# Human Organ-specific Autoimmune Disease

## Molecular Cloning and Expression of an Autoantibody Gene Repertoire for a Major Autoantigen Reveals an Antigenic Immunodominant Region and Restricted Immunoglobulin Gene Usage in the Target Organ

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## Abstract

**The most common organ-specific autoimmune disease in humans involves the thyroid. Autoantibodies against thyroid peroxidase (TPO) are present in the sera of virtually all patients with active disease. We report the molecular cloning of the genes for 30 high-affinity, IgG-class human autoantibodies to TPO from thyroid-infiltrating B cells. Analysis of the putative germline genes used for the TPO human autoantibodies suggests the use of only five different H and L chain combinations involving four H chains and three L chains. In addition, the same combination of H and L chains was found in multiple patients. The F(ab) proteins expressed by these genes define two major, closely associated domains (A and B) in an immunodominant region on TPO. These A and B domains contain the binding sites of ~ 80% of IgG-class TPO autoantibodies in the sera of patients with autoimmune thyroid disease. The present information permits analysis, not previously possible, of the relationship between autoantibody H and L chain genes and the antigenic domains on an autoantigen. Our data, obtained using target organ-derived autoantibodies, indicate that there is restriction in H and L chain usage in relation to the interaction with specific antigenic domains in human, organ-specific autoimmune disease. (*J. Clin. Invest.* 1993. 92:62–74.) Key words: autoantibody • autoantigen • autoimmunity • immunoglobulin gene • thyroid peroxidase**

## Introduction

The most common organ-specific autoimmune disease in humans involves the thyroid. Hashimoto's thyroiditis and Graves' disease, in their pure forms, represent two ends of a clinical spectrum ranging from glandular hypofunction to hyperfunction. In both diseases there is a breakdown in tolerance to a number of thyroid-specific autoantigens and the generation of an IgG antibody response. Thyrotropin receptor autoantibodies occur predominantly in Graves' disease and are responsible for hyperthyroidism (reviewed in reference 1). Autoantibodies to thyroglobulin, of uncertain pathogenetic importance, tend to be found in patients with Hashimoto's thyroiditis rather than Graves' disease. However, autoantibodies against thyroid peroxidase (TPO),[1] the cell surface enzyme responsible for thyroid hormone synthesis and previously known as the thyroid "microsomal" antigen, are present in virtually all patients with active autoimmune thyroid disease (2, 3).

TPO autoantibodies are of high affinity and predominantly IgG1 and IgG4 (4). There is evidence that these IgG1 (but not IgG4) autoantibodies may play a role in thyroid cell destruction (reviewed in reference 5). TPO autoantibodies correlate well with thyroid inflammation in autopsy studies of subjects without clinical thyroid disease (6). With newer, sensitive assays, TPO autoantibodies (of unknown affinity) are detectable, usually at low levels, in up to 25% of the adult female population (7). The present concept is that autoimmune thyroid disease is predominantly subclinical because of sufficient thyroid reserve and regeneration under the influence of thyrotropin.

Information on the genes coding for organ-specific autoantibodies, as well as knowledge of their epitopes, would be invaluable in understanding the pathogenesis of antibody-mediated autoimmune diseases. Among the major organ-specific human autoimmune diseases (thyroiditis, diabetes mellitus type I, pemphigus vulgaris, myasthenia gravis, primary biliary cirrhosis, pernicious anemia, and Addison's disease), few IgG class monoclonal autoantibodies have been produced and even fewer cloned at the molecular level (8–12). In no disease have both the autoantibody repertoire and the autoantigenic domains been defined.

We now report the molecular cloning of the genes for 30 new organ-specific (TPO) human autoantibodies. These genes all code for high-affinity IgG autoantibodies. We demonstrate that these TPO autoantibodies, which appear to utilize a restricted number of H and L chain genes, encompass a restricted immunodominant region on TPO recognized by patients with autoimmune thyroid disease.

## Methods

*Molecular cloning of TPO-binding F(ab) fragments.* In order to obtain TPO-binding F(ab)s we used thyroid tissue from patients with autoimmune thyroid disease because it is enriched, compared with blood, lymph node, and thymus, in B cells secreting TPO autoantibodies (reviewed in reference 13). In addition, previous studies have demonstrated the difficulty of obtaining thyroid-specific monoclonal autoantibodies, particularly TPO autoantibodies, by using blood versus thyroid lymphocytes (14).

We constructed five new human F(ab) combinatorial cDNA libraries from the thyroid tissue of three patients (WR, TR, and JA) with Graves' disease in the vector Immunozap (Stratacyte, La Jolla, CA) as described previously (10), with the following modifications. We pre-

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*Received for publication 13 October 1992 and in revised form 8 January 1993.*

1. *Abbreviations used in this paper:* CDR, complementarity determining region; FR, framework region; TPO, thyroid peroxidase.

*Table I. Oligonucleotide Primers Used to Amplify the H and L (κ) Chain Genes*

| Primer | Sequence | Source | Family/C region |
|---|---|---|---|
| VH region primers | | | |
| 1a | 5′-CAG GTG CAG CTC GAG CAG TCT GGG-3′ | (16) | VH1; plus others |
| 3a | G-- --- --- --- --- G-- --- --- | (16) | VH3; plus others |
| | (C-- --- --- --G C-- --- --- --) | VH1/5BACK (17) | VH1; VH5 |
| | (G-- --- --- --G C-- G-- --- GG) | VH3BACK (17) | VH3 |
| VH2 | 5′-CAG GTC AAC CTC GAG GAG TCT GG-3′ | based on (17) | VH2 |
| | (--- --- --- --G C-- --- --- --) | VH2BACK (17) | VH2 |
| VH6 | 5′-CAG GTA CAG CTC GAG CAG TCA GG-3′ | based on (17) | VH6 |
| | (--- --- --- --G C-- --- --- --) | VH6BACK (17) | VH6 |
| 1f | 5′-CAG GTG CAG CTG CTC GAG TCT GGG-3′ | (16) | VH1; VH4 |
| 3f | G-- --- --- --- --- --- --G --- | (16) | VH3; plus others |
| | (--- --- --- --- -A- --- --G --) | VH4BACK (17) | VH4 |
| VHc | 5′-AG GTG CAA CTG CTC GAG TCT GG-3′ | Immunozap | unspecified |
| VHd | -- --- --- --- --- --- --G -- | Immunozap | unspecified |
| H chain constant region primers | | | |
| CH1 | 5′-AGC ATC ACT AGT ACA AGA TTT GGG CTC-3′ | Immunozap | IgG hinge region |
| Cγ4 | 5′-GCA TGA ACT AGT TGG GGG ACC ATA TTT GGA-3′ | (18) | IgG4 hinge region |
| Vκ primer | | | |
| V(L)k | 5′-GT GCC AGA TGT GAG CTC GTG ATG ACC CAG TCT CCA | (45) | Family crosspriming |
| | (--C A-- -A- C-C --- --- --- ---) | VK1a (16) | |
| | (--A A-T -A- C-C --G --- --- ---) | VK3a (16) | |
| κ constant region primer | | | |
| C(L)k | 5′-T CCT TCT AGA CTA ACA CTC TCC CCT GTT GAA GCT CTT TGT GAC GGG CGA ACT C-3′ | Immunozap | |

Included in parentheses (for comparison) are primers used in other studies. VH families for which the primers were designed are indicated. Dashes indicate identity. Nucleotide restriction sites are underlined; XhoI, SpeI, SacI, and XbaI for VH, CH, V(L)k, and C(L)k regions, respectively.

pared cDNA by reverse transcription (First Strand Synthesis kit; Stratacyte) of mRNA obtained either by the technique of Han et al. (15) or using the QuickPrep mRNA purification kit (Pharmacia, Inc., Piscataway NJ). In an attempt to cover as wide a range of heavy chain variable region (VH) genes as possible, we used upstream primers described by Persson et al. (16), primers of Marks et al. (17) modified by introducing restriction sites, as well as the VHc and VHd region primers of Stratacyte (Table I). Two heavy (H) chain libraries were constructed from each of the WR and JA cDNA, one using the "CH1" constant region primer of Stratacyte and the other using an IgG4 constant region primer (Cγ4) based on nucleotide sequence data in the IgG4 hinge region (18) (Table I). PCR conditions were as follows: 25 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min; and 15 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. These diverse primers all produced VH products of the correct size (Fig. 1). Because TPO autoantibodies are predominantly of κ L chain type (4), we used the Immunozap V(L)κ and C(L)κ primers (Table I).

The unamplified combinatorial libraries ($0.8–2.0 \times 10^6$ recombinants) were screened in XL1-Blue cells by conventional techniques (19) using as antigen the secreted, recombinant human TPO (20) labeled with $^{125}$I to a specific activity of 10–20 μCi/μg protein by the iodogen method (21). TPO-binding plaques were cloned to homogeneity and plasmids were excised from the Immunozap bacteriophage using the helper phage R408, according to the Stratacyte protocol. Nucleotide sequencing of the cDNA inserts was performed by the dideoxynucleotide chain termination method (22).

