNA}/CCC^{DNA}) and PNA/RNA duplex B (GGG^{PNA}/CCC^{RNA}) in which the PNA strand contains consecutive guanines had 17 and 26 °C higher melting temperatures duplexes than duplexes F (CCC^{PNA}/GGG^{DNA}) and G (CCC^{PNA}/GGG^{RNA}) in which the PNA strand contained consecutive cytosines (Table 2). Comparison of analogous DNA/DNA, DNA/RNA, and DNA/PS duplexes C, D, E, H, I, and J showed much smaller differences in melting temperature as a function of context for the consecutive guanines for the DNA/RNA and DNA/PS duplexes and no difference for the DNA/DNA duplexes.

We then replaced the consecutive guanines with adenines or thymines to examine whether the difference in melting temperature was due to altered purine content within the PNA strand. If consecutive purines were enhancing the melting temperature, then the stability of a duplex containing consecutive adenines, while still expected to be lower than one containing consecutive guanines, should be higher than an analogous duplex containing thymidines. We did not observe this, as duplex M (AAA^{PNA}/TTT^{DNA}) containing consecutive adenines within the PNA strand actually has a slightly lower melting temperature than duplex K (TTT^{PNA}/AAA^{DNA}) in which the PNA strand contained consecutive thymines. Duplexes M and K exhibit higher melting temperatures than analogous DNA/DNA duplexes N and L, but the differences, 4 and 11.5 °C, respectively, are much smaller than the 24 °C difference observed between duplexes A (GGG^{PNA}/CCC^{DNA}) and C (GGG^{DNA}/CCC^{DNA}).

Finally, we replaced the middle or either of the flanking guanines within the central triplet (Table 2). All of these substitutions reduced the melting temperature for complementary PNA/DNA duplexes. When the middle guanine was replaced by adenine (O, GAG^{PNA}/CTC^{DNA}), thymidine (Q, GTG^{PNA}/CAC^{DNA}), or cytosine (S, GCG^{PNA}/CGC^{DNA}), the melting temperature was reduced approximately 7, 13, and 7 °C, respectively. The greater decrease in T_m shown by duplex Q that had a PNA strand containing GTG relative to duplex O that had a PNA strand containing GAG suggests that, at least in this context, consecutive purines may yield some stabilization. Duplex U which contains a PNA lacking any middle nucleobase of the triplet showed the largest decrease in melting temperature, emphasizing the importance of consecutive bases, even if they do not result in optimal stability. We also substituted the N- or C-terminal guanine of the triplet with cytosine to yield duplexes V (CGG^{PNA}/GCC^{DNA}) and X (GGC^{PNA}/CCG^{DNA}), and observed that these limited alterations still reduced melting temperatures by 8 °C when compared to duplex A.

Our experiments suggest that no substitution within the guanine triplet will yield a more stable PNA−DNA duplex. Strong binding to C50−52 by consecutive guanines probably contributes to the importance of recognition of these bases for PNA-mediated inhibition especially by relatively short PNAs (see below), although the importance for binding of 11−13 base PNAs is probably less. The origins of this preference will remain unknown pending

Table 2: Influence of Nearest-Neighbor Interactions on Melting Temperature; Bases Being Exchanged Are in Boldface[a]

	Duplex	T_m °C		Duplex	T_m °C
A	PNA GTTA**GGG**TTAG DNA 3'-CAAT**CCC**AATC-5'	64.0	O	PNA GTTA**GAG**TTAG DNA 3'-CAAT**CTC**AATC-5'	56.8
B	PNA GTTA**GGG**TTAG RNA 3'-CAAU**CCC**AAUC-5'	64.2	P	DNA 5'-GTTA**GAG**TTAG-3' DNA 3'-CAAT**CTC**AATC-5'	40.5
C	DNA 5'-GTTA**GGG**TTAG-3' DNA 3'-CAAT**CCC**AATC-5'	42.0	Q	PNA GTTA**GTG**TTAG DNA 3'-CAAT**CAC**AATC-5'	51.3
D	DNA 5'-GTTA**GGG**TTAG-3' RNA 3'-CAAU**CCC**AAUC-5'	34.6	R	DNA 5′-GTTA**GTG**TTAG-3' DNA 3'-CAAT**CAC**AATC-5'	40.6
E	PS 5'-GTTA**GGG**TTAG-3' DNA 3'-CAAT**CCC**AATC-5'	32.3	S	PNA GTTA**GCG**TTAG DNA 3'-CAAT**CGC**AATC-5'	57.3
			T	DNA 5'-GTTA**GCG**TTAG-3' DNA 3'-CAAT**CGC**AATC-5'	42.7
F	PNA GTTA**CCC**TTAG DNA 3'-CAAT**GGG**AATC-5'	47.0			
			U	PNA GTTA**G**/GlyGly/**G**TTAG DNA 3'-CAAT**CCC**AATC-5'	28.7
G	PNA GTTA**CCC**TTAG RNA 3'-CAAU**GGG**AAUC-5'	38.4			
H	DNA 5'-GTTA**CCC**TTAG-3' DNA 3'-CAAT**GGG**AATC-5'	42.0	V	PNA GTTA**CGG**TTAG DNA 3'-CAAT**GCC**AATC-5'	55.9
I	DNA 5'-GTTA**CCC**TTAG-3' RNA 3'-CAAU**GGG**AAUC-5'	30.0	W	DNA 5'-GTTA**CGG**TTAG-3' DNA 3'-CAAT**GCC**AATC-5'	44.1
J	PS 5'-GTTA**CCC**TTAG-3' DNA 3'-CAAT**GGG**AATC-5'	29.7	X	PNA GTTA**GGC**TTAG DNA 3'-CAAT**CCG**AATC-5'	56.1
K	PNA GTTA**TTT**TTAG DNA 3'-CAAT**AAA**AATC-5'	43.1	Y	DNA 5'-GTTA**GGC**TTAG-3' DNA 3'-CAAT**CCG**AATC-5'	44.4
L	DNA 5'-GTTA**TTT**TTAG-3' DNA 3'-CAAT**AAA**AATC-5'	31.6			
M	PNA GTTA**AAA**TTAG DNA 3'-CAAT**TTT**AATC-5'	39.1			
N	DNA 5'-GTTA**AAA**TTAG-3' DNA 3'-CAAT**TTT**AATC-5'	35.1			

