D14S13 locus (b = 0.437, 0.104, and 0.203 for the D2S44, D17S79, and D14S13 loci, respectively). The z-statistics for the D2S44, D17S79, and D14S13 loci were 0.97, 0.99, and −0.14 for the Caucasian population, −0.72, 0.90, and −1.49 for the black population, and 0.09, −1.92, and −0.58 for the Hispanic population. Note that none of these z-statistics exceed the critical value for individual tests.

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Detection of *Borrelia burgdorferi* DNA in Museum Specimens of *Ixodes dammini* Ticks

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In order to investigate the potential for *Borrelia burgdorferi* infection before the recognition of Lyme disease as a clinical entity, the polymerase chain reaction (PCR) was used to examine museum specimens of *Ixodes dammini* (deer ticks) for the presence of spirochete-specific DNA sequences. One hundred and thirty-six archival tick specimens were obtained representing various continental U.S. locations; DNA sequences characteristic of modern day isolates of *B. burgdorferi* were detected in 13 1940s specimens from Montauk Point and Hither Hills, Long Island, New York. Five archival specimens of *Dermacentor variabilis* (dog tick) from the same collection and 118 *Ixodes* specimens from other endemic and nonendemic sites were negative. These data suggest that the appearance of the Lyme disease spirochete in suitable arthropod vectors preceded, by at least a generation, the formal recognition of this disease as a clinical entity in the United States.

ALTHOUGH SUBSETS OF THE DIverse clinical manifestations of Lyme disease were recorded in Europe early in this century (*1–4*), recognition of the disease as a distinct clinical entity did not occur until the mid-1970s (*5–7*). Today, Lyme disease is the most common tick-borne zoonosis in the United States, with more than 6000 human infections reported each year (*8*). Among the factors thought to contribute to the prevalence and spread of this disease are a burgeoning deer population, a constitutive rodent reservoir, and the indiscriminate feeding habits of the major tick vector (*9, 10*). Although some epidemiologic studies have suggested a relatively recent spread from earlier enzootic foci (*11*), it is not known how long *B. burgdorferi*, the spirochetal etiologic agent of Lyme disease (*12, 13*), has existed in its reservoir or vector populations.

The risk of a human aquiring Lyme disease is dependent on an interplay of microbial, environmental, and demographic factors. Ultimately, transmission is effected by nymphal ticks of the *Ixodes ricinus* complex (*14–18*). *Ixodes dammini* is the primary vector in enzootic areas of the northeastern United States, where its distribution correlates directly with that of cases of Lyme disease in humans and domestic animals (*14, 15*). The polymerase chain reaction (PCR) can be used (*19–21*) for the direct detection of *Borrelia* DNA and can detect spirochetes in field-collected specimens of *I. dammini* (*22*). The PCR-based method appears to be at least as sensitive as direct microscopic visualization after staining with a *Borrelia*-specific fluorescent antibody (DFA). PCR can also be applied to dried or alcohol-preserved specimens; the DFA method cannot be used on such specimens because of high levels of background fluorescence. This feature, coupled with success in the use of PCR to recover nucleic acids from archeological specimens (*23, 24*), prompted us to obtain and examine museum specimens of *I. dammini* to address the possible antiquity of Lyme disease in the United States.

We obtained 102 alcohol-preserved ticks from the Museum of Comparative Zoology, Cambridge, Massachusetts, archived between 1945 and 1951. These specimens were collected from Naushon Island and Martha's Vineyard in Massachusetts, and from various locations on Long Island, New York. Three additional groups of ticks from South Carolina and Florida were accessioned in the Museum of Comparative Zoology between 1925 and 1950 (*25*). Additional preserved specimens from other areas of the United States, dating from 1924 to 1950, were obtained from the Rocky Mountain Laboratories Acarine Collection (Smithsonian Institution). All specimens were stored in 70% alcohol and were cataloged according to the source and site of collection.

