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High-affinity and specific recognition of human thyroid stimulating hormone (hTSH) by *in vitro*-selected 2′-amino-modified RNA

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ABSTRACT

RNA sequences containing 2′-amino pyrimidines that bind with high-affinity to human thyroid stimulating hormone (hTSH) were isolated from a random sequence library by an *in vitro* selection–amplification procedure. A representative RNA ligand (T-15) has an equilibrium dissociation constant (K_d) of 2.5 nM for its interaction with hTSH and can discriminate between other members of the glycohormone family; no detectable binding was observed at low micromolar concentrations of hCG (human chorionic gonadotropin), while measured K_d values for the interactions with hLH (human leutinizing hormone) and hFSH (human follicle stimulating hormone) were >1 μM and ~0.2 μM, respectively. The detection of hTSH in a dot blot assay with radiolabeled T-15 RNA was demonstrated.

INTRODUCTION

Specific molecular recognition is central to the detection of disease causing agents. Although most diagnostic tests are based on antibody ligands, other molecules capable of fulfilling the requirements for specific molecular recognition are being discovered (1–3). The *S*ystematic *E*volution of *L*igands by *EX*ponential enrichment (SELEX) process, which utilizes *in vitro* selection and amplification of nucleic acids, is a combinatorial approach for the isolation of specific sequences with unique properties of interest from random sequence libraries of oligonucleotides (4–6). The complexity (or sample size) of a typical starting library is ~10^{14} molecules. The outcome of an *in vitro* selection experiment is a population of sequences whose functional properties were dictated by the selection criterion employed in the process. Examples include high-affinity nucleic acid sequences selected for a variety of target molecules (4–19) and sequences that can either undergo autocatalysis of novel reactions (20,21) or function as an enzyme in the chemical transformation of a substrate molecule (22).

Recently, random sequence oligonucleotide libraries consisting of 2′-amino pyrimidines have been used to isolate modified oligonucleotide ligands with enhanced stability in biological fluids (12,17). Such modified oligonucleotide ligands are attractive candidates for both therapeutic and diagnostic applications. SELEX-derived small oligonucleotides (~20 kDa) typically exhibit affinities similar to those of relatively large antibodies (~160 kDa). This feature, along with the availability of modified oligonucleotide random sequence pools with enhanced nuclease stability, prompted us to exploit the SELEX process for the isolation of nucleic acid ligands with high affinity for human thyroid stimulating hormone (hTSH), a hormone commonly used as a diagnostic marker for thyroid abnormalities.

hTSH (M_r 27.7 kDa) is a glycohormone secreted by the pituitary that controls the synthesis of hormones by the thyroid gland. Measurement of serum hTSH levels is important in the diagnosis of both pituitary and thyroid disorders such as hyperthyroidism and hypothyroidism (23,24). Pituitary-derived hTSH, hLH (leutinizing hormone) and hFSH (follicle stimulating hormone), along with hCG (chorionic gonadotropin) secreted by placenta, constitute a family of glycohormones (23–27). Members of this glycohormone family have very similar structural properties. Each hormone is a heterodimer composed of non-covalently associated subunits (α and β). All four members have an identical α subunit. Each member of the family exerts its biological response via interacting with a cell surface receptor. While the β subunits confer specificity, there is a high degree of sequence similarity among the members. The sequence similarity within the first 114 amino acids of the β-subunit of hCG is 85% with hLH, 46% with hTSH and 36% with hFSH (27). Further, the β-subunit specific to one member and the α-subunit derived from a different member have been reconstituted into a heterodimer that elicited the biological response of the hormone derived from the β-subunit (23,25). Due to these structural similarities, the development of immunological assays for the specific detection of each hormone has been challenging (25).

In this study, the SELEX process was used to isolate high affinity RNA ligands for hTSH from a random sequence RNA pool containing 2′-amino pyrimidines with a complexity of ~10^{14} individual sequences. After nine rounds of selection and amplification of hTSH-bound RNA, two families of sequences emerged. A representative RNA ligand (T-15) of one family characterized by a predicted stem–loop structure bound hTSH

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with an equilibrium dissociation constant (K_d) of 2.5 nM. Importantly, this ligand bound much more weakly to other members of the glycohormone family. The use of radiolabeled ligand T-15 to detect hTSH in a dot blot assay suggests that SELEX-derived oligonucleotide ligands have potential use as diagnostic reagents.

MATERIALS AND METHODS

DNA sequences were synthesized by standard cyanoethyl phosphoramidite chemistry. After deprotection, DNA sequences were purified by gel electrophoresis under denaturing conditions. 2′-NH_2-modified UTP and CTP were synthesized as described previously (28). All RNA sequences used in the study were prepared by *in vitro* transcription of DNA templates (29) and purified by denaturing gel electrophoresis. hLH (M_r 35 500) and hFSH (M_r 38 250) were from Becton Dickinson (Research Triangle Park, NC). hTSH (M_r 27 700; 8 IU/mg) was from either Becton Dickinson or Vitro Diagnaostics (Littleton, CO). hCG (M_r 42 000; 14 000 IU/mg) was from Vitro Diagnaostics. The α-subunit and the β-subunit of hTSH were obtained from Calbiochem (La Jolla, CA). Enzymes and other chemicals of analytical grade were purchased from commercial sources.