[a] PNAs are listed from N to C termini. All melting temperatures are ±0.9 °C or less.

structural analysis, but PNA−DNA duplexes have been observed by NMR to be relatively flexible (Brown *et al.*, 1994; Eriksson *et al.*, 1996) and this may lead to a more pronounced dependence of melting temperatures on nearest-neighbor effects. An alternative explanation for enhanced stability is formation of triplex or higher order structures that are favored by consecutive guanines. Regardless of the mechanisms that contribute to the strength of PNA binding, it is likely to be generally true that predictions of the stability of PNA hybridization will have to be made cautiously, and that mismatch detection schemes that target consecutive guanines may maximize stability differences between fully complementary and mismatched sequences (Figure 3B).

Use of Critical Binding Determinants To Identify Minimal PNA Inhibitors. Our earlier studies of PNA-mediated inhibition of telomerase demonstrated efficient inhibition by PNAs 11−13 bases in length, with 1000−10 000-fold lower inhibition by shorter PNAs (Norton *et al.*, 1996). However, none of the shorter PNAs assayed in the previous study had been designed to recognize both critical determinants for inhibition identified by our PNA scanning: the 3′ end of the template, C56; and the internal cytidine triad, C50−52. We used this information to synthesize a series of PNAs 6−10 bases in length (PNAs 18−22, Table 3), that were anchored at A49, directly adjacent to the 5′ side of the cytidine triad, and moved progressively toward the 3′ end of the template.

In all cases, the measured melting temperatures for duplexes containg PNA and complementary DNA (35−59 °C) or RNA (47−64 °C) were substantially above the assay temperature of 25 °C (Table 2). PNAs 18 and 19 containing 6 or 7 bases were poor inhibitors, while PNAs 20−22 containing 8, 9, or 10 bases inhibited with IC_{50} values of 300, 40, and 10 nM, respectively (Table 3). The additional inhibition observed upon recognition of C56 and U57 demonstrates that these nucleotides are available for recognition. We conclude that recognition of C50−52, while necessary, is not sufficient for optimal inhibition by PNAs, and that inhibition can be increased by also recognizing C56 and U57. The value of identification of accessible determinants for inhibitor design is emphasized by the finding

Table 3: Effect of PNA Size and Complementarity on Inhibition of Telomerase Activity and Melting Temperature (T_m) with DNA and RNA[a]

	Sequence	IC_{50} (μM)	T_m°C (DNA)	T_m°C (RNA)
	60 55 50 45			
	3'-AAGAGU**C**AAU**CCC**AAUCUG-5'			
18	Gly-TAGGGT	30	35	47
19	Gly-TTAGGGT	20	39	51
20	Gly-GTTAGGGT	0.3	47	57
21	Gly-AGTTAGGGT	0.04	54	60
22	Gly-CAGTTAGGGT	0.01	59	64
23	Gly-TAGGGTTAG	1	58	
24	GTTAGGGT	0.2	52	
25	Ac-GTTAGGGT	0.3	54	
26	Bz-GTTAGGGT	0.2	50	
27	Acr-GTTAGGGT	0.3	60	
28	AGTTAGGG	0.03	47	58
29	CAGTTAGG	0.4	40	52
30	TCAGTTAG	>30	33	38
31	GGGTTAGA	3	51	51

[a] PNAs are listed N to C termini. Underlined and boldfaced nucleotides are those suggested during initial PNA scanning (Table 1) as being critical for optimal inhibition. All PNAs contained a C-terminal lysine. N-Terminal modifications, if any, are noted. Ac, acetyl; Bz, benzoyl; Acr, acridinyl. Numbering of telomerase RNA is noted (Feng *et al.*, 1995).

that eight base PNA 20 (T_m for DNA = 47 °C) that recognizes both C50−52 and C56 inhibits better than nine base PNA 23 (T_m for DNA = 58 °C) that recognizes C50−52 but not C56 (Table 3).