*Preparation of soluble F(ab) fragments.* F(ab)s were expressed as soluble proteins in XL1-Blue cells, as previously described (11). In brief, protein synthesis was induced with 1 mM isopropyl-thio-galactopyranoside (Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C. The cells were then pelleted, resuspended in 0.02 times the original volume of 10 mM Tris, pH 8.0, containing 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (all from Sigma Chemical Co.). After one freeze-thaw, the suspension was sonicated, membranes pelleted by centrifugation at 4,000 *g* and the F(ab)s were affinity-purified from the supernatant using a Protein G sepharose column (Pharmacia, Inc.).

*Direct F(ab) binding of $^{125}$I-TPO.* As previously described (11), F(ab)s diluted in assay buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20, and 0.5% BSA) were incubated with $^{125}$I-TPO (15,000–20,000 cpm) and mouse monoclonal antibody to human κ light chains (QE11, Recognition Sciences, Birmingham, UK) in a total

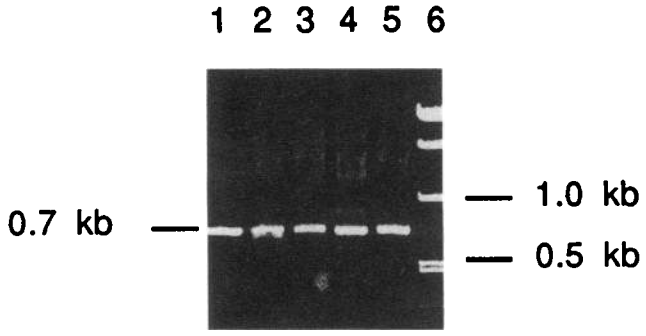



*Figure 1.* VH gene products generated by the PCR in a representative experiment. PCR conditions were as described in Methods using the following oligonucleotides in conjunction with the CH1 primer (Table I): lane *1*, VH6; lane *2*, VH2; lane *3*, VHc/d; lane *4*, VH1a/3a; lane *5*, VH1f/3f; lane *6*, PM2 marker digested with Hind III.

volume of 200 μl. After 1 h at room temperature, 100 μl of donkey anti-mouse Sac-cel (IDS, Boldon, Tyne and Wear, UK) was added, and the incubation was continued for 30 min. After addition of 1 ml of assay buffer and vortexing, the mixture was centrifuged for 5 min at 1,000 *g* to sediment the immune complexes which were then counted to determine the percent radiolabeled TPO bound. The affinities of the F(ab)s for TPO were determined by Scatchard analysis (23) from values obtained in the presence of increasing concentrations of unlabeled TPO.

*Competition between F(ab)s for binding to TPO.* One F(ab) was immobilized by incubation (total volume of 200 μl) with murine mAb anti-human κ (QE11) for 1 h at room temperature. After incubation with 100 μl of Sac-cel (30 min at room temperature), the immobilized F(ab) complexes were diluted in assay buffer (see above) and centrifuged at 1,000 *g* (5 min at 4°C). The pellets were resuspended in normal human serum diluted 1:30 in assay buffer to saturate remaining anti-κ binding sites. In a separate set of tubes, increasing concentrations of "free" F(ab) were preincubated with $^{125}$I-TPO for 1 h at room temperature. Aliquots (100 μl) were then incubated for 30 min with the immobilized F(ab) pellets and washed with assay buffer, and the radioactivity bound to the Sac-cel was counted. Nonspecific binding (~ 2% of total counts added) was subtracted to provide values for specific binding to TPO.

*Competition studies between F(ab) fragments and serum TPO autoantibodies.* Sera from 10 patients with autoimmune thyroid disease were studied. All sera contained high levels of TPO autoantibodies (detectable by ELISA [24] at dilutions of 1:1,000 or greater). Binding of $^{125}$I-TPO by serum autoantibodies was measured by precipitating the antigen-antibody complex with Protein A (Pansorbin, Calbiochem-Behring Corp., La Jolla, CA) (2) in the presence of increasing concentrations of F(ab) fragments. The F(ab) fragment–TPO complex, lacking the CH2 domain of the Fc region, is not precipitated by Protein A. Use of Protein A is effective for serum TPO autoantibodies (2) because of the near absence of IgG3 TPO autoantibodies (4, 25).

Duplicate aliquots of sera were incubated for 1 h at room temperature with $^{125}$I-TPO, alone or with F(ab) fragments. Pansorbin (100 μl) was added and the incubation was continued for 30 min. After addition of 1 ml assay buffer (see above), the mixture was vortexed and centrifuged for 30 min at 1,000 *g* (4°C), supernatants were removed by aspiration, and TPO remaining in the pellets was counted. In preliminary experiments, we determined serum dilutions needed to provide binding values of 15–20% in the absence of F(ab) fragments. These dilutions ranged from 1:1,000 to 1:7,000. Nonspecific $^{125}$I-TPO binding in the presence of control serum without TPO antibodies was 2–5% of total cpm added. This value was subtracted from the values obtained with patients' sera in calculating the percent inhibition by the F(ab) fragments.

## Results

*Frequencies and subclass of TPO-specific F(ab)s.* Screening 5 F(ab) combinatorial libraries from three patients (WR, TR, and JA) yielded 30 TPO-binding clones which were plaque-purified. The frequencies of TPO-binding F(ab)s (Table II) differed markedly between patients. Far more TPO-specific F(ab)s were obtained from patients TR and WR than from patients SP and JA These frequencies refer only to TPO-binding F(ab)s and are *not* a measure of the frequency of the individual VH and VL chains in the libraries.

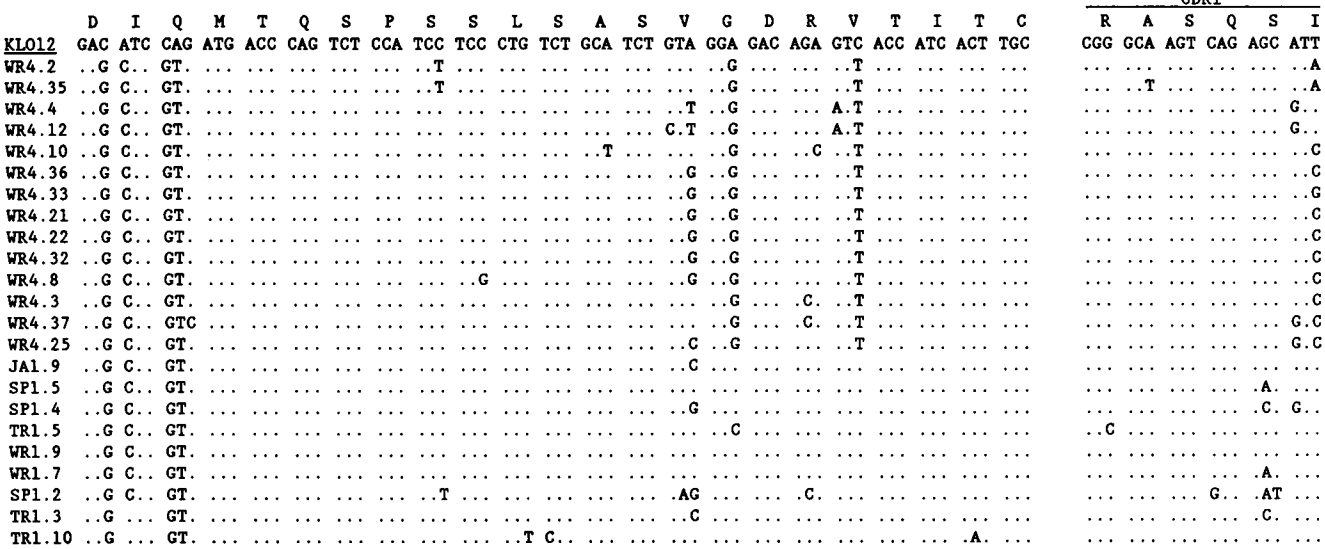
For two patients, two different libraries were prepared. The WR I, TR I, JA I, and the previously reported (10) SP I libraries used the "CH1" primer (Stratacyte) which we subsequently observed crossprimes for both IgG1 and IgG4 (26). The WR IV and JA IV libraries used an IgG4 specific primer (Table I). Both IgG1 and IgG4 F(ab)s were isolated from the WR I and SP I libraries, whereas only IgG4 F(ab)s were obtained from the WR IV library and at a higher frequency. In contrast, the TR I library yielded only IgG1 F(ab)s. The large number of TPO-specific IgG4 F(ab)s obtained from the WR I library and the lack of such F(ab)s from the TR I library is consistent with the greater contribution of Ig4 to TPO autoantibodies in the serum of patient WR relative to patient TR (data not shown).