The SELEX process

Five nanomoles of gel-purified, synthetic template DNA containing 40 nucleotide (nt) contiguous random sequence flanked by defined primer annealing sequences [5′-GGGAGGACGATGCGG-$(N)_{40}$-CAGACGACTCGCCCGA-3′] were amplified by four cycles of the polymerase chain reaction (PCR) with primers 5′-TAATACGACTCACTATAGGGAGGACGATGCGG-3′ and 5′-TCGGGCGAGTCGTCTG-3′. Approximately 800 pmol of the PCR-derived template DNA (~5 × 10^{14} molecules) were transcribed *in vitro* by T7 RNA polymerase (1000 U) in a 3-ml transcription reaction consisting of 40 mM Tris–HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM Spermidine, 5 mM DTT, 0.002% Triton X-100 (v/v), 4% polyethylene glycol (w/v) and 2 mM each of ATP, GTP, 2′-NH_2CTP and 2′-NH_2UTP (17). Full-length transcription products were purified on 8% polyacrylamide gels under denaturing conditions, suspended in TEM buffer (binding buffer; 10 mM Tris–HCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, pH 6.8), heated to 70°C and chilled on ice. The RNA was then incubated with hTSH at 37°C for 15 min. The RNA–protein mixture was filtered through a pre-wet nitrocellulose filter and the filter was immediately washed with 5 ml binding buffer. Bound RNAs were eluted from the filter (6), recovered by ethanol precipitation and reverse transcribed by avian myeloblastosis virus reverse transcriptase (Life Sciences) at 48°C for 45 min with the DNA sequence 5′-TCGGGCGAGTCGTCTG-3′ as the primer. Following PCR amplification of the cDNA, the resulting duplex DNA template was transcribed *in vitro* to obtain RNA for the next round of selection.

The concentration of hTSH in the binding reaction was decreased gradually in successive rounds from 3 µM to 200 nM to progressively increase selective pressure. The selection process was repeated until the affinity of the enriched RNA pool for hTSH was substantially increased. At that point, cDNA was amplified by PCR with primers 5′-CCGAAGCTTAATACGACTCACTATAGGGAGGACGATGCGG-3′ and 5′-GCCGGATCCTCGGGCGAGTCGTCTG-3′, which introduced *Bam*HI and *Hin*dIII restriction sites (underlined) at the 5′- and 3′-ends of the PCR products, respectively. The PCR products were digested with *Bam*HI and *Hin*dIII and cloned into pUC18 that had been digested with the same enzymes. Individual clones were screened and sequenced using standard techniques (30).

Equilibrium dissociation constants (K_d values)

Internally-labeled RNA was synthesized by using [α-^{32}P]ATP in *in vitro* transcription reactions. Full-length transcripts were purified by polyacrylamide gel electrophoresis under denaturing conditions. RNA suspended in TEM buffer (~5 nM) was heated to 80°C, chilled on ice and then transfered to room temperature. Protein binding was carried out with low concentrations of radiolabeled RNA (typically ~10 pM) to satisfy saturation binding at protein excess conditions. RNA was incubated with varying amounts of hormone in 50 µl TEM buffer containing 0.01% hSA for 10 min at 37°C. RNA–protein mixtures were passed through pre-wet nitrocellulose filters (0.2 µm) and the filters were immediately washed with 5 ml binding buffer. Radioactivity retained on filters was quantitated by liquid scintillation counting. The quantity of RNA bound to filters in the absence of hormone was used for background correction. The percentage of input RNA retained on each filter was plotted against the corresponding log protein concentration. The non-linear least-squares method was used to obtain the dissociation constant (K_d) (11).

Thermal melting

For thermal melting, the full-length sequence T-15 was synthesized by *in vitro* transcription and purified by denaturing polyacrylamide gel electrophoresis. RNA was heated in TEM or sodium phosphate buffer to 95°C and cooled to room temperature prior to the determination of its melting profile. Thermal melting profiles of RNA were obtained on a Cary Model 1E spectrophotometer by recording the absorbance at 260 nm while the sample was heated at 1°C/min. The melting point (T_m) was calculated using the first derivative values.

Dot blot assay

hTSH was suspended in TEM buffer (300 µl) containing 0.1% hSA (w/v) and applied to pre-wet nitrocellulose filters (0.45 µM; BioRad) under suction. Gel-purified, internally labeled RNA T-15 was then applied to the blots in 50 µl TEM buffer (~0.5 pmol/µl) and filtered gently. Filters were immediately washed three times with 300 µl of the same buffer followed by two times with 300 µl 0.5 M urea in the same buffer to eliminate most of background binding of RNA to nitrocellulose filters. The blots were dried and analyzed with a PhosphorImager and by autoradiography.

RESULTS

Selection

A selection carried out in the low ionic strength TEM buffer resulted in a progressive increase in affinity of the enriched RNA pools for hTSH. Sequences obtained from the 9th round PCR products are shown in Figure 1. There were 21 unique sequences among the 37 sequences analyzed. The selected purine-rich RNA sequences share a somewhat conserved 9 nucleotide sequence;

