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A Combinatorial Approach Toward DNA Recognition

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A combinatorial approach has been used to identify individual RNA molecules from a large population of sequences that bind a 16–base pair homopurine-homopyrimidine DNA sequence through triple-helix formation. Fourteen of the seventeen clones selected contained stretches of pyrimidines highly homologous to the target DNA sequence (T·AT and C^+·GC). In addition, these RNA molecules contained hairpin loops, interior loops, and nonstandard base triplets [C^+(or C)·AT, U·GC, G·GC, and A·AT] at various positions. Affinity cleavage experiments confirmed the ability of selected sequences to bind specifically to the target DNA. Systematic variation in both the target DNA sequence and buffer components should provide increased insight into the molecular interactions required for triple-helix–mediated recognition of natural DNA.

THE POWER OF COMBINATORIAL strategies for generating molecules with novel properties has been exploited in the generation of catalytic antibodies (*1*) and more recently in the study of protein-ligand interactions (*2–5*). We report application of this approach to the molecular recognition of double-helical DNA through the synthesis and screening of large RNA libraries for selective triple-helix formation. The sugar-phosphate backbone of nucleic acids provides a natural framework for building specific hydrogen-bonding interactions with functional groups in the major groove of DNA (*6*). This structural motif, however, is currently limited to the recognition of predominantly homopurine-homopyrimidine tracts of DNA. Polypyrimidine oligonucleotides bind specifically in the major groove, parallel to the purine strand, through Hoogsteen T·AT and C^+·GC base triplets (*7, 8*). Polypurine oligonucleotides bind in the major groove, parallel to the pyrimidine strand, through G·GC and A·AT triplets (*9*). Recently, it has been shown that a G·TA triplet can also be formed in a polypyrimidine oligonucleotide, although the interaction is considerably weaker than the T·AT triplet (*10*). Given the conformational flexibility associated with nucleic acids, as well as the effects of pH, salt, and small molecules on nucleic acid structure, it was of interest to determine whether additional hydrogen-bonding schemes might be associated with triple-helix formation. The extension of triple-helix formation to the recognition of mixed DNA sequences would likely find applications in the analysis of nucleic acid structure and function (*11*) as well as in the development of therapeutic agents based on selective gene inactivation (*12*).

Because a general solution to triple-helix–mediated recognition of natural DNA sequences might involve relatively complex rules (including context effects, looped structures, and conformational isomers), we chose a strategy that samples large numbers of diverse sequences for binding to a defined DNA duplex. Analysis of those sequences that form the most stable triple-stranded structures, coupled with systematic variations in the target sequence, should provide increased insight into the molecular interactions responsible for triple-helix formation. In order to test the feasibility of this approach, an RNA library has been generated and screened against a homopurine-homopyrimidine target DNA, for which the recognition "rules" are known (T·AT and C^+·GC) (*7, 8*).

The library of randomized RNA molecules was generated from a 106-nucleotide (nt) DNA template containing: (i) a T7 promoter sequence; (ii) a 16-nt priming site for the polymerase chain reaction (PCR); (iii) a 50-nt random sequence (synthesized from an equimolar mixture of the four bases); and (iv) another 23-nt priming site for reverse transcription and PCR amplification of the in vitro transcripts (Fig. 1A) (*2, 3*). In vitro transcription of the template with a 33-nt primer (Fig. 1A) and T7 RNA polymerase (*13*) affords an 89-nt RNA transcript. Based on the amount of the DNA template used in the initial transcription reaction, the complexity of the RNA pool generated from this template is on the order of 10^{10} to 10^{12} individual primary sequences, a small fraction of all possible sequences (*3*). In addition to sequence diversity, randomized 50-nt oligoribonucleotides should be able to form a large number

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of secondary and even tertiary structures. Sequencing analysis of 20 cDNA clones of the transcribed RNA library indicates that the base composition is essentially random, with a slight excess of purines over pyrimidines.