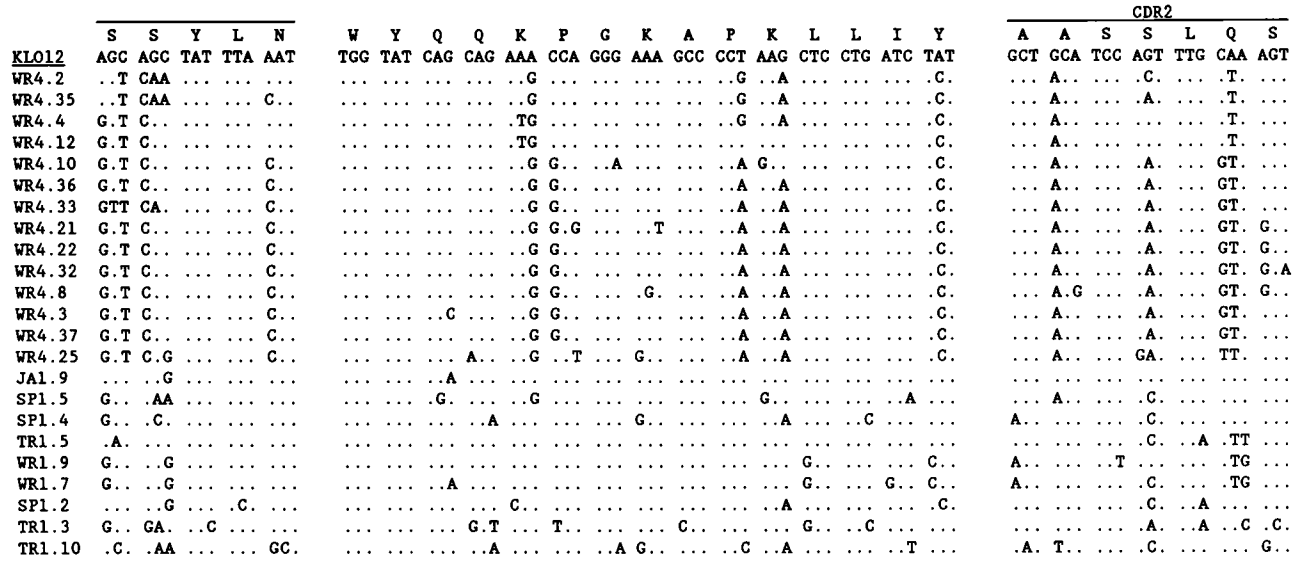
*Table II. Summary of TPO-specific Human F(ab)s Obtained from Four Graves' Patients*

| | | | H chain | | | L chain | | |
|---|---|---|---|---|---|---|---|---|
| Library (frequency)* | Subclass | Clone | Germline‡ | VH | JH | Germline‡ | VK | JK |
| WR I (10/90,000) | 1 | 1.7; 1.9 | V1-3b | 1 | 4 | KL012 | 1 | 1 |
| | 4 | 4.2–5; 4.8 | HV1L1 | 1 | 4 | KL012 | 1 | 2 |
| | | 4.10; 4.12 | | | | | | |
| WR IV (12/30,000) | 4 | 4.21; 4.22; 4.25–35 | HV1L1 | 1 | 4 | KL012 | 1 | 2 |
| TR I (7/90,000) | 1 | 1.3; 1.5 | 8-1B | 3 | 4 | KL012 | 1 | 2 |
| | 1 | 1.6; 1.8 | HV1263 | 1 | 3 | A3 | 2 | 2 |
| | 1 | 1.9; 1.13 | V1-3b | 1 | 4 | A' | 1 | 4 |
| | 1 | 1.10 | V1-3b | 1 | 4 | KL012 | 1 | 1 |
| JA I (1/200,000) | 1 | 1.9 | HV1L1 | 1 | 6 | KL012 | 1 | 4 |
| JA IV (0/200,000) | — | — | | | | | | |
| SP I§ (3/180,000) | 1 | 1.2 | HV1L1 | 1 | 6 | KL012 | 1 | 2 |
| | | 1.4; 1.5 | HV1L1 | 1 | 6 | KL012 | 1 | 1 |
| | 4 | 4.6 | HV1L1 | 1 | 4 | KL012 | 1 | 2 |

Combinatorial libraries were constructed from mRNA prepared from intrathyroidal B cells (Methods). * Frequency of TPO-specific clones. ‡ Putative germline genes based on data presently available. § Previously published (11). Clone SP4.6 was obtained by recombining the SP1.2 L chain with the parent SP I H chain library (26).

**A**




*TPO-specific F(ab) gene usage.* The nucleotide sequences (Figs. 2 and 3) and derived amino acid sequences (Figs. 4 and 5) of the 30 new and 4 previously reported TPO-specific F(ab)s from patient SP (10, 11, 26) were compared to the closest, presently known germline genes. It must be emphasized that, even with the recent expansion of VH germline sequence information (27), the germline assignments are putative and cannot be regarded as definitive without characterization of the respective genes in these patients. Homologies are shown simply as a means to categorize the large body of data.

The 34 TPO human autoantibodies appeared to use a relatively restricted number and combination of H and L chain genes (Figs. 2–5, summarized in Table II). In particular, L chain putative germline gene KL012 (28), a VK1, is used in TPO autoantibodies from all four patients (Figs. 2 and 4). In three patients, KL012 was the only L chain obtained. However, for this very large group of L chains, the possibility of other germline gene usage cannot be excluded. Indeed the nucleotide sequence pattern (Fig. 2) of the WR4 L chains differs from those of the other KL012-related L chains. In one patient (TR), two other TPO-specific L chains were obtained; A′ (29) is a VK1 (like KL012) and A3 (29) is a VK2.

The TPO-specific F(ab) H usage appears to be slightly less restricted than that of the L chain (Figs. 3 and 5, summarized in Table II). Four different groups of H chains were used, involving three VH1 and one VH3 germline genes. One group of H chains related to HV1L1 (30) was used in three patients and another smaller group similar to V1-3b (31) was used in two patients. Both H chains are members of the VH1 family and are used in combination with putative L chain germline genes KL012 or A′. Two of the TPO-specific H chains, related to the VH3 gene 8-1B (32) and the VH1 gene HV1263 (33), were found in a single patient. One of these H chains, related to 8-1B, was found in combination with a KL012-like L chain. Out of a total of five H and L chain combinations, only one was unique for both H and L chains (HV1263/A3). The D regions could not be clearly assigned to any known germline sequences.

**B**

| | G | V | P | S | R | F | S | G | S | G | S | G | T | D | F | T | L | T | I | S | S | L | Q | P | E | D | F | A | T | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KL012 | GGG | GTC | CCA | TCA | AGG | TTC | AGT | GGC | AGT | GGA | TCT | GGG | ACA | GAT | TTC | ACT | CTC | ACC | ATC | AGC | AGT | CTG | CAA | CCT | GAA | GAT | TTT | GCA | ACT | TAC |
| WR4.2 | ... | ... | ... | ... | ... | ... | ... | ... | TC. | ... | ... | ... | ... | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | GT. | ... |
| WR4.35 | ... | ... | ... | ... | ... | ... | ... | ... | .C. | ... | ... | ... | ... | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | .T. | ... |
| WR4.4 | ... | ... | ... | ... | ... | ... | ... | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | ... | ... | TC. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| WR4.12 | ... | ... | ... | ..T | ... | ... | ... | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | ... | ... | TC. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| WR4.10 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | .T. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.36 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.33 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | .G. | ... | ... | ... | ..T | ... | ... |
| WR4.21 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.22 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.32 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.8 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ..T | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... |
| WR4.3 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | ... |
| WR4.37 | ... | ... | ... | ..G | ... | ... | ..C | ... | .C. | ... | ... | .A. | ... | ..C | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | .GT | ... | ... |
| WR4.25 | ... | ... | ... | ..G | ..A | ... | ..C | ... | .C. | ... | ... | .A. | ... | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | T.. | ... |
| JA1.9 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | G.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | A.. | ... | ..G | ... |
| SP1.5 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| SP1.4 | ... | ... | ... | ... | ... | ... | ... | ..G | ... | ..C | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| TR1.5 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..A | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| WR1.9 | ... | ... | ... | ... | ... | ... | ... | ... | TCC | ... | ... | .A. | ... | ... | ... | ... | ... | ... | G.. | ... | ... | ... | ... | ... | ..G | ... | ... | ..C | ... | ... |
| WR1.7 | ... | ... | ... | ... | ... | ... | ... | ... | .CC | ... | ... | ... | ... | ..C | ... | ... | ... | ... | G.. | ... | ... | ..A | ... | ..C | ... | ... | ..C | ..C | ... | ..T |
| SP1.2 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | .A. | ... | ... | ... | ... | .G. | ... | ... | ... | ... | ... |
| TR1.3 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.C | ... | ... | ... | ... | ... | ... | .A. | ... | ..G | ... | ... | ... | ... | ... | ... | ... |
| TR1.10 | ... | ... | ... | ... | ..A | ... | GT. | ... | ... | ... | ... | ... | ... | ..C | ... | ... | ... | ... | ... | .C. | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... |