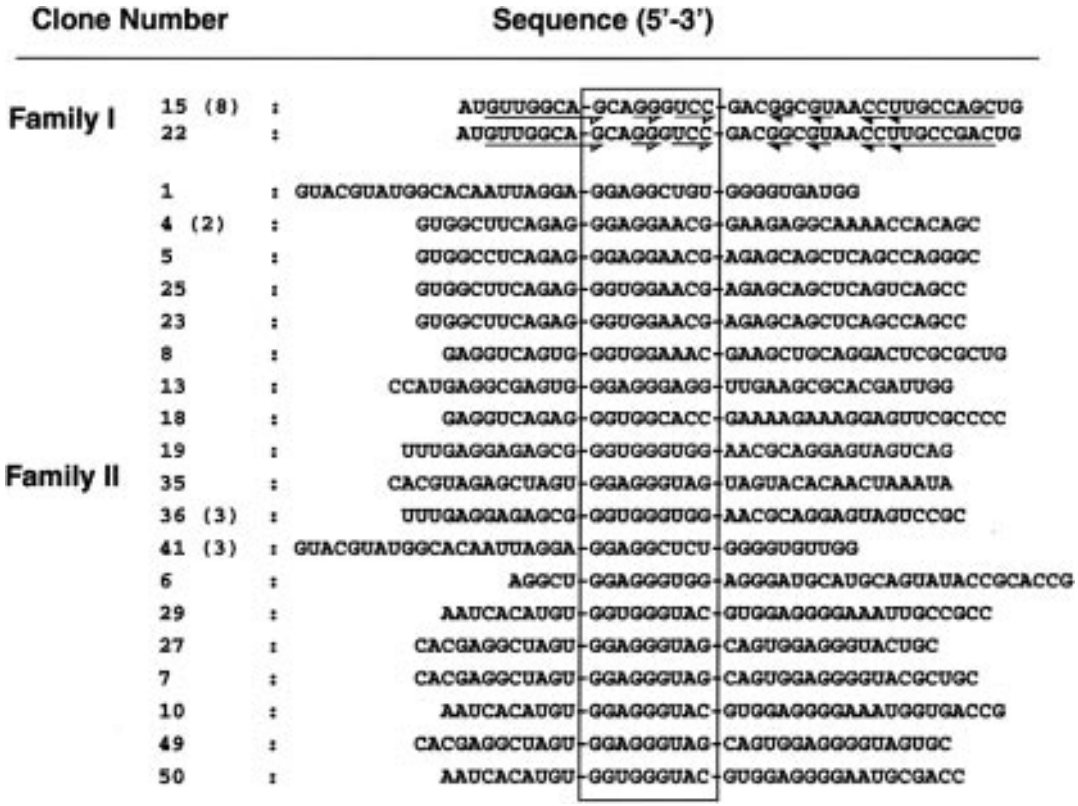

Figure 1. Sequences isolated by affinity selection for hTSH. For each clone, only the variable 40 nt region is shown. The number of clones identified with the same sequence is shown in parenthesis. In full-length RNA molecules, the variable sequence is flanked by 5′-fixed (5′-GGGAGGACGAUGCGG--) and 3′-fixed (--CAGACGACUCGCCCGA-3′) sequences. All pyrimidines have 2′-NH_2-modified sugars. The boxed region indicates the loose consensus sequence identified in both families. Half arrows in family I sequences indicate potential base pairing between the 5′ and the 3′ regions.

5′-GG/$_C$A/$_U$GGG/$_{AC}$U/$_A$A/$_{GC}$G/$_C$-3′ (boxed in Fig. 1). These sequences were classified into two families based on predicted secondary structures. The 5′ and 3′ halves of the variable (or randomized) regions of sequences grouped into family I share a high degree of complementarity in base pairing (indicated by half arrows in Fig. 1), suggesting their possible existence as a stem–loop structure in solution. A portion of the 5′ half of the variable region of sequences classified into family II are partially complementary to the 3′ fixed sequence (primer annealing site). The predicted family II stem–loop structures have less structured stems and larger loops than the corresponding structures predicted for family I RNA sequences.

Affinity and specificity

A nitrocellulose filter binding technique was used to screen several representative ligands from the two sequence families for their ability to interact with hTSH. The calculated K_d values of these ligands range from 2.5 to 100 nM, whereas the K_d of the unselected random sequence pool is ≥2.5 μM. Of the sequences analyzed, RNA sequence 15 (referred to as ligand T-15) exhibited the highest affinity for hTSH (K_d = 2.5 ± 0.6 nM). Ligand T-15, the most abundant sequence that represented 24% of the affinity-selected pool was chosen for further study, based on its high-affinity. Ligand T-15 bound hTSH with an at least three orders of magnitude higher affinity than the unselected random sequence pool (Fig. 2a). This ligand did not exhibit high-affinity binding to hTSH in the absence of Mg^{2+} ions (Fig. 2a), indicating the crucial role played by Mg^{2+} ions in the RNA–protein interaction.

The specificity of ligand T-15 was tested by analyzing its affinities for other members of the glycohormone family. There is no measurable binding of the ligand to hCG at concentrations up to 1 μM (Fig. 2b). The K_d values for the ligand T-15 binding to hLH and hFSH are > 2 μM and ~200 nM, respectively (Fig. 2c and d). These data indicate that ligand T-15 is specific for hTSH. Since all four members of the glycohormone family share an identical α-subunit, the lack of high-affinity binding to other members implies that ligand T-15 does not bind exclusively to the α-subunit. This is indeed what we observed when the binding of ligand T-15 to the isolated α-subunit was investigated (K_d is in the μM range; Fig. 2e). The RNA ligand also did not bind with high affinity to the isolated β-subunit of hTSH (Fig. 2f). Thus, it is possible that either the RNA binding site on hTSH includes residues from both subunits or the individual subunits undergo a structural change upon their physical separation, preventing RNA binding with high-affinity.

The interaction between ligand T-15 and hTSH is salt and pH dependent. The binding of this RNA to hTSH markedly decreases above pH 7.5 in both TEM and MEM (10 mM MES, 0.1 mM EDTA and 2 mM $MgCl_2$) buffers. The pK_a of the 2′-amino group has been measured to be between 6.2 and 6.5 (31,32), and hence the distribution of protonated and unprotonated amine groups in an RNA molecule is sensitive to the pH of the medium, especially near pH 6–7. Furthermore, folding of RNA molecules may force certain 2′-amino groups into microenvironments whose pH may