In vitro selection was carried out with a 25-bp DNA fragment containing a 16-bp homopurine-homopyrimidine target site (Fig. 1B). This sequence was immobilized onto a thiol-Sepharose support through a disulfide bond at the 3′ terminus of the pyrimidine strand (*14*). This linkage strategy avoids secondary components (such as streptavidin-biotin) that might also bind the RNA library. In order to insure selection for triple-helix formation over simple Watson-Crick duplex formation, the following control experiment was carried out. Two oligonucleotides (5′-AGAGAGAGAGAGGGA-3′ and 5′-TCTCTCTCTCTCCCT-3′) were synthesized that can form either a Watson-Crick duplex with the immobilized pyrimidine strand or a triplex structure with the immobilized duplex DNA, respectively. The oligonucleotides were labeled with ^{32}P at their 5′ termini and assayed for binding to the 25-bp DNA affinity column. The pyrimidine oligonucleotide was retained by the support when loaded and washed with 2 M NaCl, pH 5.5 buffer, conditions known to favor triple-helix formation (*15*). When the DNA affinity column was subsequently eluted with 50 mM tris·HCl buffer (pH 8.0), the bound pyrimidine oligonucleotide was eluted from the column. In contrast, the purine oligonucleotide showed no significant binding to the DNA affinity column under either high or low pH and salt concentration.

Selection was carried out by incubating the in vitro RNA transcripts with the DNA affinity column at low pH (pH 5.5) and high salt concentration (2.0 M NaCl) for 1 hour at room temperature (*16*). The support was washed exhaustively with the same buffer to remove unbound RNA; bound RNA was then eluted with a low-salt buffer (50 mM tris·HCl, pH 8.0, 1 mM EDTA). The eluted RNA molecules were used as templates for cDNA synthesis and subsequent amplification by PCR reaction (*17*). Because the 33-nt 5′ primer (Fig. 1A) also contains a promoter sequence for T7 RNA polymerase, the amplified cDNA library could be used directly as a template for the next round of selection against the immobilized DNA fragment. During the third, fourth, and fifth rounds of selection, bound RNA transcripts eluted from the DNA affinity column (positive selection) were subsequently passed through an underivatized thiol-Sepharose column under the same conditions in order to remove RNA specific for the thiol-Sepharose support (negative selection) (*18*). After five successive rounds of selection, the RNA transcripts were transcribed into cDNAs and amplified. The duplex cDNAs were digested with restriction enzymes Eco RI and Hind III, and inserted into Eco RI–Hind III fragment of plasmid pDP24 (*19*).

Sequencing of 17 clones of the cDNA library revealed that 14 clones contained stretches of homopyrimidine sequences that are highly "complementary" to the homopurine-homopyrimidine target DNA (T·AT and C^+·GC) (Fig. 1C). Clones 1 and 2 are complementary to 16 and 15 base pairs of the target, respectively, but contain one interior loop each in the RNA strand. Clone 3 has a single C^+(or C)·AT nonstandard triplet, and clones 4, 7, and 8 have three or four nonstandard triplets [C^+(C)·AT, U·GC, A·AT, and G·GC] each at various positions. Clone 9 contains a single C^+(C)·AT triplet as well as deletions of one or two nucleotides at both the 5′ and 3′ termini of the putative recognition motif of the RNA, whereas clone 10 contains three nonstandard triplets and a single nucleotide interior loop. Clones 5 and 6 contain 7- to 8-nt hairpin loops as well as one or two nonstandard triplets and interior loops within the pyrimidine tracts. Clones 11 to 14 contain a combination of interior loops, nonstandard triplets, and truncations in the recognition motifs. Examination of the remaining clones showed no obvious homology to the target sequence based on known triple-helix motifs. Careful analysis of a larger set of sequences may reveal new bonding schemes not yet evident in these clones. Interestingly, all 17 clones sequenced contain truncations in the random sequence region between the PCR primers (which rendered these transcripts 14 to 30 nt shorter than the expected length) that occurred during the process of repeated transcription, selection, and reverse transcription. This phenomenon has been observed by us in a number of libraries (*20*) and by others (*21*) and likely reflects an enzyme-associated selective pressure.