| | | | CDR3 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Y | C | Q | Q | S | Y | S | T | P |
| KL012 | TAC | TGT | CAA | CAG | AGT | TAC | AGT | ACC | CCT |
| WR4.2 | ... | ... | ... | ... | ... | ... | .T. | ... | ..G |
| WR4.35 | ... | ... | ... | ... | ... | ... | .T. | ... | ..G |
| WR4.4 | ... | ... | ... | ... | ... | .T. | .C. | ... | ..G |
| WR4.12 | ... | ... | ... | ... | ... | .T. | .C. | ... | ..G |
| WR4.10 | ... | ... | ... | ..A | ..C | .T. | .CA | ... | ..G |
| WR4.36 | ... | ... | ... | ..A | ... | ... | .CA | ... | ..G |
| WR4.33 | ... | ... | ... | ..A | ... | ... | .CA | ... | ..G |
| WR4.21 | ... | ... | ... | ... | ... | ... | .CA | ..G | ... |
| WR4.22 | ... | ... | ... | ... | ... | ... | .CA | ..G | ... |
| WR4.32 | ... | ... | ... | ... | ... | ... | .CA | ..G | ... |
| WR4.8 | ... | ... | ... | ... | ... | ... | .CA | ..G | ... |
| WR4.3 | ... | ... | ... | ..A | ... | ... | .CA | ..G | ..G |
| WR4.37 | ... | ... | ... | ..A | ... | ... | .CA | ..G | ..G |
| WR4.25 | ... | ... | ... | ..A | ... | .T. | .CA | ... | ... |
| JA1.9 | ... | ... | ... | ... | ... | ... | .A. | ... | ... |
| SP1.5 | ... | ... | ... | ... | ... | ... | ... | ... | ..G |
| SP1.4 | ... | ... | ... | ... | ... | ... | ... | ... | ..G |
| TR1.5 | ... | ... | ... | ... | ... | ... | .C. | ..G | ..G |
| WR1.9 | ... | ... | ... | ... | ..C | ... | GA. | ... | ..G |
| WR1.7 | ... | ... | ... | ... | G.. | ... | GA. | ... | ..G |
| SP1.2 | ... | ... | ... | ... | .C. | ... | ... | T.. | ..G |
| TR1.3 | ... | ... | ... | ... | ... | ... | GC. | ... | ..G |
| TR1.10 | C.. | ... | ..G | ... | GC. | ... | .CG | ..G | --- |

*Figure 2.* Nucleotide sequences of TPO-specific F(ab) L chains. Designation of the framework (FR) and CDRs and the JK germline genes are according to Kabat et al. (29). The sources of the closest currently known VK germline genes are KL012 (28) and A′ and A3 (29). Sequences with identical nucleotides or silent substitutions were as follows: WR4.35–WR4.5; WR4.32–WR4.31; WR4.8–WR4.2/28.34. The nucleotide sequences shown here and in Fig. 3 have been submitted to GenBank (Accession No. L12061–L12103).

The large group of F(ab)s which are most closely related to the germline gene HV1L1 require further comment because they appear to fall into two groups. The WR4 clones and SP4.6 are of subclass IgG4 while SP1.2 and JA1.9 are of subclass IgG1 (Table II). There are consistent nucleotide (Fig. 3) and amino acid (Fig. 5) differences in the framework and complementarity determining regions (CDRs), which suggest that these two groups may not be derived from the same VH germline genes. Although HV1L1 is the closest available germline gene currently available, it may not be the precursor of either group of F(ab)s. Finally, the D and J regions of the two F(ab) groups are different. It should be stressed that each of these unique VH, D, and J combinations was obtained from combinatorial libraries prepared from three different patients.

*F(ab) affinities for TPO.* We expressed recombinant F(ab)s representative of the different H and L chain permutations (Table III). Despite the use of multiple bacterial hosts and culture conditions, only small amounts (up to ~ 40 μg purified protein per liter) of purified protein could be obtained. Nevertheless, this amount was sufficient for further studies. Calculation of the affinities ($K_d$) for human TPO revealed all F(ab)s to bind with high affinity (~ $10^{-10}$ M) (Table III), comparable to autoantibodies in patients' sera (2).

*Domains on TPO recognized by F(ab)s.* Competition between pairs of F(ab)s for binding to human TPO was used to define their respective binding domains. In this approach, increasing concentrations of one F(ab) were preincubated with radiolabeled TPO and then added to a second, immobilized F(ab). Representative experiments are shown in Fig. 6. For example (Fig. 6 *A*), TR1.8 and TR1.9 each completely inhibited TPO binding to immobilized TR1.9. In contrast, preincubation of WR1.7 and SP1.5 with radiolabeled TPO did not prevent subsequent TPO binding to immobilized TR1.9. Despite these differences in their ability to compete for TPO binding to TR1.9, all *free* F(ab)s were capable of binding comparable amounts of radiolabeled TPO in separate, concurrent direct binding assays (Fig. 6 *B*).

The above experiment (Fig. 6, *A* and *B*) demonstrates overlap in the areas on TPO recognized by TR1.9 and TR1.8 but not between TR1.9 and WR1.7 or SP1.5. Similarly, there was

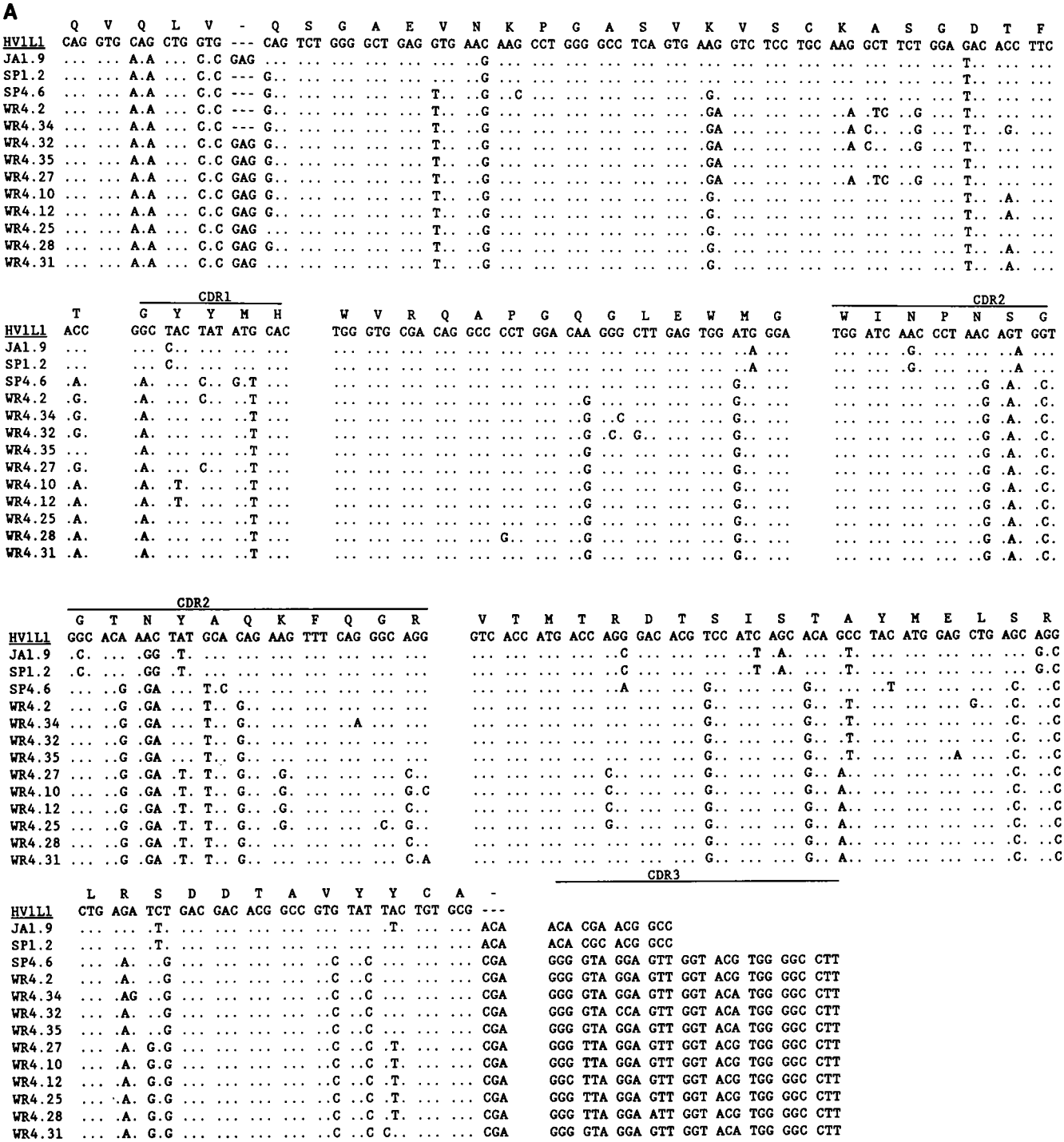



*Figure 3.* Nucleotide sequences of TPO-specific F(ab) H chains. Designation of the FR and CDRs is according to Tomlinson et al. (27). The sources of the closest, presently known VH germline genes are HV1L1 (30), HV1263 (33), V1-3b (31), and 8-1B (32). SP4.6 is a F(ab) obtained recently from patient SP (26). Sequences with identical nucleotides or silent substitutions were as follows: WR4.2–WR4.3, 4, 5, 8, 11, 21, 22, 33, 36, 37 with the exception that WR4.3, 5 and 8 have CAG, not GAG at codon 7; SP1.2–SP1.4, 5 (11); TR1.9–TR1.13.

overlap in the WR1.7 binding domain with those of TR1.8 and SP1.5 but not with TR1.9 (Fig. 6 *C*). The SP1.5 binding domain overlapped that of TR1.3 and WR1.7 but not TR1.9 (Fig. 6 *D*). The SP1.5 domain appeared to overlap to a small extent with that of TR1.8 (Fig. 6 *D*). It is important to note that these differences between F(ab) binding domains are not related to differences in their affinities for TPO (Table III).