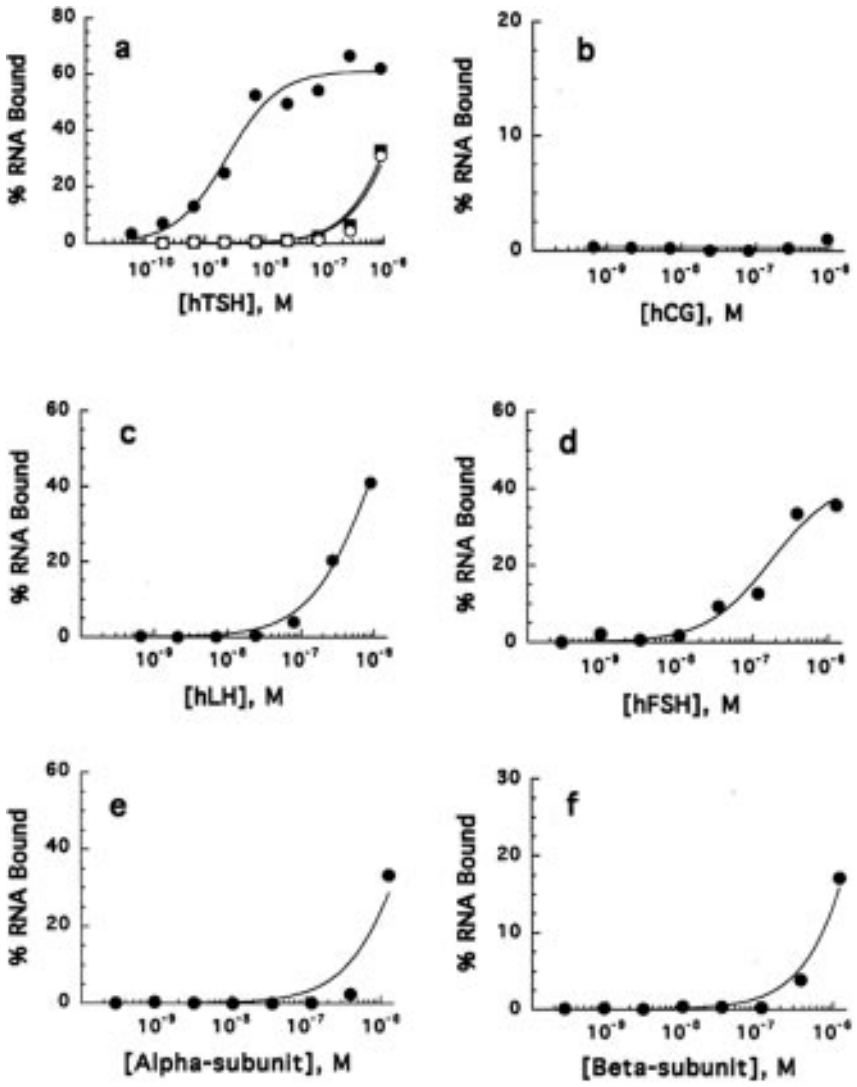

Figure 2. Nitrocellulose filter binding analyses of ligand T-15 to members of the glycohormone family. All binding reactions were carried out in TEM buffer except for one case (open circles) as described in the Materials and Methods. (**a**) Binding of ligand T-15 to hTSH in either TEM buffer (closed circles; K_d = 2.5 ± 0.6 nM) or in TEM + 4 mM EDTA buffer (open circles; K_d > 2.5 μM). Squares indicate the binding of the unselected random sequence RNA pool to hTSH. Binding of ligand T-15 to hCG (**b**); to hLH (**c**); to hFSH (**d**); to the α subunit (**e**) and to the β-subunit of hTSH (**f**).

be quite different from that of the medium. The observed pH sensitivity on protein recognition is therefore not unexpected for sequences bearing 2′-amino modification, and in fact, has been previously observed (17). In addition to the changes in the charge distribution of RNA, the surface charge of hTSH may also change with pH, contributing to the overall bimolecular interaction.

The binding also decreases with increasing concentrations of monovalent cations, especially >50 mM (data not shown). The observed salt sensitivity of the RNA–protein interaction suggests that the majority of factors contributing to hTSH-ligand T-15 interaction are electrostatic in nature and the shielding of charges on the RNA, the protein, or both, would have a profound effect on the overall interaction. Low affinity and non-specific protein–nucleic acid interactions are sensitive to the presence of competing nucleic acids in binding reactions (33,34). As shown in Figure 3a, the addition of >1000-fold molar excess of tRNA decreased the overall binding of ligand T-15 to hFSH, suggesting that the interaction between the RNA ligand and hFSH is likely to be non-specific. Contrary to the interaction with hFSH, the presence of a large excess tRNA had little effect on the high-affinity binding of ligand T-15 to hTSH (Fig. 3b).

Secondary structure

As discussed above, due to the presence of complementary bases in the 5′ and the 3′ regions of the variable 40 nt stretch, a stem–loop structure can be predicted for ligand T-15 (Fig. 4a). Alternatively, the existence of several guanine dinucleotide repeats in the sequence may allow the RNA to fold into intermolecular or intramolecular G-quartet structures. Among other secondary structures such as stem–loops and pseudoknots, G-quartet structures were also common in ligands that have been identified by *in vitro* selection (9,16–18,35). Guanines involved in G-quartet structures are resistant to RNAse T1 cleavage (17), especially under conditions that favor G-quartet formation (36,37). To investigate whether ligand T-15 assumes G-quartet