In order to determine whether these sequences do in fact specifically bind duplex

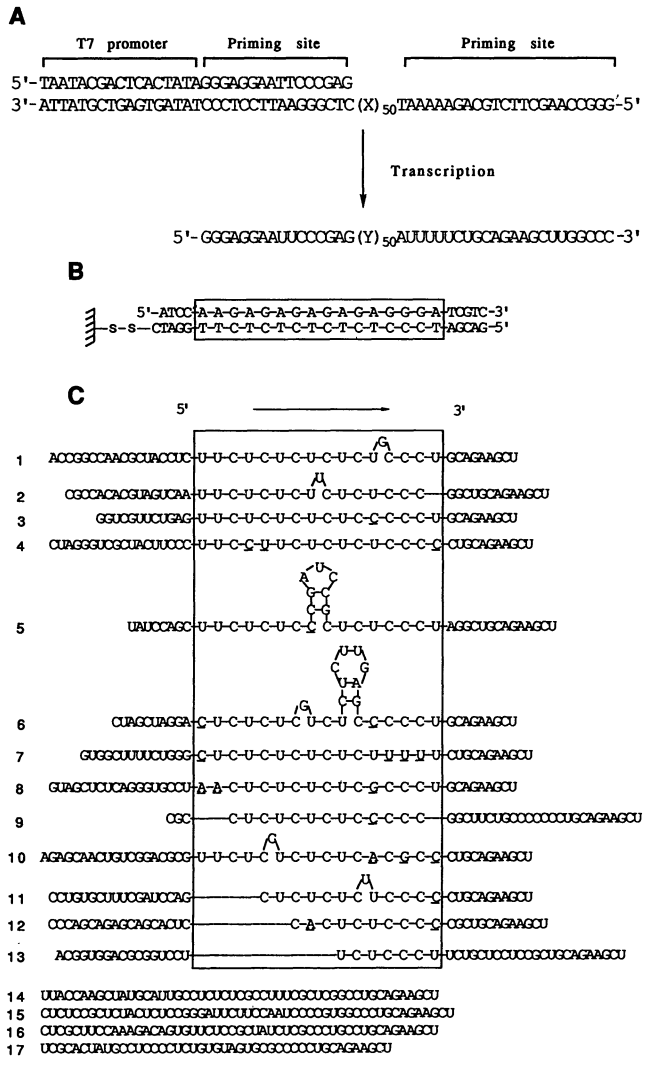

Fig. 1. (**A**) The 106-nt DNA template, 33-nt primer containing the T7 promoter, and 89-nt in vitro RNA transcript. The 50-nt random sequence is denoted by $(X)_{50}$ and $(Y)_{50}$ in DNA and RNA, respectively. (**B**) DNA affinity column with the homopurine-homopyrimidine target sequence boxed. (**C**) Sequences of 17 clones of the selected RNAs transcribed from Hind III–linearized pDP24 containing the cDNA insert (*19*). The putative recognition motifs (boxed) of clones 1 to 14 are aligned with the 16-bp target DNA. The secondary structures are depicted to show maximum sequence homology between the putative recognition motifs of RNAs and the target DNA. Underlined bases indicate nonstandard base triplets. Clones 15 to 17 show no apparent homology to the target DNA and thus were not aligned with the target.

DNA, we examined their ability to direct affinity cleavage of the target DNA. This method involves conjugation of synthetic DNA cleaving agents (*22*) or nonspecific phosphodiesterases (*23, 24*) to the DNA binding molecules of interest. Analysis of the DNA cleavage patterns generated by the conjugate provides information about the sequence specificity, binding orientation, and groove preference of the associated DNA binding molecules. A 17-nt oligoribonucleotide corresponding to the sequence of clone 1 (5′-UUCUCUCUCUCUGCCCU-3′) was chemically synthesized (Applied Biosystems) with a thiol-derivatized phosphoramidite coupled to the 5′ terminus (*25*). After deprotection and purification (*26*), the oligoribonucleotide (which contains a sulfhydryl linked to the 5′ terminus by a tether of six methylenes and a phosphate) was coupled to a $Lys^{84} \rightarrow Cys$ (K84C) mutant staphylococcal nuclease by a disulfide exchange reaction (*24*). The resulting oligoribonucleotide–staphylococcal nuclease conjugate was assayed for its ability to cleave a 269-bp 3′-^{32}P end-labeled restriction fragment containing the 16-bp homopurine-homopyrimidine target sequence. Examination of the cleavage pattern (Fig. 2, lane 5) reveals that cleavage occurred over three A·T base pairs at the 5′ terminus of the homopurine target site (pH 5.7, 100 mM NaCl, 2 mM spermine, 20% ethylene glycol) [in accord with our earlier semisynthetic nuclease work (*24*)]. This result confirms that the pyrimidine oligoribonucleotide forms a triple-helical structure with the target DNA sequence, parallel to the Watson-Crick purine strand.