The above competition studies and others not shown, covering all permutations of immobilized and free F(ab)s, suggest that there are two domains, A and B, recognized on human TPO. The extent to which the F(ab)s interact with each domain is summarized in Table IV and is shown schematically in Fig. 7. The binding sites of SP1.5 and WR4.5 lie completely within the TPO A domain. TR1.9 binds entirely to the B domain. TR1.8 interacts predominantly with the B domain but overlaps slightly with the A domain. Conversely, WR1.7 binds

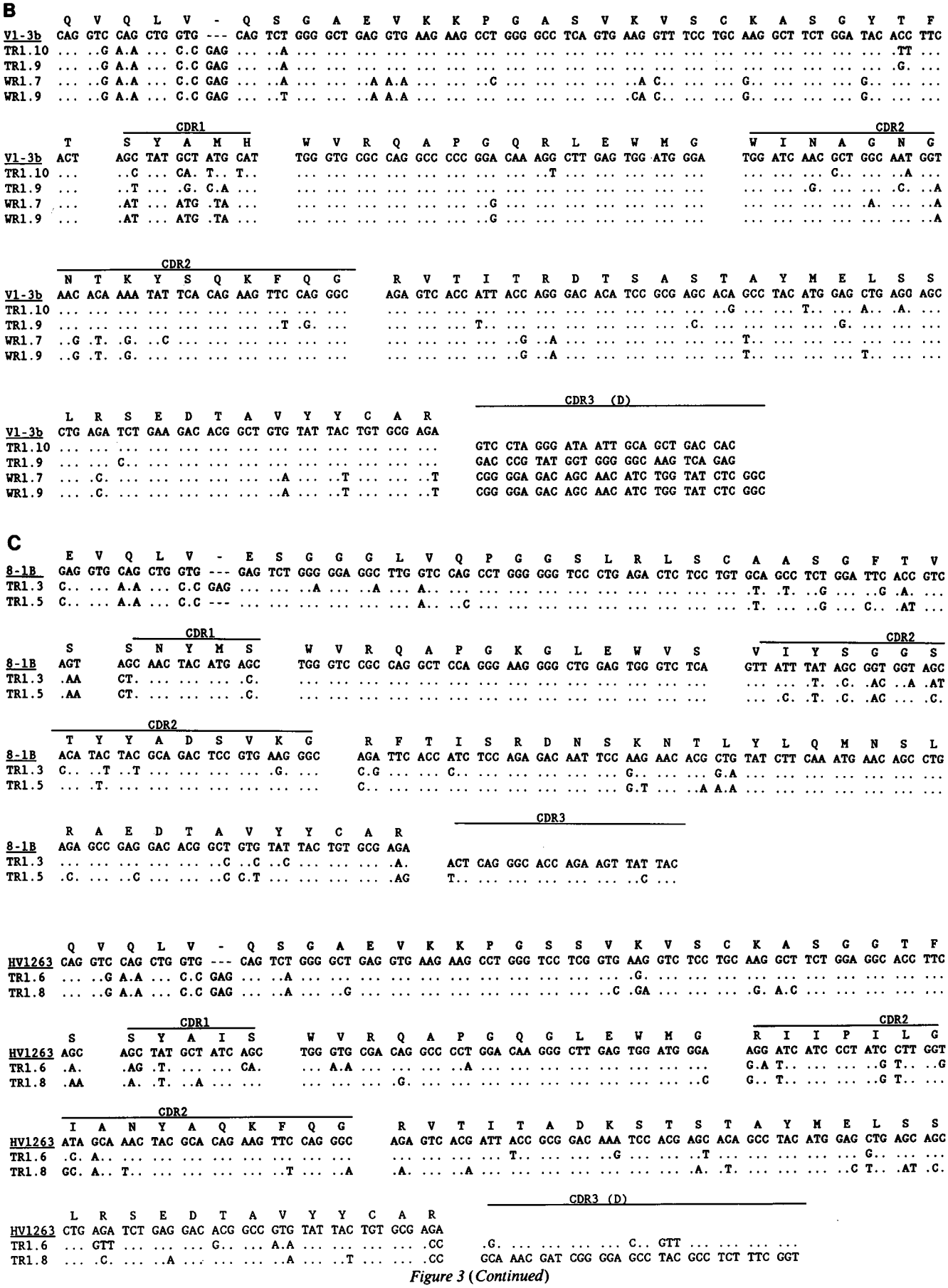



*Figure 3 (Continued)*

| | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | JKappa |
|---|---|---|---|---|---|---|---|
| KL012 | DIVMTQSPSSLSASVGDRVTITC | RASQSISSYLN | WYQQKPGKAPKLLIY | AASSLQS | GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC | QQSYSTP | |
| TR1.10 | EL.....P.....N. | .....TK..A | .....E..... | DS.T..G | .....V.....T.....H. | ..A.T.- | 1 |
| JA1.9 | EL..... | .....R... | ..... | ..... | .....A.....I..... | ....N.. | 4 |
| SP1.4 | EL..... | ....TVGT... | .....E..... | T..T... | .....R..... | ..... | 2 |
| TR1.5 | EL..... | .....N.... | ..... | ...T.L. | ..... | ....T.. | 2 |
| TR1.3 | EL..... | ....T.GD... | ...D.S..P..V... | ...N.HT | .....H.....N..... | ....A.. | 1 |
| SP1.5 | EL.....S. | ....N.GK... | ..R.....E.... | GT.T... | ..... | ..... | 2 |
| WR1.9 | EL..... | .....GR... | .....V..H | T....L | .....E.....V..... | ....D.. | 1 |
| WR1.7 | EL..... | ....N.GR... | .....V.VH | T..T.L. | .....T.....V..... | ..G.D.. | 1 |
| WR4.2 | EL..... | .....Q... | .....S | .T.T.L. | .....S.....V... | ....I.. | 2 |
| WR4.35a | EL..... | .....Q..H | .....S | .T.N.L. | .....T.....S.....I... | ....I.. | 2 |
| WR4.33 | EL..... | .....MVH..H | .....A.....S | .T.N.V. | .....T..E...S.....R..... | ....T.. | 2 |
| WR4.12 | EL.....L...I.... | .....VGR... | ....M.....S | .T...L. | .....T..E...S..... | ...FT.. | 2 |
| WR4.4 | EL.....I.... | .....VGR... | ....M.....S | .T...L. | .....T..E...S..... | ...FT.. | 2 |
| WR4.25 | EL..... | .....VGR..H | ...K...E.....S | .T.D.L. | .....T..E...S.....S... | ...FT.. | 2 |
| WR4.3 | EL.....T..... | .....GR..H | ..H..A.....S | .T.N.V. | .....T..E...S..... | ....T.. | 2 |
| WR4.37 | EL.....T..... | .....VGR..H | .....A.....S | .T.N.V. | .....T..E...S.....G.... | ....T.. | 2 |
| WR4.32b | EL..... | .....GR..H | .....A.....S | .T.N.VG | .....T..E...S..... | ....T.. | 2 |
| WR4.22 | EL..... | .....GR..H | .....A.....S | .T.N.VG | .....T..E...S..... | ....T.. | 2 |
| WR4.8c | EL..... | .....GR..H | .....A.R.....S | .T.N.VG | .....T..E...S..... | ....T.. | 2 |
| WR4.36 | EL..... | .....GR..H | .....A.....S | .T.N.V. | .....T..E...S..... | ....T.. | 2 |
| WR4.21 | EL..... | .....GR..H | .....A.N.....S | .T.N.VG | .....T..E...S..... | ....T.. | 2 |
| WR4.10 | EL.....L.....S.... | .....GR..H | .....A....E...S | .T.N.V. | .....T..E...S.I..... | ...FT.. | 2 |
| SP1.2 | EL.....E..T..... | ...EN..R.S. | ....Q.....S | ...T... | .....H.....N..... | ..T..S. | 2 |
| | | | | | | | |
| A' | AIQLTQSPSSLSASVGDRVTITC | RASQGISSALA | WYQQKPGKAPKLLIY | DASSLES | GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC | QQFNSYP | |
| TR1.13 | ELVM..... | .....RG.. | ..... | ...T... | ....S..... | ..... | 4 |
| TR1.9 | ELVM.....N.A. | ..... | .....R.... | ...N... | .....I... | ..... | 4 |

| | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | |
|---|---|---|---|---|---|---|---|
| A3 | DIVMTQSPLSLPVTPGEPASISC | RSSQSLLHSNGYNYLD | WYLQKPGQSPQLLIY | LGSNRAS | GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC | MQALQTP | |
| TR1.6 | EL..... | .....G..... | ..... | ..... | ..... | .....P. | 2 |
| TR1.8 | EL.....F..... | ..... | ..... | .S.S... | ..... | .....P. | 2 |

*Figure 4.* Derived amino acid sequences of TPO-specific F(ab) L chains. Designation of the FR and CDRs and sources of VK germline genes are as in Fig. 2. (*a*) Same as WR4.5; (*b*) same as WR4.31; (*c*) Same as WR4.27, 28, 34.

primarily to the A domain but also overlaps with TR1.8 (but not TR1.9) in the B domain. The binding site of TR1.3 spans the A and B domains equally.

