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RNAs with Dual Specificity and Dual RNAs with Similar Specificity

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The biological role of RNA is delimited by its possible reactions, which can be explored by selection. A comparison of selected RNAs that bind one ligand with those that bind two related ligands suggests that a single nucleotide substitution can expand binding specificity. An RNA site with dual (joint) specificity has adenine and cytosine bases whose pK_a's appear shifted upward, thereby mimicking an efficient general acid-base catalyst. The joint site also contains two conserved, looped arginine-coding triplets implicated in arginine site formation. Two selected joint RNAs are identical in some regions and distinct in others. The distinct regions, like some peptides, seem to function similarly without being similar in primary structure.

A bulged helix within the *Tetrahymena* group I intron binds guanosine during the intron excision reaction (*1*). It also specifically binds L-arginine, but not other normal amino acids (*2*). This dual affinity suggested that comparison of RNA sites binding related ligands might show how an RNA site changes specificity. Here we used selection-amplification to isolate sites that bind both guanosine and arginine and sites that bind guanosine alone. Selection-amplification has been used to obtain RNA molecules capable of binding various proteins (*3*), dye affinity ligands (*4*), free arginine (*5*), and adenosine triphosphate (ATP) (*6*).

Joint arginine-guanosine sites were isolated by requiring two related affinities from a limited number of randomized nucleotides. We first selected by arginine affinity chromatography on RNA with 25 randomized nucleotides (*5*, *7*) and elution with L-arginine. This column was alternated with a guanosine affinity column (*7*) eluted with guanosine monophosphate (GMP). After the seventh selection, pooled RNA was rerun on the guanosine column. The GMP-eluted RNA was reverse transcribed, amplified, and cloned in pUC19 for sequencing.

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For the selection of guanosine-binding RNAs by affinity chromatography we used the same guanosine diphosphate (GDP)–agarose and elution (Fig. 1) (*8*). Ligand-specific elution of RNA in both selections virtually ensures that selected RNAs bind the free ligand and its immobilized affinity derivative at the same site.

The predominant RNA sequences from guanosine and joint arginine-guanosine selections are identical, except at two locations (Fig. 2A). The joint site consensus has a 5′ extension and also conserves C10 and A11. The C10 (Fig. 2B) is not the only nucleotide that occurs at this position; one to two nucleotides of any kind except U appear here in the guanosine RNA. In contrast, only A occurs at position 11 of the joint site and the guanosine-binding RNA never contains a purine here.

Binding specificity is determined by this internal-loop CA (Fig. 2A). A truncated joint motif transcript spanning positions W and Z (Fig. 2C) binds to both guanosine and arginine columns with dissociation constant (K_d) values 1.4 times that of the full RNA. A transcript spanning positions X and Z binds to the guanosine and arginine columns with, respectively, 1.8 and 2.3 times the K_d of the full-length RNA. A truncate spanning positions Y and Z showed no measurable affinity for the arginine column and minimal affinity for the guanosine column (20 times the K_d of the full-length