Our primary target for PCR detection of *Borrelia*-specific sequences was the gene encoding the major outer surface protein (OspA) of *B. burgdorferi* reference strain B31 (*26–28*). The *ospA*-specific primers (ospA2 and ospA4) were first used to test for the presence of *Borrelia* sequences in 15 specimens of nymphal *I. dammini* from Montauk Point, Long Island, New York, collected from a gray squirrel (*Sciuris carolinensis*) in the 1940s and in 5 contemporaneous nymphal ticks obtained from a vole (*Microtus pennsylvanicus*) on Naushon Island. Amplification products of the size expected for the *ospA* gene, 156 bp in length, were present in 3 of the 15 Montauk Point specimens by agarose gel electrophoresis (Fig. 1A). To verify the presence of *Borrelia*-specific DNA in these specimens, we used a second set of reagents designed to detect the flagellin gene (*fla*) of strain B31 (*29*). This primer pair produces a 200-bp genus-specific amplification product. *Borrelia* species of many types, including the relapsing fever agents *Borrelia hermsii* and *Borrelia recurrentis*, are detected with these primers, but not *Treponema*, *Leptospira*, or several exoflagellum-bearing organisms (*30*). The results of amplification of archival tick extracts with this primer pair are shown in Fig. 1B; the same three specimens previously identified with the *ospA* primer set also gave rise to an amplification product of the size expected for the *Borrelia fla* gene. Neither of the two target sequences was present at detectable concentrations in five specimens from Naushon Island dating to 1942. The identities of both the *ospA* and *fla* gene amplification products were confirmed by slot-blot hybridization with internal oligonucleotide probes (Fig. 2, a and b).

Analysis of the remaining specimens with the *ospA*- and *fla*-specific primer pairs indicated a relatively high prevalence of *Borrelia*-infected ticks from two eastern Long Island

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sites in the 1940s (Table 1). Other positive specimens from this location include one of ten nymphal ticks collected from the cottontail rabbit (*Sylvilagus floridanus*) and five of ten specimens from the white-footed mouse (*Peromyscus leucopus*). Two adult specimens swept from vegetation in 1946 obtained from the Rocky Mountain Laboratories Acarine Collection were also positive. Consistent with the geographic clustering of the positive specimens, two of five specimens collected from such mice trapped near Hither Hills State Park, Long Island, New York (adjacent to Montauk Point), were also positive for spirochete DNA. Negative results were obtained for nymphal *Ixodes dentatus* specimens from what are now enzootic areas of Orient, Long Island, New York, and Oak Bluffs, Martha's Vineyard, Massachusetts [although *I. dentatus* rarely feeds on mammals other than the cottontail rabbit, it maintains the Lyme spirochete in a cryptic cycle (*31–33*)] or from nymphal *I. dammini* ticks from Naushon Island and adult specimens from East Sandwich, Massachusetts.

Several groups of specimens were intended as negative controls for these experiments. Specimens of the noncompetent tick, *Dermacentor variabilis* (*34*), collected simultaneously with infected *I. dammini*, were negative. Similarly, infection was not detected in contemporaneous specimens of *Ixodes scapularis*, a competent vector taken from sites where Lyme disease is not endemic, and a few *Ixodes pacificus* from northern California, where less than 5% of such ticks are infected (*34, 35*).

We sought to exclude the possibility that the spirochete sequences detected in these specimens were due to the presence of its closest known relative, *Borrelia hermsii*, which causes relapsing fever (*36*). Although *Borrelia* species other than *B. burgdorferi* have not been documented in ticks from the Northeast, some *B. hermsii* isolates may, in theory, be detectable with primer pairs designed to identify *B. burgdorferi* (*22, 36*). We isolated and sequenced a portion of the 16S ribosomal gene of a cloned reference strain (B31) of *B. burgdorferi*. PCR primers were then designed to produce a 77-bp amplification product that is specific to the Borreliae; DNA samples from other non-*Borrelia* spirochetes do not give rise to this product (*37*). Although amplification of both *B. hermsii* fragments, we analyzed the amplification products in an area separate from the one where PCR reaction tubes were set up. We prepared reagents to be used in sample setup in a separate room, and used positive displacement pipettes or a dedicated set of pipetters for the preparation of both samples and reagents. With these precautions, none of the five negative controls included with each run showed a detectable amplification product in any of our experiments.