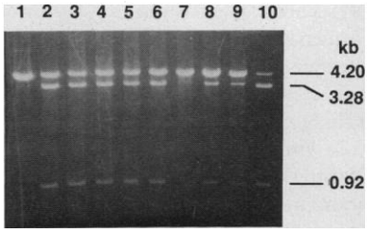

Fig. 3. A 1% ethidium bromide–stained agarose gel showing cleavage of plasmid pDP26 DNA. Lane 1, Sca I–linearized pDP26 DNA; lane 2, DNA treated with the oligodeoxyribonucleotide–staphylococcal nuclease conjugate **7**; lanes 3 to 8, DNA treated with the oligodeoxyribonucleotide-nuclease conjugates **1** to **6**, respectively; lane 9, DNA treated with the oligoribonucleotide-nuclease conjugate; lane 10, Sca I–Sph I digest of pDP26 DNA (3.28 kb and 0.92 kb). Circular DNA was first linearized with Sca I and then used in affinity cleavage reactions. The reaction conditions were similar to that described in Fig. 2, except that the reaction mixture, which contained higher concentrations of DNA (1.4 μg, 50 nM) and nuclease conjugate (100 nM), was incubated for 20 min before and after the addition of Ca^{2+}.

In order to determine whether the corresponding oligodeoxyribonucleotide also binds the 16-bp target sequence, we synthesized a nuclease conjugate (**1**) containing the DNA analogue (5′-TTCTCTCTCTCT-GCCCT-3′) of the clone 1 RNA sequence. For comparison, a nuclease conjugate (**7**) containing the consensus 16-nt homopyrimidine oligodeoxyribonucleotide (5′-TTC-TCTCTCTCTCCCT-3′) was also constructed. Both oligodeoxyribonucleotide-nuclease conjugates cleaved the 269-bp restriction fragment with efficiencies and specificities similar to those of the oligoribonucleotide analogue, suggesting that in this clone the 2′-hydroxyl group of the third strand does not interact significantly with the duplex DNA (Fig. 2, lanes 4 and 6). Consequently, in order to facilitate the affinity cleavage experiments, the cleavage properties of the oligodeoxyribonucleotide-nuclease conjugates **2** to **6** (corresponding to the recognition motifs of clones 2 to 6) were assayed.