*Domains on TPO recognized by autoantibodies in patients' sera.* The question arises as to what extent the TPO binding domains A and B reflect the binding domains of TPO autoantibodies in patients' sera. F(ab)s WR1.7 and TR1.9 were selected for competition studies with serum TPO autoantibodies because their binding sites do not overlap yet extensively cover the A and B domains.

A spectrum of competition patterns was observed in 10 randomly selected sera of patients with autoimmune thyroid disease. In the representative examples shown, TPO autoantibodies in patients' sera were inhibited preferentially by WR1.7

| | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 (D) | |
|---|---|---|---|---|---|---|---|
| HV1L1 | QVQLV-QSGAEVNKPGASVKVSCKASGDTFT | GYYMH | WVRQAPGQGLEWMG | WINPNSGGTNYAQKFQG | RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR | | |
| WR4.10 | ..K.LEE....LK.....R.....YN.N | DF.I. | .....V | .....KNA..RFSER.. | G.....A..AT....TS..A.....F... | GLGVGTWGL | JH4 |
| WR4.12 | ..K.L.E....LK.....R.....YN.N | DF.I. | .....V | .....KNA..RFSER.. | .....A..AT....TS..A.....F... | ..... | . |
| WR4.28 | ..K.L.E....LK.....R.....YN.N | D..I. | .....A.....V | .....KNA..RFSE.... | .....A..AT....TS..A.....F... | ...I..... | . |
| WR4.27 | ..K.L.E....LK.....R....V..Y..S | D.HI. | .....V | .....KNA..RFSER.. | .....A..AT....TS..A.....F... | ..... | . |
| WR4.34 | ..K.L-E....LK.....R....P..YS.S | D..I. | .....V | .....KNA..R.SE.... | .....A..AV....TS..... | .V..... | . |
| WR4.35 | ..K.L.E....LK.....R.....Y..T | D..I. | .....V | .....KNA..R.SE.... | .....A..AV....TS..... | .V..... | . |
| WR4.32 | ..K.L.E....LK.....R....P..Y..S | D..I. | .....AV..V | .....KNA..R.SE.... | .....A..AV....TS..... | .VP..... | . |
| WR4.31 | ..K.L.....LK.....R.....YN.N | D..I. | .....V | .....KNA..RFSE.... | .....A..AT....TS..A.....H... | .V..... | . |
| WR4.25 | ..K.L.....LK.....R.....Y..N | D..I. | .....V | .....KNA..RFSER.A | G....G..A..AT....TS..A.....F... | ..... | . |
| WR4.2[a] | ..K.L-E....LK.....R....V..Y..S | D.HI. | .....V | .....KNA..R.SE.... | .....A..AV...VTS..... | .V..... | . |
| SP4.6 | ..K.L-E....LKN.....R.....Y..N | D.HV. | .....V | .....KNA..R.SQ.... | .....A..A.....TS.K..... | .V..... | . |
| JA1.9 | ..K.LEE.....K.....Y... | .H... | .....I | ...S..R.A.RF..... | .....S....N.V.....G..F.....T | TRTA | JH6 |
| SP1.2[b] | ..K.L-.....K.....Y... | .H... | .....I | ...S..R.A.RF..... | .....S....N.V.....G..F....F..T | .... | . |
| | | | | | | | |
| V1-3b | QVQLV-QSGAEVKKPGASVKVSCKASGYTFT | SYAMH | WVRQAPGQRLEWMG | WINAGNGNTKYSQKFQG | RVTITRDTSASTAYMELSSLRSEDTAVYYCAR | | |
| WR1.7 | ..K.LE.....I.....L..E...D... | N.MI. | ..... | .....D..KIR..... | .....S.....T.....S | RGDSNIWYLG | JH4 |
| WR1.9 | ..K.LE.....I.....TL..E...D... | N.MI. | ..... | .....KIR..... | .....S.....T..... | ..... | . |
| TR1.9[c] | ..K.LE.....S.. | ..GL. | ..... | ....S..T.....R. | ...F.....T....G.....P..... | DPYGGGKSE | . |
| TR1.10 | ..K.LE.....I.. | ..HL. | Y.....S.... | .....P.K..... | .....L.MN..... | VLGIIAADH | . |
| | | | | | | | |
| 8-1B | EVQLV-ESGGGLVQPGGSLRLSCAASGFTV | SSNYM | SWVRQAPGKGLEWV | SVIYSGGSTYYADSVK | GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR | | |
| TR1.3 | Q.K.LE.....I.....VV..LN. | KL... | T..... | ...FTD.NP.....R | ....L.....E..V.....K | TQGTRSYY | JH4 |
| TR1.5 | Q.K.L-.....IH.....V...LN. | KL... | T..... | ..TFTD.T.F..... | .....D..I.....T.D...L....K | S..... | . |
| | | | | | | | |
| HV1263 | QVQLV-QSGAEVKKPGSSVKVSCKASGGTF | SSYAI | SWVRQAPGQGLEW | MGRIIPILGIANYAQKF | QGRVTITADKSTSTAYMELSSLRSEDTAVYYCAR | | |
| TR1.6 | ..K.LE.....R..... | NKF.. | H.I..... | ..GF..MF.TT..... | .....S.....V...V...A.I....T | GNDRGPVASFG | JH3 |
| TR1.8 | ..K.LE.....R...RT..... | KNF.. | ....R..... | ..GF..MF.ATY..... | ..K.....NI...D.NT.T.....T | A....AY.... | . |

*Figure 5.* Derived amino acid sequences of TPO-specific F(ab) H chains. Designation of the FR and CDRs and sources of the VH germline genes are as in Fig. 3. SP4.6 is a F(ab) obtained recently from patient SP (26). The Q to K substitution at position 3 represents the restriction site introduced by the PCR oligonucleotide. The presence of an E at position 6 (not in the putative germline gene) results from the use of the 1a/3a oligonucleotide primers (see Table I). (*a*) Same as WR4.3, 4, 5, 8, 11, 21, 22, 33, 36, 37 with the exception that WR4.3, 5, and 8 have Q (for E) at position 7; (*b*) same as SP1.4, 5 (11); (*c*) Same as TR1.13.