Effect of N-Terminal Modification on PNA Inhibition. We attempted to further enhance inhibition by synthesizing PNAs 25−27 containing chemically modified N-termini. Our hypothesis was that these modifications might form hydrophobic interactions with either the RNA or the protein component that would act to stabilize PNA binding. While the acridinyl-modified PNA did possess a higher melting temperature to its complementary DNA oligomer (Table 3) than did unmodified PNA 24, inhibition of human telomerase was not significantly enhanced. Apparently either this chemical modification cannot make the contacts responsible for increasing hybridization to an isolated complementary oligonucleotide, or it can make contacts but these are not relevant for enhancing inhibition. While these experiments are inconclusive regarding the potential for terminal modifications to enhance inhibition of telomerase activity, the fact that PNAs can be readily modified suggests that as human telomerase becomes available in larger quantities PNAs may be a valuable tool for delivery of affinity labels to map reactive amino acids and nucleotides near the RNA active site.

Optimization of Inhibition by Taking Advantage of the Known Determinants C50−52, C56, and U57. Both PNA length and position relative to the RNA active site are critical for optimal inhibition. Once we had established that C50−52, C56, and U57 were determinants for PNA-mediated inhibition, we synthesized an eight base PNA (PNA 28) to span the region they define. This PNA exhibited an IC_{50} value of 30 nM, the lowest for any eight base PNA assayed and 30-fold lower than nine base PNA 23 that only recognized C50−52 (Table 3). This result demonstrates that PNA inhibition can be progressively refined as determinants of recognition are identified. It is essential to note that this potent inhibition is achieved in spite of PNA 28 possessing a melting temperature of 47 °C for DNA, 11°C lower than that of PNA 23. Therefore, while the high affinity that we describe for hybridization of PNAs containing consecutive guanines undoubtedly plays a role in efficient inhibition by the shorter PNAs described in this study, the exact location is equally if not more important, even for relatively short PNAs.

Identification of a highly inhibitory eight base PNA allowed further demonstration of the importance of binding to individual template nucleotides. We synthesized a series of PNAs eight bases in length (PNAs 29−31, Table 3) to further assay the importance of binding to C50−52. PNA 29 which uncovered C50 yielded an IC_{50} value 10-fold higher than for PNA 28. No inhibition was observed for PNA 30 which uncovered determinant C51 as well as C50. These results are consistent with those observed with the longer PNA inhibitors (PNAs 2−4 and 9−11) that indicated template bases C50−C52 must be recognized for optimal inhibition and confirms the specific importance of binding to C50 and C51. As we would predict, the melting temperature of PNAs 29 and 30 decreased as the potential for binding to consecutive cytidines was removed, and this is likely to contribute to the reduced IC_{50} values. PNA 31 covered C50-C52, but was extended in the 5′ direction of the RNA template and no longer had the potential to bind C56 and U57 (Table 3). It inhibited telomerase with an IC_{50} that was 100-fold higher than the IC_{50} value for PNA 28. These results further demonstrate that recognition of C50−52 is important, but not sufficient, for optimal inhibition and that binding to multiple separate determinants is necessary for optimal inhibition by PNAs. Binding to the 5′ side of the template relative to C50−52 is less important for inhibition than binding to the 3′ side containing C56 and U57.

Importance of Other Nucleotides within the RNA Active Site. PNA scanning confirms the accessibility of nucleotides C50−52, C56, and U57 for base-pairing. Why should recognition of some nucleotides be especially critical for PNA-mediated recognition? The RNA active site must function in conjunction with the protein subunits, and may be involved in structural interactions with these subunits or with other regions of the RNA. As a result, all 11 nucleotides may not be equally available for optimal base-pairing with inhibitory oligomers when the ribonucleoprotein is not bound to substrate. Furthermore, once bound, some PNAs may be more susceptible than others to displacement during polymerization. Our results do not exclude the possibility that other nucleotides play important roles in oligomer recognition, and scanning strategies using shorter PNAs or PNAs containing systematically placed mismatches can be used to identify and rank more subtle contributions to inhibitor recognition. Additional studies of the kinetics of PNA inhibition may also allow an understanding of the reasons for poor inhibition by some PNAs. Results gained from probing with PNAs should complement those gained from mutagenesis and mapping using nucleases or metal-dependent cleavage to afford a more definitive picture of RNA accessibility, structure, and function.

Conclusion. Our studies introduce PNA scanning as an approach for systematically mapping the recognition of complex nucleic acids. Series of PNAs can be readily synthesized, and this method should be widely applicable. We find that the importance of recognition of RNA nucleotides within the RNA active site of human telomerase to inhibitors differs and that cytidines 50−52 and 56 and uridine 57 are important determinants for inhibition of telomerase. At a minimum, these results indicate that these nucleotides

are physically exposed for inhibitor binding. Cytidines 50−52 occur at the functional boundary between the alignment and elongation domains, and may represent a physical boundary element as well. The accessible nucleotides we identify facilitated the design of small PNA inhibitors, and we anticipate that they can be key recognition elements for inhibition of human telomerase by even smaller molecules.

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