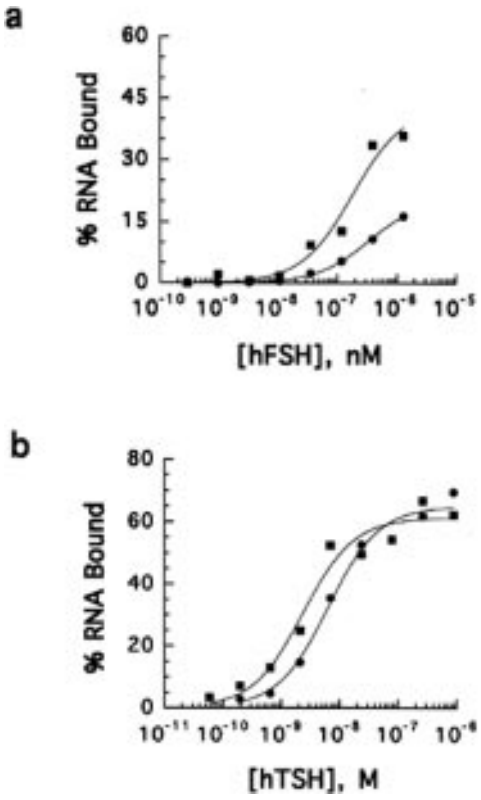

Figure 3. Ligand binding in the presence of non-specific nucleic acid. Binding of ligand T-15 to hFSH (**a**) and hTSH (**b**) in the presence and absence of excess tRNA. Binding reactions were carried out in TEM buffer (as described in Fig. 2) in the absence or presence of 10 mg/ml tRNA. Squares indicate the binding in the absence of tRNA, whereas circles indicate the binding in the presence of tRNA.

structures, RNAse T1 cleavage patterns were obtained in several buffers [with KCl (facilitates G-quartet formation) and LiCl (does not facilitate G-quartet formation)] as described in (17). The cleavage pattern of ligand T-15 was identical in all three buffers examined (standard RNase T1 buffer containing urea, and TEM buffer containing either KCl or LiCl) (data not shown), suggesting that this ligand does not assume the G-quartet structure. As an alternative to RNAse T1 cleavage susceptibility, chemical probing with dimethyl sulfate (DMS), which reacts primarily at the N7 of guanines (38), can be used to confirm the possible existence of G-quartet structures (36,37). However, DMS treatment of the T-15 RNA resulted in smeared bands on sequencing gels, hampering the interpretation of results (data not shown). This unexpected observation could be due to the 2′-amino groups in RNA that may potentially react with DMS.

Ligand T-15 containing 2′-amino pyrimidines has a T_m (the temperature at which 50% of RNA is denatured) of 42°C in 0.1 M sodium phosphate buffer (Fig. 4b; dotted line), indicating that the RNA is folded into a secondary structure. A similar T_m value was observed in TEM buffer, but the melting transition occurs over a broader range than that observed in the phosphate buffer (Fig. 4b; solid line).

Detection

We investigated whether the RNA T-15 ligand could be used in a dot blot assay to detect hTSH, analogous to immunoblots in which antibodies are used to detect and quantitate their cognate targets. Different concentrations of hTSH [from 800 nM (177 µIU/ml) to 50 nM (11 µIU/ml)] were applied to a nitrocellulose membrane. The membrane was then exposed to radiolabeled RNA (either affinity-selected T-15 or unselected random sequence library). A representative autoradiogram of a dot blot is shown in Figure 5a. PhosphorImager quantitation of

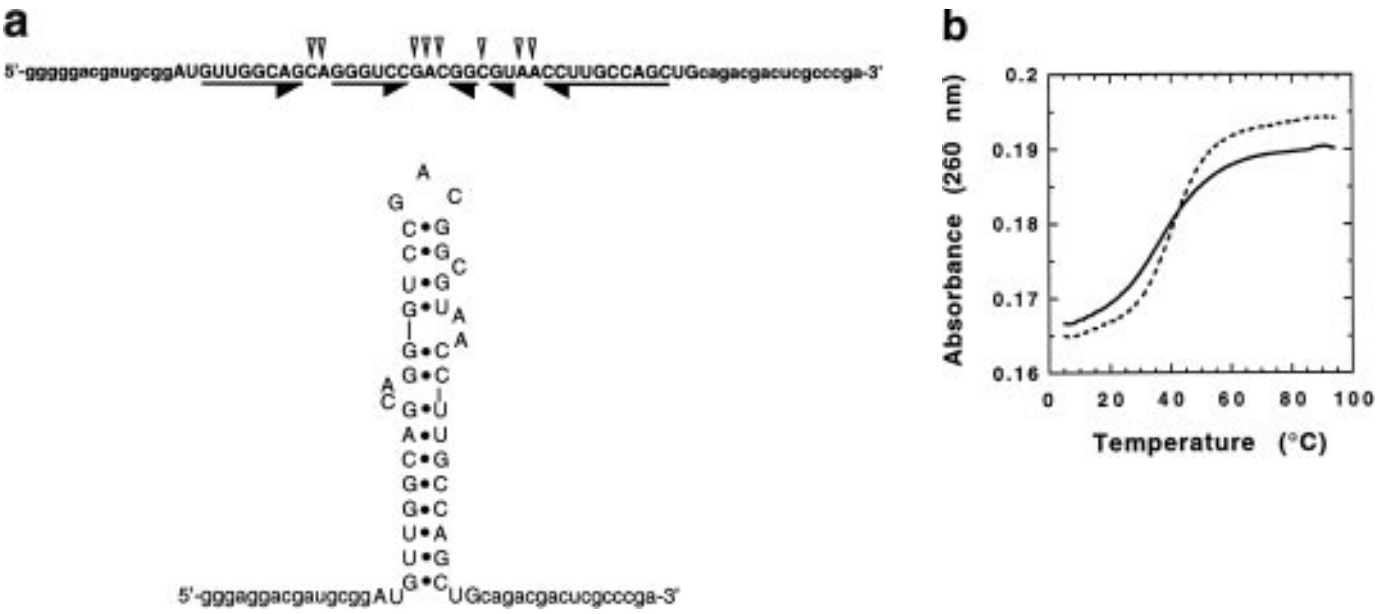

Figure 4. A predicted secondary structure for ligand T-15. (**a**) The primary structure of ligand T-15 is shown on the top. Lower case letters denote the 5′- and 3′-fixed regions. Half arrows indicate the base complementarity that potentially permits the formation of the secondary stem–loop structure shown below. The triangles indicate unpaired nucleotide bases. (**b**) Melting profile of ligand T-15 carried out in either 100 mM sodium phosphate buffer (dotted line) or TEM buffer (solid line).