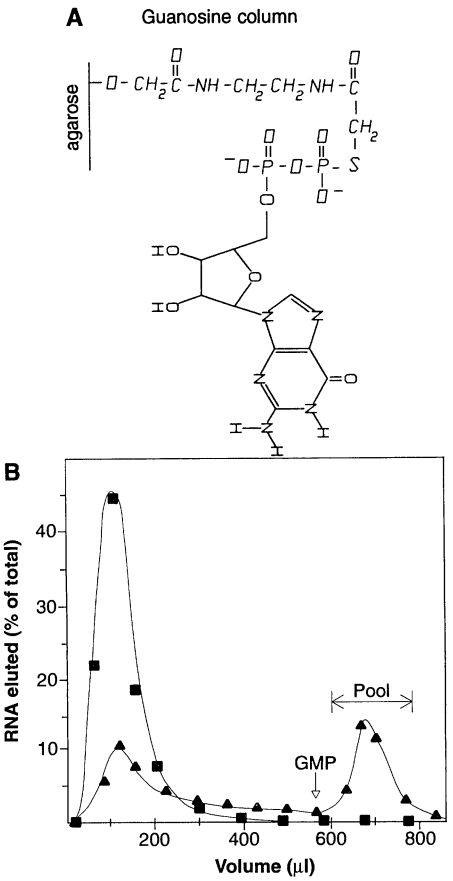


Fig. 1. Guanosine affinity chromatography. (**A**) Structure of the guanosine affinity resin. (**B**) Elution profiles (*7*) for cycle 1 (■) and cycle 5 (▲) of the guanosine selection are shown.

transcript). These boundary determinations indicate that the first nucleotide of the joint consensus may contribute slightly to binding, but not significantly to specificity. The A5 of the joint consensus is present in some guanosine motif representatives (Fig. 2B) and therefore is not specific either. The remaining characteristic structure found in the joint site, the internal-loop CA, previously appeared in three independent arginine-binding RNAs (*5*). Taken together, these observations hint that DNA sites may be assembled from fixed smaller units.

Elution with nucleoside analogs (*9*, *10*) suggests that the binding site in both RNAs

wraps closely around the guanosine base but has few ribose contacts. RNA sites approach or contact the bound base's C-6 O, N-1 H, N-2 H, and N-7 atoms (Fig. 3). Guanine C-6 O makes a crucial contact with both RNAs; even sulfur substitution (6-thioguanosine) disrupts binding. The N-1 and N-7 positions either contact the RNAs or are hindered; binding is inhibited when they are methylated. Both RNAs contact G's C-2 amino; inosine monophosphate, which lacks this group, fails to measurably interact. Unlike the group I intron (*1*), these sites do not depend on ribose 2′(3′)-OH. Neither RNA distinguishes dideoxyguanosine triphosphate (ddGTP), dGTP, or GTP. Nor are 5′ substitutions strongly distinguished: Guanosine binds better than GMP, but GMP, GDP, and GTP are indistinguishable. No discernible interaction occurs with adenosine monophosphate (AMP), uridine monophosphate (UMP), or cytidine monophosphate (CMP).

The guanosine and joint sites differ around the guanine base. The joint RNA hinders guanine C-8 more than guanosine RNA (compare K_d for 8-mercaptoguanosine and guanosine; Fig. 3). The opposite occurs at N-1 where methylation disrupts guanosine RNA binding but less severely reduces joint RNA binding.

Arginine competes with guanosine for the binding sites. The joint binding RNA has a specific affinity for the guanidino group common to both ligands. Methylation of the guanidinium of arginine (N^G-monomethyl-arginine) decreases binding, and methyl guanidinium has an affinity similar to arginine. In contrast, no interaction with lysine can be detected (Table 1).

Despite affinity for methyl guanidinium, we suggest that the arginine α-amino group contributes to binding. Replacing α-amino with hydroxyl (L-2-hydroxy-5-guanidinovaleric acid), though preserving an H-bonding possibility, makes binding undetectable. Furthermore, the α-amino must compensate for electrostatic repulsion of the carboxylate. Elimination of this negative charge by amidation (argininamide) or by decarboxylation (agmatine) increases binding by about twofold (Table 1). A weak preexistent arginine affinity of the guanosine RNA argues for interaction with the common guanidino group. Thus, the joint site's internal-loop CA, conserved in other arginine sites (5), may provide electrostatic or H-bonding contacts for α-amino and thereby increase binding.