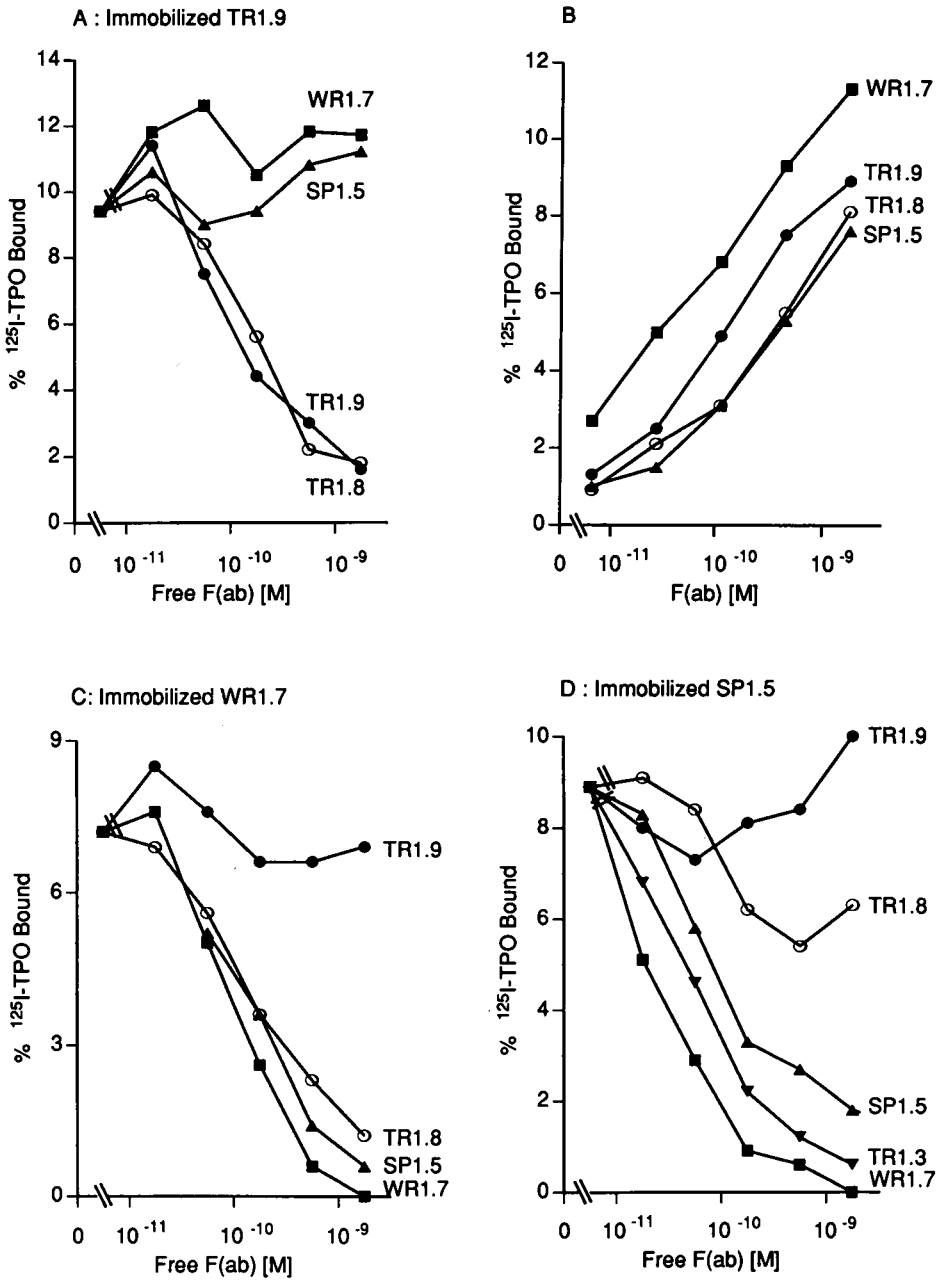



*Figure 6.* Domains on TPO recognized by F(ab)s. Increasing concentrations of one F(ab) were preincubated with radiolabeled TPO and then added to a second, immobilized F(ab) (Methods). The immobilized F(ab) was TR1.9 (panel *A*), WR1.7 (panel *C*), and SP1.5 (panel *D*). Confirmation of the direct binding potency of the *free* F(ab)s was determined concurrently in each experiment. A representative control (panel *B* for the experiment in panel *A*) is shown.

(Fig. 8 *A*), preferentially by TR1.9 (Fig. 8 *B*) or in a more balanced proportion by both F(ab)s (Fig. 8 *C*). Overall, of the 10 sera, five were inhibited preferentially by WR1.7, two by TR1.9, and three to approximately the same extent by WR1.7 and TR1.9.

Of greater importance was the efficacy of the combination of the WR1.7 and TR1.9 F(ab)s in competing for serum TPO autoantibody binding. In the 10 sera, this combination inhibited TPO autoantibody binding by 83±5% (mean±SEM). These figures may underestimate the full extent of the inhibition because of limitations to the highest concentrations of F(ab)s which could be used for competition.

## Discussion

There is evidence that the ability to produce TPO autoantibodies is inherited as an autosomal dominant trait in women with incomplete penetrance in men (34, 35). The location and nature of the gene or gene cluster responsible for this inheritance are unknown. Polymorphisms at the VH locus are associated with autoantibody production (31, 36). Therefore, knowledge of the genetic background of TPO-specific autoantibodies may provide insight into the basis for the inheritance of thyroid autoimmunity.

The present report identifies and characterizes the genes coding for a comprehensive panel of high-affinity human autoantibodies to a major, organ-specific autoantigen. These recombinant F(ab)s cover a region on thyroid peroxidase recognized by a majority (~ 80%) of autoantibodies in the sera of patients with thyroiditis, the most common autoimmune disease. In the more intensively studied connective tissue diseases, genes for numerous autoantibodies have been characterized. Initially, the "natural" and disease-associated autoantibodies cloned were IgM, many of low affinity and with polyspecificity (37,

*Table III. Affinities for Human TPO of Expressed F(ab) Fragments*

| H chain | L chain | Clone | Affinity |
|---|---|---|---|
| | | | $K_d$ (M) |
| HV1L1 | KL012 | WR4.5 | $3.0; 3.2 \times 10^{-10}$ |
| | | SP1.2* | $8.0 \pm 1.0 \times 10^{-11}$ |
| | | SP1.4* | $2.0; 2.0 \times 10^{-10}$ |
| | | SP1.5* | $8.0 \pm 1.0 \times 10^{-11}$ |
| | | SP4.6* | $1.0 \pm 0.2 \times 10^{-10}$ |
| V1-3b | KL012 | WR1.7 | $1.2; 2.9 \times 10^{-10}$ |
| | | TR1.10 | $1.7; 1.4 \times 10^{-10}$ |
| 8-1B | KL012 | TR1.3 | $5.1 \pm 0.1 \times 10^{-10}$ |
| HV1263 | A3 | TR1.8 | $2.7 \pm 0.1 \times 10^{-10}$ |
| V1-3b | A′ | TR1.9 | $1.5 \pm 0.2 \times 10^{-10}$ |

Classification is according to their putative germline genes based on presently available data. Values from duplicate or triplicate experiments were calculated by Scatchard analysis (23). * Previously reported (11, 26).

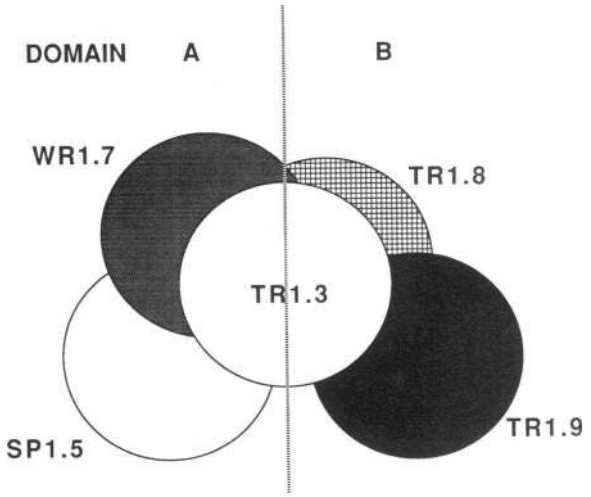



*Figure 7.* Schematic representation of the binding domains on TPO for the expressed F(ab)s.

38). More recently, high-affinity IgG-class rheumatoid factors and antibodies to double-stranded DNA have been produced and defined (39–42). However, the combination of a comprehensive autoantibody repertoire and its antigenic domain(s) has not been defined previously in either organ-specific or non-organ-specific autoimmune disease in humans.

From thyroid tissue-infiltrating B cells from three patients with autoimmune thyroiditis, we have cloned and determined the nucleotide sequences of 30 new TPO-specific F(ab)s. Previously, we had reported information on four antibodies obtained from another patient (10, 11, 26). The most remarkable finding from analysis of the putative germline genes used for TPO human autoantibodies is the relatively restricted number and combinations of H and L chain genes. Similarly, there is evidence for restricted T cell receptor V gene usage in the early stages of autoimmune thyroid disease (reviewed in reference 43). In our study of 34 recombinant F(ab)s, we found a total of only five different H and L chain combinations involving four H chains and three L chains. In addition, one combination of H and L chains (HV1L1 and KL012) was obtained from combinatorial libraries prepared from three patients. Another H and L chain combination (V1-3b and KL012) was found in libraries prepared from two patients. These combinations involved different H chains (HV1L1 and V1-3b) with the same L chain (KL012). As stated previously, these apparent H and L chain restrictions are based on presently available information on germline genes in the population. Without data on the germline genes in the individual patients, it is possible, particularly for the L chains, that additional germline genes may be involved.

*Table IV. Domains on TPO Recognized by F(ab)s as Determined by Competition between Pairs of F(ab)s for Antigen Binding*

| | TPO domains | | | |
|---|---|---|---|---|
| Clone | A | B | H chain | L chain |
| SP1.5 | ++++ | − | HV1L1 | KL012 |
| WR4.5 | ++++ | − | HV1L1 | KL012 |
| WR1.7 | ++++ | ++ | V1-3b | KL012 |
| TR1.3 | ++++ | ++++ | 8-1B | KL012 |
| TR1.8 | + | ++++ | HV1263 | A3 |
| TR1.9 | − | ++++ | V1-3b | A′ |

Classification is according to their putative germline genes based on presently available data. ++++, complete; −, no overlap with the indicated domain. + or ++, partial overlap.

Studies of "natural" autoantibodies and autoantibodies to erythrocyte antigens have shown over-representation of VH4 family genes (38, 44). In contrast, four-fifths of our TPO-specific F(ab) combinations were derived from VH1 family germline genes. The other F(ab) utilized a VH3 germline gene. Among the VH1-encoded TPO F(ab)s, the most abundant were related to the recently described HV1L1 germline gene, which is utilized in a rheumatoid factor (30). Because of the pattern of nucleotide differences between our TPO F(ab)s and HV1L1, it is possible that the former are not derived from HV1L1 but from another, as yet undescribed, germline gene.