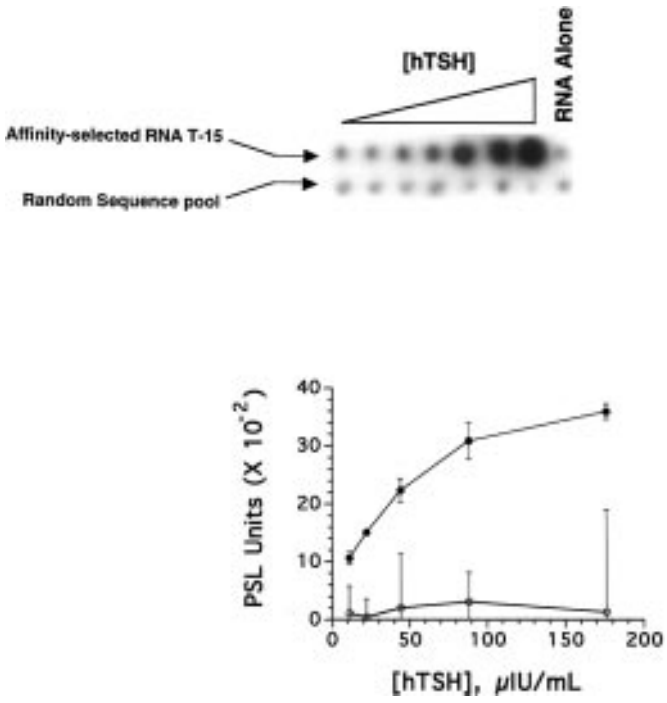

Figure 5. Detection of hTSH by radiolabeled RNA on dot blots. Varying concentrations of hTSH mixed with 0.1% hSA were blotted and detected with radiolabeled RNA (either unselected random sequence pool or affinity-selected ligand T-15). (**a**) An autoradiogram of a representative dot blot. (**b**) A PhosphorImager quantitation of the radioactivity plotted against the concentration of hTSH. All data are expressed as the mean ± SE of the background corrected mean of quadruplicate samples.

the signal as a function of the input concentration of hTSH is shown in Figure 5b. The signal obtained with the radiolabeled unselected random sequence pool (used as a control) did not correlate with the amount of hTSH on the blot (Fig. 5b; open circles). However, with the affinity-selected radiolabeled ligand T-15, the signal correlated with the concentration of hTSH used. A linear relationship between the signal and the amount of hTSH up to 50 μIU/ml was observed.

RNA ligands containing 2′-amino modified pyrimidines have been shown to be resistant to both alkali hydrolysis and nuclease digestion (39,40), and hence have exhibited increased survival time in biological fluids (12,17). Consistent with these studies, we noticed that the half lifes of the ligand T-15 in ~95% human urine and ~95% human serum were at least 300-fold higher than the corresponding values of all 2′-hydroxy form of the same sequence (data not shown) when analyzed as described in (17).

DISCUSSION

In spite of the high degree of structural similarity present among the four members of the glycohormone family, they elicit different biological effects by interacting with three distinct receptors. HCG and hLH bind to the same receptor (27). The existence of hormone-specific receptors (natural ligands) suggests that each member contains a unique set of epitopes for specific molecular recognition; feasibly even with artificial ligands. In fact, with a great deal of effort, antibodies specific for each member have been raised (25). The identification of such antibodies has led to the development of diagnostic assays for the detection of the individual hormones in biological fluids.

The work described here was initiated to use the SELEX process to select oligonucleotide ligands that bind hTSH with high affinity and specificity. Clone 15 (T-15), the highest affinity ligand in the selected pool, has a K_d of 2.5 nM for its interaction with hTSH. The specificity of ligand T-15 for hTSH was indicated by its inability to bind with high-affinity to hCG, hLH and hFSH, especially in the presence of competing tRNA.

These results were obtained under direct selection conditions, where no specific counterselection against ligands with affinity for closely related members of the glycohormone family was incorporated. This outcome is intriguing, especially given the existence of an identical α-subunit in all four hormones. Furthermore, the crystal structure of one of the member (hCG) indicates that the two subunits are highly structured and have similar topology (27). Affinity selection described in this study demonstrates that the RNA selected for hTSH recognizes a site that is unique to this hormone and does not readily identifiable in other members. It should be noted that under similar conditions, without counterselection, RNA ligands isolated by affinity-selection for hCG bound hLH with equal affinity (in addition to the presence of identical α-subunits, the β-subunits of the two hormones are ~85% similar), but with low affinity to the other two members (Y. Lin, D. Nieuwlandt and S. D. Jayasena; unpublished results). The selection of RNA ligands specific for hCG required a counterselection procedure to eliminate RNAs with affinity for hLH (West *et al.*, in preparation). The counterselection strategy has been successfully used to isolate RNA ligands highly specific for theophylline and can discriminate between binding to caffeine, an analogue that differs from theophylline by a single methyl group (13), as well as ligands that discriminate in binding to arginine and citrulline (19,41). These studies suggest that a direct SELEX process with no counterselection may be sufficient for the isolation of oligonucleotides that can discriminate between related targets, but highly discriminatory ligands to very similar targets may require counterselection strategies.