The parental RNAs include fixed nucleotides from the 3′ polymerase chain reaction (PCR) primer complement (Fig. 2, B and C). To determine the role of these nucleotides, we randomized 13 positions of the joint RNA (Fig. 2C) and reselected them on alternating arginine and guanosine

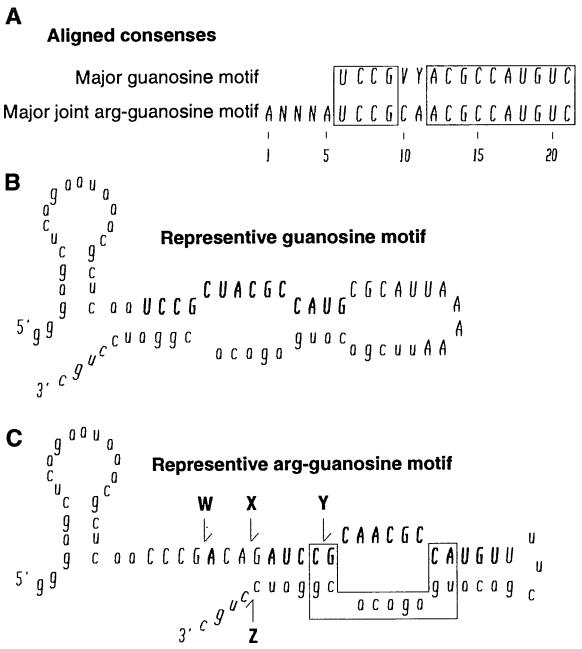


Fig. 2. (**A**) Consensus sequences for ligand-binding RNAs. Identical positions are boxed; V represents 1 to 2 nts of any identity except uridine; Y is a pyrimidine; and N is any nucleotide. Consensus means that χ^2 analysis yielded a probability for nonrandomness exceeding 99%. Guanosine binding consensus was determined from 14 independent isolates (61% of the sequenced clones), and the joint motif was determined from 31 independent isolates (55% of sequenced clones). (**B** and **C**) Energy-minimized secondary structures. Sequences are those used for subsequent studies; the calculated standard free energies of formation for the illustrated guanosine and joint motif structures are −8.4 and −14.5 kcal/mol, respectively (*27*). Length and sequence of hairpin loops in both motifs are highly variable. Consensus sequences are boldface, uppercase marks the 25 initial randomized positions, and lowercase indicates flanking PCR primer sites. Nucleotides of the joint motif randomized for subsequent reselection are boxed. Boundaries of truncated transcripts are indicated with capitals W, X, Y, and Z; these contained three added 5′ G residues for efficient transcription (*28*).

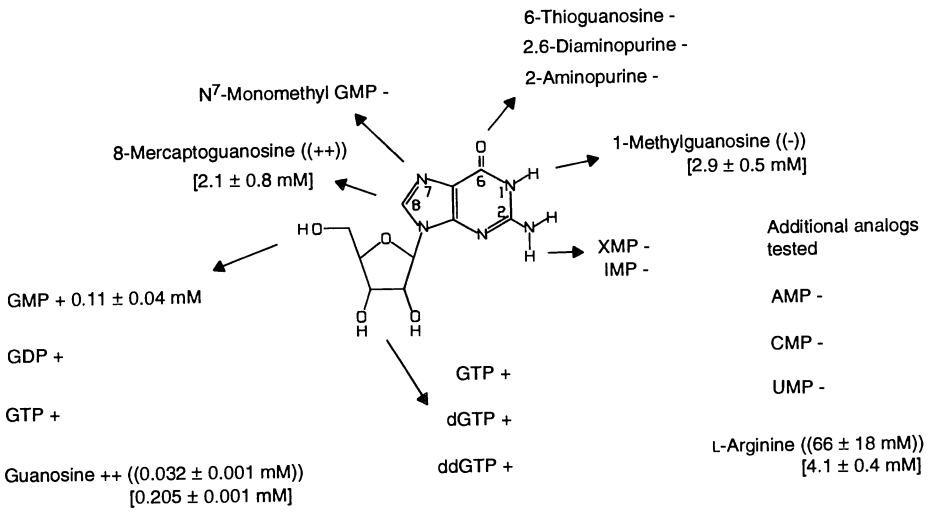


Fig. 3. Affinity of major guanosine and joint arginine-guanosine RNAs for ligands. Where the two motifs differ, K_d values for the guanosine RNA are in double parentheses and joint motif data within single brackets. No detectable affinity (−); same affinity as GMP (+); and same affinity as guanosine (++).