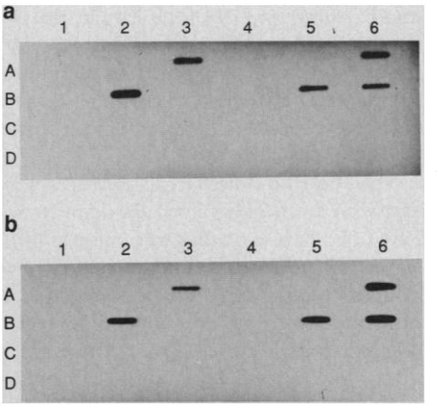

Fig. 2. Slot-blot analysis of *Borrelia*-specific amplification products. *ospA*- and *fla*-specific amplification products from the 15 specimens from Montauk Point (Fig. 1) were blotted on a nylon membrane and probed with ^{32}P end-labeled oligonucleotide probes specific for the *ospA* (**a**) and *fla* (**b**) amplification products by methods described previously (*22*). Two positive controls [consisting of 1 ng of genomic *B. burgdorferi* DNA (derived from cloned strain N40)] and five negative controls (no DNA added) were included for each experiment. Samples 1 to 5 (from Fig. 1), slots A1 to A5; samples 6 to 10, slots B1 to B5; samples 11 to 15, slots C1 to C5; positive control, slots A6 and B6; negative controls, slots D1 to D5.