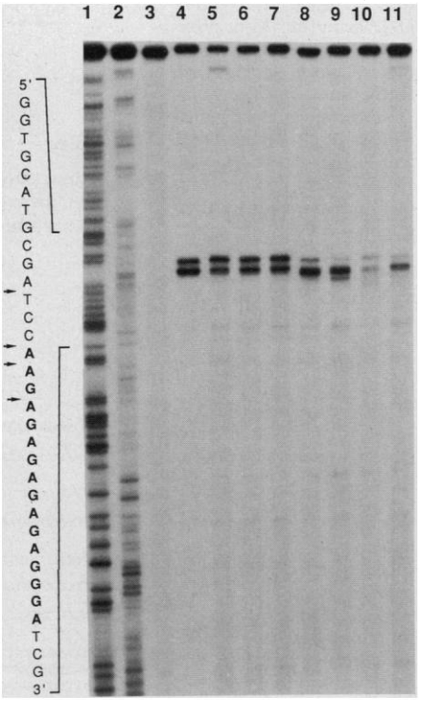

Fig. 2. Autoradiogram of a denaturing 8% polyacrylamide gel showing affinity cleavage of the 3′-^{32}P end-labeled 269-bp Nhe I/Hinc II restriction fragment of plasmid pDP26 (*28*). Lane 1, Maxam-Gilbert G reaction; lane 2, cleavage of the 269-bp DNA with free K84C staphylococcal nuclease (1 μM, room temperature, 10 s); lane 3, DNA treated in the absence of nucleases; lanes 4 and 5, DNA treated with the oligodeoxyribonucleotide–staphylococcal nuclease conjugate **7** and the oligoribonucleotide-nuclease conjugate, respectively; lanes 6 to 11, DNA treated with oligodeoxyribonucleotide-nuclease conjugates **1** to **6**, respectively. The homopurine target site is in bold face; arrows indicate cleavage sites. Cleavage reactions were carried out in a reaction mixture (total volume 10 μl) containing 25 mM tris acetate (pH 5.7), 2 mM spermine, 20% ethylene glycol (v/v), 100 mM NaCl, 10 μg of poly(A) (6.6 mM in nucleotides), 3′ end-labeled DNA (4 nM, ~10,000 cpm), and the nuclease conjugate (50 nM). After incubation of the reaction mixture for 15 min at room temperature, the reaction was initiated by the addition of 1 μl of 100 mM $CaCl_2$ solution and terminated after 5 min by the addition of 20 μl of 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid (pH 7.5). The cleavage products were precipitated with ethanol and analyzed by gel electrophoresis.

The combinatorial approach described here could be extended to the screening of both RNA and DNA libraries against duplex DNA targets of mixed sequences. The effects of pH,

salt, temperature, and cofactors on triplex formation as well as differences in oligoribonucleotide and oligodeoxyribonucleotide triplex formation can also be investigated. Sequence analysis of a large population of ligands, coupled with detailed biophysical characterization of the complexes, may provide insight into a general recognition motif for duplex DNA. Similarly, peptide and protein libraries may help define the nature of protein-DNA recognition.