Three factors could contribute to the apparent TPO-specific VH and VL gene restriction that we observed: (*a*) the use of thyroid-infiltrating B cells, (*b*) limitations imposed by the oligonucleotide primers used in the PCR, and (*c*) overrepresentation of particular VH and VL genes in our libraries. First, regarding the source of the B cells, our panel of TPO-specific F(ab)s was only obtained because we used lymphocytes from the thyroid, which is enriched with TPO-specific B cells (reviewed in reference 13). Information on TPO autoantibodies generated from extrathyroidal sources would be of interest but would be beyond the scope of the present study. Further, this goal may not be feasible given the statistics of the H and L chain combinatorial approach and the ethical impossibility of boosting our patients with TPO, as was done with tetanus toxoid (16, 45).

It is unlikely that the TPO-specific F(ab) H and L chain gene restriction can be attributed to limitations in the oligonucleotide primers used in the study. In the case of the H chains, we used a wide range of variable region primers designed to

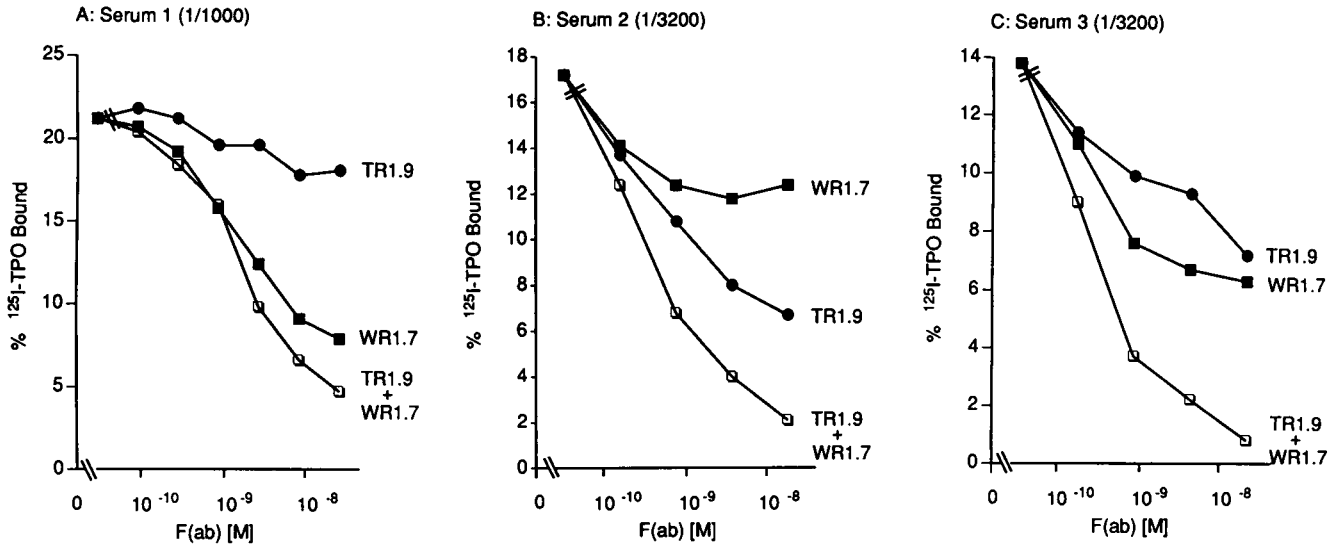



*Figure 8.* Domains on TPO recognized by autoantibodies in three representative sera (panels *A*, *B*, and *C*) from patients with autoimmune thyroid disease. F(ab)s WR1.7 and TR1.9, alone or in combination, were used to compete for serum autoantibody binding to radiolabeled TPO (Methods).

cover all six VH families (Table I). In addition, the F(ab)s obtained used variable regions genes from more than one VH (VH1, VH3) and VK (VK1, VK2) family. Our libraries also contain VH4 genes as we obtained this family in a F(ab), the sequence of which is not included in the results because we could not express the protein.

We have evidence against the possibility of over-representation in our libraries of particular VH and VL genes. In separate studies (26), we observed that functional HV1L1 VH and KL012 VL genes in a library are relatively rare, ~ 1:5,000 and 1:500, respectively. Finally, the most important evidence suggesting that the TPO-specific VH and VL gene restriction observed is not a consequence of all three potential limitations discussed above is that the F(ab)s expressed in our repertoire represent more than 80% of TPO autoantibodies present in patients' sera.

Whether or not the combinatorial library approach can generate the H and L chain combinations observed in vivo is under debate (46, 47). The same pairing of H and L chains was observed in murine monoclonal antibodies (specific for influenza hemagglutinin) generated by both conventional hybridoma technology and the combinatorial recombinant F(ab) approach (48). In contrast, in a study of hapten antibodies, the in vivo H and L chain pairing was not attained by the combinatorial approach (46). In addition, some studies have shown H chain promiscuity in pairing with L chains (47). Therefore, we cannot be certain that the TPO-specific H and L chain combinations reflect the in vivo situation. However, using a TPO-specific H (or L) chain to randomly search for other L (or H chains) from the same patient capable of forming F(ab)s with high affinity for TPO, we did not observe promiscuity and only obtained closely related L and H chains from the same VK and VH families (26). Further, our F(ab)s had very high affinities for TPO (~ $10^{-10}$ M), comparable with those of serum TPO autoantibodies (2).

Turning to the other major aspect of our study, the majority of TPO autoantibodies in patients' sera interact with the protein in its native, undenatured state (11, 49, 50). Despite studies using different approaches (reviewed in reference 5), the number of epitopes recognized by TPO autoantibodies, or even the number of antigenic domains involved, was unknown. Previous evidence, with which the present data are in agreement, suggested that the human autoimmune response to TPO (51, 52), like that to thyroglobulin (53), is limited to a few epitopic domains.

Using a comprehensive repertoire of recombinant human F(ab)s, we now define two major contiguous epitopic areas, A and B, on TPO. Most IgG-class serum TPO autoantibodies interact with the A and B domains. TPO is a large (933 amino acids, 107 kD) (54) globular protein relative to the size of the F(ab) binding site (55). For this reason and because a combination of only two different F(ab)s can compete for binding by most TPO autoantibodies, the autoimmunogenic region comprises a single, relatively small area (domains A + B). It is possible that serum TPO autoantibodies to other regions of TPO, including linear epitopes (reviewed in reference 5), exist but these will comprise a small proportion of the repertoire (11, 56).

The present information now permits analysis of the relationship between H and L chain genes and the antigenic domains on TPO recognized by TPO autoantibodies. Such an analysis has not previously been possible in an autoimmune disease. The clearest association is between the L chain gene KL012 and the A domain on TPO (Table IV). In contrast, the B domain is most closely associated with the L chains A′ and A3. The H chains are more diverse and may alter the fine specificity of the F(ab) binding site by interacting with antigenic areas adjacent to the L chain binding site. In some instances, exemplified by TR1.3 (VH 8-1b/VK KL012), the H chain appears to shift the F(ab) binding from the A domain to cover both A and B domains.

Of interest is the observation that F(ab)s of both subclass IgG1 and IgG4 can interact with the A domain on TPO. All of the IgG4 and some of the IgG1 F(ab)s appear to be related to the VHI germline gene HV1L1. Use of the same VH germline gene by F(ab)s of two IgG subclasses raises the possibility of subclass switching. In rheumatoid factors, the same germline gene has clearly been shown to code for a low-affinity IgM and

subsequently for an affinity-matured IgG molecule (42). In contrast, observations of crossreactive idiotypes in human antibodies to *Haemophilus influenzae* b polysaccharide indicate independent B cell lineages in IgG1 and IgG2 antibodies (57). However, in the case of the TPO-specific IgG1 and IgG4 F(ab)s, we feel that subclass switching is unlikely. First, there are significant differences between the VH1 components of the IgG subclasses. Second, these H chains have totally different D and J regions.

Studies in mice have provided evidence for (58–60) and against (61) restricted H and L chain usage in rheumatoid factors and DNA autoantibodies. Similarly, a diverse array of immunoglobulin genes occurs in human rheumatoid factors derived from rheumatoid synovial tissue (62). A possible explanation for these divergent observations is that the autoantibodies are interacting with a wide range of epitopes. For example, thyroglobulin autoantibodies, induced by immunization in mice, interact with many epitopes and are derived from a large number of V region gene segments (63). In contrast, our data in an organ-specific autoimmune disease, indicate that disease-associated autoantibodies recognize a limited antigenic region on TPO. Such autoantibodies do, indeed, appear to be associated with restricted H and L chain usage.

## Acknowledgments

We thank Dr. Orlo Clark and Dr. Quan Duh (University of California, San Francisco) and Dr. Sebastiano Filetti (University of Catanzaro, Italy) for providing the Graves' thyroid tissue used in this study.

This research was supported by National Institutes of Health grants DK-36182 and EY-09498.

*Note Added in Proof.* We note the recent report by Hexham et al. (1992. *Autoimmunity.* 14:169–172) of the H and L chain variable region sequences of a human monoclonal TPO autoantibody generated by fusion of Hashimoto thyroid lymphocytes with a mouse myeloma line.

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