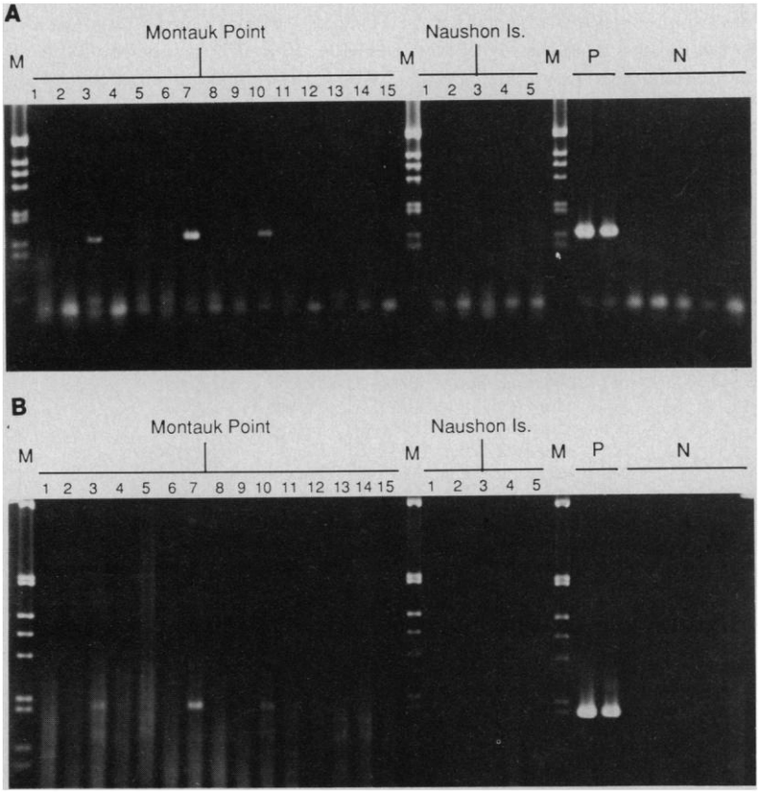

Fig. 1. Detection of *Borrelia*-specific DNA sequences in museum specimens of *I. dammini*. Fifteen specimens collected from squirrels at Montauk Point between 1945 and 1951 and five specimens collected from field voles on Naushon Island were analyzed for the presence of *ospA*-related sequences (**A**) and *fla* sequences (**B**). M indicates the lanes containing DNA size standards; the numbers at the left indicate fragment sizes in base pairs. P and N indicate the lanes containing the positive (1 ng of genomic DNA, strain N40) and negative (no DNA added) controls, respectively. Specimens were removed from the 70% ethanol storage solution with a flame-sterilized forceps, reidentified, and placed into 70% ethanol until further analysis. For PCR analysis, samples were first air-dried on filter paper disks for 5 min; 200 μl (dry volume) of 0.5-m glass beads (Biospec Products, Bartlesville, Oklahoma) were incubated in 1.0 ml of 1% bovine serum albumin in distilled water at 37°C for 30 min and then washed twice in 1.0 ml of distilled water. Ticks were placed whole into 0.6-ml microcentrifuge tubes containing a slurry (20 μl) of the treated glass beads. Specimens were crushed into the beads with a disposable plastic dowel for 30 to 45 s (to liberate the midgut contents), and 25 μl of PCR buffer (10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% gelatin, 0.5% NP40, and 0.5% Tween 20) was added. Samples were boiled for 5 min, then quenched immediately on ice; 5-μl portions of the broth were removed for amplification by PCR and subsequent electrophoretic analysis as described (*22*). The sequences of the primers used for amplification are as follows. The *ospA* gene target: ospA2, 5′-GTT TTG TAA TTT CAA CTG CTG ACC-3′; ospA4, 5′-CTG CAG CTT GGA ATT CAG GCA CTT C-3′; and ospA3 (probe), 5′-GCC ATT TGA GTC GTA TTG TTG TAC TG-3′. The *fla* gene target: fla1, 5′-GAT GAT GCT GCT GGC ATG GGA GTT TCT GG-3′; fla3, 5′-CTG TCT GCA TCT GAA TAT GTG CCG TTA CCT G-3′; and fla2 (probe), 5′-ATT CAG ACA ACA GAA GGG AAT TTA AAT GAA GTA G-3′. To reduce the risk of contamination of our samples with PCR

and *B. burgdorferi* DNA samples give rise to a similar-sized amplification product, the two species can be distinguished by hybridization to an end-labeled internal oligonucleotide probe because of sequence heterogeneity in the product.

The 16*S* ribosomal DNA sequences were amplified from geographically distinct isolates of *Borrelia*, including five cultures of *B. burgdorferi* and seven cultures made from five isolates of *B. hermsii*. Also included in this experiment were two extracts of archival *I. dammini* from Montauk Point; these specimens were previously shown to contain *Borrelia ospA*- and *fla*-specific sequences. The amplification products were blotted and hybridized with an internal oligonucleotide probe specific for *B. burgdorferi* strain B31. As shown in Fig. 3, ribosomal DNA (rDNA)–specific amplification products were present for all 12 of the *Borrelia* isolates tested; as expected, extracts from the 2 1940s ticks also contained such sequences. However, after a 1-hour high-stringency wash, the signal generated by the seven *B. hermsii* cultures was lost, whereas the amplification products from the five *B. burgdorferi* isolates and the archival tick specimens retained the probe. Thus, in this region of the spirochetal rDNA, sequences present in tick extracts from the 1940s were more characteristic of *B. burgdorferi* than *B. hermsii*.

These data demonstrate the presence of *B. burgdorferi* DNA sequences in museum specimens of *I. dammini* ticks that were collected from eastern Long Island in the 1940s, 25 to 35 years before Lyme arthritis was recognized as a distinct clinical illness in residents of Lyme, Connecticut. All of the 13 positive specimens were from the adjacent communities of Montauk Point and Hither Hills (Table 1). Although it is tempting to draw conclusions regarding an "original focus" of Lyme disease in the United States, the numbers of samples from other current foci of disease such as Naushon Island, Oak Bluffs, and Lyme are small or lacking. Thus, demonstration of the presence of *Borrelia*-specific sequences in the museum specimens has allowed us to ascertain the presence of spirochetes in the past, but we cannot yet draw conclusions regarding the time or place of introduction of the pathogen on this continent.

The dominant role of the white-footed mouse, *P. leucopus*, as a major reservoir host of the agent of Lyme disease in the northeastern United States (*38*) suggests that ticks removed from such hosts might more frequently contain recently ingested spirochetes than would ticks removed from other hosts. Ticks removed from deer, however, would be infected less frequently (*39*). Although the small number of available specimens allows only a cautious interpretation of the results, our mouse-derived ticks contained spirochete DNA sequences more frequently than the other ticks. The observed tick and host-associations and the geographical clustering of infection in two eastern Long Island sites are consistent with the current paradigm of the ecology of *B. burgdorferi* (*10, 17, 31*).