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14. The 26-nt pyrimidine-rich strand was synthesized with thiol-derivatized CPG, deprotected, purified by high-performance liquid chromatography (HPLC), and converted to the *S*-thiopyridyl disulfide (*24*). The oligonucleotide was then annealed with a threefold excess of the 25-nt complementary strand, and the resulting duplex DNA was incubated with dithiothreitol (DTT)-treated thiol-Sepharose (Pharmacia) in 50 mM tris·HCl, pH 8.0 buffer at room temperature for 48 hours with shaking. After removel of unreacted DNA, the column loading (3.0 nmol of DNA per milliliter) was determined by reducing an aliquot of the Sepharose support with DTT and assaying DNA concentration.
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16. Approximately 18 μg (600 pmol) of the 89-nt RNA transcripts in 200 μl of 50 mM sodium acetate, pH 5.5, 1 mM $MgCl_2$, 2 M NaCl buffer was heated to 65°C for 5 min, allowed to cool to room temperature (RT), and loaded onto a column containing 150 μl of the DNA-derivatized support (~450 pmol of duplex DNA). After incubation at RT for 1 hour, the column was washed with 3.0 ml of the above buffer and bound RNA was eluted with 1.0 ml of 50 mM tris·HCl, pH 8.0, 1 mM EDTA buffer. The RNA was recovered by ethanol precipitation with glycogen (50 μg/ml) as the carrier.
17. Complementary DNAs were prepared from RNAs with M-MuLV reverse transcriptase and a 17-nt primer (5′-GGGCCAAGCTTCTGCAG-3′) as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989), sections 8.60–8.62]. The cDNAs were then amplified to ~5 μg of double-stranded DNA by PCR with the 33-nt and 17-nt oligonucleotides as primers. The PCR reaction (100 μl) was carried out in a buffer containing 67 mM tris·HCl, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 μM of each primer, 250 μM of each dNTP, and 2.5 units of Taq polymerase for 15 cycles (1 min at 91°C, 2 min at 52°C, and 1 min at 72°C).
18. A selection carried out with a 25-nt random RNA library, in the absence of the negative selection, failed to generate triplex-forming sequences.
19. Plasmid pDP24 is a derivative of pUC119 [J. Vieira and J. Messing, *Methods Enzymol.* **153**, 3 (1987)] with a 41-bp synthetic DNA fragment containing the T7 promoter sequence (5′-AATTGTAATACGACTCACTATAGGGAGGAATTCCTCGAGGA-3′) inserted into the Eco RI–Hind III fragment. Insertion of a PCR amplified cDNA fragment into the Eco RI–Hind III site of pDP24 places the cDNA under the control of the T7 promoter. Subsequent runoff transcription of Hind III–linearized plasmid generates RNA transcripts identical to those generated from the synthetic 106-nt template.
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25. [1-(Benzyldithio)hexyl]-6-oxy-cyanoethyl-*N*, *N*-diisopropylamino-phosphoramidite (**1**) was synthesized by reacting 1-(benzyldithio)hexan-6-ol (**2**) with two equivalents of 2-cyanoethyl-*N*, *N*-diisopropylchlorophosphoramidite (Aldrich) in anhydrous acetonitrile containing four equivalents of diisopropylethylamine at RT for 30 min. The product was extracted with 10% aqueous sodium bicarbonate solution, dried, and used directly on an automated DNA synthesizer. After the oligoribonucleotide was cleaved from the solid support and deprotected (*26*), the benzylmercaptan group was removed with 20 mM DTT in a 50 mM tris·HCl (pH 8.0), 1 mM EDTA buffer to regenerate the 5′-sulfhydryl. For the synthesis of compound **2**, see T. Muaiyama and K. Takahashi, *Tetrahedron Lett.* **56**, 5907 (1968).
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27. The cleavage pattern produced by conjugate **5** contains cleavage sites both to the 5′ side of the homopurine target site and within the homopurine binding site. This result suggests that two different binding modes may be possible, one involving the hairpin loop structure and a second that involves 5′-TTCTCTCCC-3′ binding to the 3′ half of the homopurine target.
28. Plasmid pDP26 was constructed by inserting a synthetic DNA fragment (5′-GATCCGACGATCCCTCTCTCTCTCTTGGATCGCATG-3′) containing the target DNA sequence into the large Bam HI–Sph I fragment of pBR322. The 269-bp Nhe I/Hinc II fragment used in cleavage reactions was labeled with ^{32}P by filling in the Nhe I 5′ overhang with Sequenase version 2.0 (USB).
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30. We thank A. Andrus and R. S. Vinayak at Applied Biosystems for providing the 17-nt RNA. Supported by NIH grant R01GM41679 and an NSF Waterman Award to P.G.S.

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Oligoclonal Expansion and CD1 Recognition by Human Intestinal Intraepithelial Lymphocytes

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A human intestinal intraepithelial lymphocyte (IEL) T cell line was established from jejunum to characterize the structure and function of the αβ T cell antigen receptors (TCRs) expressed by this population. Single-sided polymerase chain reaction (PCR) amplification cloning and quantitative PCR amplification of the TCR chains from the cell line and from fresh IELs demonstrated that IELs were oligoclonal. The IEL T cell line exhibited CD1-specific cytotoxicity and a dominant IEL T cell clone was CD1c-specific. Thus, human jejunal intraepithelial lymphocytes are oligoclonal and recognize members of the CD1 gene family.

THE EPITHELIAL SURFACE OF THE intestine contains a distinct population of IELs, the function of which is unknown (*1*). Human and murine intestinal IELs are enriched for T lymphocytes that express the γδ TCR (*1*). However, the majority of human intestinal IELs express the αβ TCR, and most of these human αβ IELs express the CD8 accessory molecule (*2*). The predominant expression of CD8 by human intestinal αβ IELs distinguishes them from T cells in the lamina propria and peripheral blood and suggests that their TCRs are directed at major histocompatibility complex (MHC) class I or class I–like molecules. The TCRs expressed by murine γδ IELs have been shown to utilize a small number of variable region genes but to have extensive junctional diversity (*3*). No comparable analysis, however, has been

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