Ligand T-15 did not show high-affinity binding to individual subunits of hTSH, indicating the requirement for the intact heterodimer for effective binding. Antisera specific for a given member have been raised by using the respective β-subunits (25). The difference in the molecular recognition properties of antibodies and SELEX-derived nucleic acid ligands may be due to the way in which the target is presented during the two selection processes. Most antibodies are known to recognize linear epitopes within a protein, presumably due to the presentation of peptide fragments by antigen-presenting cells. However, in the SELEX process an intact protein is repeatedly presented to increasingly enriched pools of oligonucleotides. Hence, SELEX-derived oligonucleotide ligands tend to recognize conformational epitopes.

Ligand T-15 can potentially form a stem with 14 base pairs of which 9 base pairs form an uninterrupted helix. The melting temperature observed for this ligand is substantially lower than what would be observed for an unmodified 2′-OH RNA sequence. In agreement with the T_m observed for sequence T-15 containing 2′-amino pyrimidines, the presence of 2′-amino pyrimidines in oligonucleotides has been shown to lower their melting transitions significantly (42,43). Decreased stability of helices containing 2′-amino pyrimidines may direct SELEX-derived ligands to adopt alternate structures such as G-quartets (17) or may not assume a known secondary structure (12). However,

as evidenced by ligand T-15, the selection of ligands which adopt helical structures is also possible from random sequence pools containing 2′-amino pyrimidines, suggesting that this particular modification may not significantly decrease the shape repertoire available for molecular recognition.

Affinity selections on a single target have been carried out with chemically different oligonucleotide libraries such as DNA, RNA and those with other chemical modifications (8,9,15,17,18,35,44). 'Winning' ligands resulting from such selections were different with respect to their nucleotide sequence and predicted secondary structures. Furthermore, RNA versions of DNA ligands (or vice versa) were not functional in binding to the cognate target (17,35,44). Similar to these previous observations, the high-affinity binding of ligand T-15 was abolished when the 2′-amino pyrimidines of the sequence were replaced by 2′-hydroxy pyrimidines; the K_d of T-15 in its all 2′-hydroxy form is similar to that of the unselected random sequence pool (data not shown). These results indicate that the chemical composition of the initial library that governs the folding of individual sequences remains critical for the function of selected ligands. The effect of particular chemical modifications, 2′-amino modification in the present study, on target recognition may be a direct participation in the interaction with the target and/or an indirect effect contributing to the overall folding of the ligand.

The linear sequence information of oligonucleotide probes is used in the detection of complementary sequences in northern and Southern blotting. This technique has been somewhat modified to detect proteins that naturally bind to nucleic acid sequences on transfer blots by using nucleic acid probes (45). The SELEX process permits the isolation of high-affinity nucleic acid sequences for a wide range of targets, including non-nucleic acid binding proteins. Such ligands can potentially be useful for the detection of non-nucleic acid binding proteins on blots. In this report, we tested this idea by demonstrating the detection of hTSH by RNA ligand T-15. Using a dot blot format, native hTSH was detected with the radiolabeled ligand as the signal.

Chemilumeniscent substrates have been useful for the detection of alkaline phosphatase at 10^{-19}–10^{-15} M (46). Synthetic oligonucleotides conjugated to alkaline phosphatase have been used in non-radioactive hybridization assays (47). Thus, the sensitivity of the dot blot assay described here can potentially be increased by using RNA–enzyme conjugates that utilize fluorescence or luminescence substrates.

The results presented in this report demonstrate that the SELEX process can be used to identify nucleic acid ligands with high affinity and specificity for a given member of a protein family. Data presented here as well as accumulating evidence (48,49) suggest that SELEX-derived nucleic acid ligands can be used in diagnostic assays either in place of or in combination with antibodies. Analytes that are not readily immunogenic as well as those to which high-affinity antibodies are not currently available may be attractive targets for the SELEX process. The ability to obtain amplifiable oligonucleotide libraries decorated with both hydrophilic and hydrophobic functional groups (44,50) offers an exciting opportunity to identify candidate ligands for both diagnostic and therapeutic applications (51).