The presence of *Borrelia*-specific sequences in archived specimens capable of infecting human hosts suggests that Lyme disease itself may have been present on this conti-

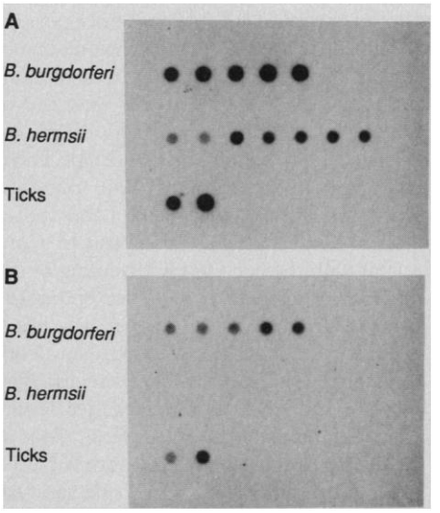

Fig. 3. Differential hybridization of an oligonucleotide probe to 16*S* rDNA amplification products. (**A**) First wash. (**B**) Extended wash at 60°C. Top row in both (A) and (B): *B. burgdorferi* isolates, from left to right: American Type Culture Collection (ATCC) 35211 (B31, Shelter Island, New York), ATCC 35210 (German skin isolate), 25015 [atypical isolate from Millbrook, New York (*43*)], N40 (Westchester County, New York), and SON328 (Sonoma, California). Middle row in both (A) and (B): *B. hermsii* isolates, from left to right: ATCC 35209, *B. hermsii* isolates passaged in three independent laboratories (originally from Washington State), and NC-1 to NC-4 (independent northern California human isolates). Bottom row: rDNA amplification products from two archived tick specimens from Montauk Point, AT-1, nymphal *I. dammini* collected from rabbit (left) and AT-2, adult *I. dammini* swept from vegetation (right). *Borrelial* DNA (10 fg to 1 ng) or the tick broth (5 μl) prepared above was added to 100 μl of PCR buffer with 62.5 μM each of deoxynucleoside triphosphate, 7% dimethyl sulfoxide, 1.8 mM $MgCl_2$, and 50 pmol of each primer. Components were cycled between 94° and 60°C with a 25-s plateau at each temperature for 45 cycles, followed by extension at 72°C for 5 min. The amplification products were dot-blotted onto a nylon membrane (Plasco, Woburg, Pennsylvania) and hybridized with an end-labeled oligonucleotide probe (DD04) in 5× standard sodium phosphate–EDTA buffer (SSPE), 5× Denhardt's solution, and 0.1% SDS for 2 hours at 60°C, washed in 2× SSPE at increasing stringency, then exposed to film for 48 hours at −70°C. The sequences of the 16*S* rDNA-specific primers and probe are as follows: DD02, 5′-CCC TCA CTA AAC ATA CCT-3′; DD03, 5′-CAC CCG AGT TGA GGA TAC C-3′; and DD04 (probe), 5′-CCC GTA AGG GAG GAA GGT AT-3′.

Table 1. Infection of archived tick specimens with the Lyme disease spirochete.

Kind of tick	Location		Year	Host	Stage of tick	Number of ticks	
	State	City or county				Examined	Infected
Ixodes dammini	NY	Montauk Point	1945–51	Skunk	Nymph	10	0
	NY	Montauk Point	1945–51	Mouse	Nymph	10	5
	NY	Montauk Point	1945–51	Squirrel	Nymph	15	3
	NY	Montauk Point	1945–51	Deer	Nymph	5	0
	NY	Montauk Point	1945–51	Rabbit	Nymph	10	1
	NY	Montauk Point	1945–51	Fox	Nymph	5	0
	NY	Montauk Point	1945–51	Catbird	Nymph	5	0
	NY	Montauk Point	1946	Swept*	Adult	2	2
	NY	Hither Hills	1945–51	Mouse	Nymph	5	2
	NY	Hither Hills	1945–51	Chipmunk	Nymph	5	0
	NY	Hither Hills	1945–51	Squirrel	Nymph	5	0
	NY	Suffolk County	1948	NI†		3	0
	MA	Naushon Island	1942	Vole	Nymph	5	0
	MA	Naushon Island	1945–51	Deer	Nymph	2	0
	MA	Chatham	1949	Dog	Adult	2	0
	MA	East Sandwich	1950	Swept	Adult	8	0
Ixodes dentatus	NY	Orient	1948	Rabbit	Nymph	5	0
	NY	Montauk Point	1945–51	Rabbit	Nymph	5	0
	MA	Oak Bluffs	1930–40	Rabbit	Nymph	5	0
Ixodes scapularis	FL	Palm Beach	1924	Swept*	Adult	5	0
	FL	Edgewater	1920–30	Swept*	Adult	5	0
	SC	Dewees Island	1929	Swept*	Adult	5	0
Ixodes pacificus	CA	Monterey	1932	Lizard	Nymph	4	0
Dermacentor variabilis	NY	Hither Hills	1945–51	Swept	Adult	5	0

*Swept from vegetation. †NI, host not indicated.

nent longer than previously thought. The work leading to the formal recognition of the disease was begun in November 1975 (*5*). Subsequently, earlier cases were identified retrospectively that fulfilled the clinical criteria for Lyme disease in Wisconsin and on Cape Cod, Massachusetts (*40, 41*). Reports from senior medical practitioners in eastern Long Island indicate that "Montauk knee" and "Montauk spider-bite" have been in the vernacular of the local residents for many years (*42*). These descriptive terms could have referred respectively to the arthritic and dermatologic manifestations of Lyme disease, but they seem not to have been linked to each other or to tick-bite. Application of the same technology to preserved, archived human samples, such as paraffin-embedded tissue sections, may someday provide additional clues to the antiquity of Lyme disease.

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An Insertion in the Human Thyrotropin Receptor Critical for High Affinity Hormone Binding

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Thyrotropin (TSH), lutenizing hormone (LH), and chorionic gonadotropin (CG) are structurally related glycoprotein hormones, which bind to receptors that share a high degree of sequence similarity. However, comparison of the primary amino acid sequences of the TSH and LH-CG receptors reveals two unique insertions of 8 and 50 amino acids in the extracellular domain of the TSH receptor. The functional significance of these insertions were determined by site-directed mutagenesis. Deletion of the 50–amino acid tract (residues 317 to 366) had no effect on TSH binding or on TSH and thyroid-stimulating immunoglobulin (TSI) biological activities. In contrast, either deletion or substitution of the eight–amino acid region (residues 38 to 45) abolished these activities. This eight–amino acid tract near the amino terminus of the TSH receptor appears to be an important site of interaction for both TSH and TSI.

THYROTROPIN (TSH) REGULATES thyroid cell function by interacting with the TSH receptor on the plasma membrane (*1*). Auto-antibodies against the TSH receptor (thyroid-stimulating immunoglobulins, TSI) are important in the development of the hyperthyroidism associated with Graves' disease (*2*). The sites on the TSH receptor that interact with TSH and TSI are unknown. The TSH and LH-CG receptors each bind specific members of a family of glycoprotein hormones that are structurally related, suggesting that the receptors may be highly similar. The molecular cloning of the complementary DNA that encodes these receptor proteins has allowed comparison of the primary amino acid sequences of the TSH (*3–6*) and LH-CG (*7, 8*) receptors. The two receptors are highly related except for two unique insertions of 8 and 50 amino acids in the extracellular domain of the 764–amino acid TSH receptor (*3*). We have therefore focused on these two regions and used site-directed mutagenesis to determine their functional significance.

The cDNA that encodes the two unique segments in the extracellular domain of the human TSH receptor, amino acids 38 to 45 and 317 to 366 (*3*), were deleted by site-directed mutagenesis, singly or together (Fig. 1). These segments are near the NH_2- and COOH-terminal regions of the 418–amino acid extracellular domain of the TSH receptor (*3*), respectively (Fig. 1). Chinese hamster ovary (CHO) cells were stably transfected with the cDNAs that encoded mutated TSH receptors and were then tested for their ability to bind TSH and to respond to TSH stimulation, measured by an increase in intracellular cyclic adenosine monophosphate (cAMP) content.

Although the 50–amino acid deletion (hTSHR-D1) represented 12% of the 418–amino acid extracellular domain of the TSH receptor, high-affinity TSH binding to the recombinant human TSH receptor was not reduced (Table 1). Consistent with this high-affinity TSH binding, TSH addition stimulated cAMP production in cells that

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