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REFERENCES

1 Rebek,J.J. (1987) *Science*, **235**, 1478–1483.
2 Cram,D.J. (1988) *Science*, **240**, 760–767.
3 James,T.D., Sandanayaka,K.R.A.S. and Shinkai,S. (1995) *Nature*, **374**, 345–347.
4 Gold,L., Allen,P., Binkley,J., Brown,D., Schneider,D., Tasset,D. and Eddy,S.R. (1993) In Gesteland,R. and Atkins,J. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 497–509.
5 Gold,L., Polisky,B., Uhlenbeck,O. and Yarus,M. (1995) *Annu. Rev. Biochem.*, **64**, 763–797.
6 Tuerk,C. and Gold,L. (1990) *Science*, **249**, 505–510.
7 Tuerk,C., MacDougal,S. and Gold,L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6988–6992.
8 Sassanfar,M. and Szostak,J.W. (1993) *Nature*, **364**, 550–553.
9 Bock,L.C., Griffin,L.C., Latham,J.A., Vermaas,E.H. and Toole,J.J. (1992) *Nature*, **355**, 564–566.
10 Bartel,D.P., Zapp,M.L., Green,M.R. and Szostak,J.W. (1991) *Cell*, **67**, 529–536.
11 Jellinek,D., Lynott,C.K., Rifkin,D.B. and Janjic,N. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 11227–11231.
12 Jellinek,D., Green,L.S., Bell,C., Lynott,C.K., Gill,N., Vargeese,C., Kirschenheuter,G., McGee,D.P.C., Abeysinghe,P., Pieken,W.A., Shapiro,R., Rifkin,D.B., Moscatelli,D. and Janjic,N. (1995) *Biochemistry*, **34**, 11363–11372.
13 Jenison,R.D., Gill,S.C., Pardi,A. and Polisky,B. (1994) *Science*, **263**, 1425–1429.
14 Ellington,A.D. and Szostak,J. (1990) *Nature*, **346**, 818–822.
15 Kubik,M.F., Stephens,A.W., Schneider,D., Marlar,R.A. and Tasset,D. (1994) *Nucleic Acids Res.*, **22**, 2619–2626.
16 Wiegand,T.W., Williams,P.B., Dreskin,S.C., Jouvin,M.-H., Kinet,J.-P. and Tasset,D. (1996) *J. Immunol.*, **157**, 231–238.
17 Lin,Y., Qiu,Q., Gill,S.C. and Jayasena,S.D. (1994) *Nucleic Acids Res.*, **22**, 5229–5234.
18 Lin,Y., Padmapriya,A., Morden,K.M. and Jayasena,S.D. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 11044–11048.
19 Geiger,A., Burgstaller,P., von der Eltz,H., Roeder,A. and Famulok,M. (1996) *Nucleic Acids Res.*, **24**, 1029–1036.
20 Bartel,D.P. and Szostak,J.W. (1993) *Science*, **261**, 1411–1418.
21 Lehman,N. and Joyce,G.F. (1993) *Nature*, **361**, 182–185.
22 Prudent,J.R., Uno,T. and Schultz,P.G. (1994) *Science*, **264**, 1924–1927.
23 Labrie,F. (1990) In Baulieu,E.-E. and Kelly,P.A. (eds), *Hormones from Molecules to Disease*. Herman Publishers, New York, NY.
24 Kourides,I.A. (1981) In Abraham,G.A. (ed.), *Radioassay Systems in Clinical Endocrinology*. Marcel Decker, Inc., New York, NY.
25 Rathnam,P. (1981) In Abraham,G.A. (ed.), *Radioassay Systems in Clinical Endocrinology*. Marcel Decker, Inc., New York, NY.
26 Pierce,J.G. and Parsons,I.F. (1981) *Annu. Rev. Biochem.*, **50**, 465–495.
27 Lapthorn,A.J., Harris,D.C., Littlejohn,A., Lustbader,J.W., Canfield,R.E., Machin,K.J., Morgan,F.J. and Isaacs,N.W. (1994) *Nature*, **369**, 455–461.
28 McGee,D.P., Vargeese,C., Zhai,Y., Kirschenheuter,G.P., Siedem,C.R. and Pieken,W.A. (1995) *Nucleosides Nucleotides*, **14**, 1329–1339.
29 Milligen,J.F., Groebe,D.R., Witherell,G.W. and Uhlenbeck,O.C. (1987) *Nucleic Acids Res.*, **15**, 8783–8798.
30 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989), *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., pp. C1.
31 Guschlbauer,W. and Jankowski,K. (1980) *Nucleic Acids Res.*, **8**, 1421–1433.
32 Verheyden,J.P.H., Wagner,D. and Moffatt,J.G. (1971) *J. Org. Chem.*, **36**, 250–254.
33 Heaphy,S., Dingwall,C., Ernberg,I., Gait,M.J., Green,S.M., Karn,J., Lowe,A.D., Singh,M. and Skinner,M.A. (1990) *Cell*, **60**, 685–693.
34 Daly,T.J., Doten,R.C., Rusche,J.R. and Auer,M. (1995) *J. Mol. Biol.*, **253**, 243–258.
35 Huizenga,D.E. and Szostak,J.W. (1995) *Biochemistry*, **34**, 656–665.
36 Williamson,J.R., Raghuraman,M.K. and Cech,T.R. (1989) *Cell*, **59**, 871–880.
37 Sundquist,W.I. and Klug,A. (1989) *Nature*, **342**, 825–829.

38 Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
39 Hobbs,J., Sternbach,H., Sprinzl,M. and Eckstein,F. (1973) *Biochemistry*, **12**, 5138–5145.
40 Pieken,W.A., Olsen,D.B., Benseler,F., Aurup,H. and Eckstein,F. (1991) *Science*, **253**, 314–317.
41 Yang,Y., Kochoyan,M., Burgstaller,P., Westhof,E. and Famulok,M. (1996) *Science*, **272**, 1343–1347.
42 Miller,P.S., Bhan,P. and Kan,L.-S. (1993) *Nucleosides Nucleotides*, **12**, 785–792.
43 Aurup,H., Williams,D.M. and Eckstein,F. (1992) *Biochemistry*, **31**, 9636–9641.
44 Latham,J.A., Johnson,R. and Toole,J.J. (1994) *Nucleic Acids Res.*, **22**, 2817–2822.
45 Bowen,B., Steinberg,J., Laemmeli,U.K. and Weintraub,H. (1980) *Nucleic Acids Res.*, **8**, 1–20.
46 Bronstein,I., Voyta,J.C., Thorpe,G.H.G., Kricka,L.J. and Armstrong,G. (1989) *Clin. Chem.*, **35**, 1441–1446.
47 Jablonski,E., Moomaw,E., Tullis,R. and Ruth,J. (1986) *Nucleic Acids Res.*, **14**, 6115–6128.
48 Drolet,D.W., Moon-McDermott,L. and Romig,T.S. (1996) *Nature Biotech.*, **14**, 1021–1025.
49 Davis,K.A., Abrams,B., Lin,Y. and Jayasena,S.D. (1996) *Nucleic Acids Res.*, **24**, 702–706.
50 Eaton,B.E. and Pieken,W.A. (1995) *Annu. Rev. Biochem.*, **64**, 837–863.
51 Gold,L. (1995) *J. Biol. Chem.*, **270**, 13581–13584.