columns. Two different motifs appeared in 20 of 21 reselected RNAs (Fig. 4). Except for one base pair, one motif reproduces the parental sequence. Although the second RNA appears to have a different primary structure, it seems likely to be isomorphous in tertiary structure [comparable with two types of tetraloops (*11*)] because representatives retain both parental activities, binding to guanosine and arginine columns with parental K_d (within twofold). Because the bottom loop of both reselected motifs is highly conserved, loop nucleotides contribute specifically to affinity, though they were fixed during the first selection.

The reselected parental joint motif was probed (*12*) with dimethyl sulfate (DMS) to identify bases that either make contact with arginine or are required for folding of the binding site. Addition of arginine protects several positions in native RNA from modification: A38, A58, and A60 (Fig. 4). The protection at A60 is reproducible, but it is superposed on a DMS-independent reverse transcriptase stop and is therefore less evident. In addition, C40 becomes more reac-

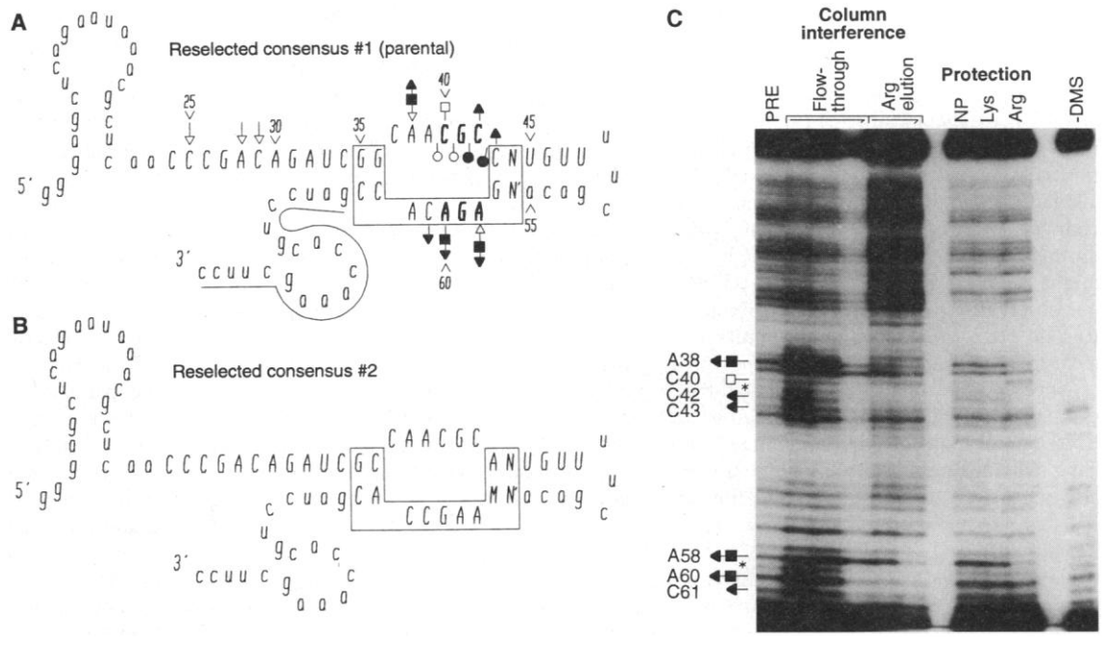


Fig. 4. (**A** and **B**) Predicted secondary structures from reselection of an arginine-guanosine motif (*27*). Thirteen randomized nucleotides (Fig. 2) are boxed. Because part of the original 3′ primer sequence was randomized, the 3′ region was extended for amplification. The new PCR primer region is indicated by a line. N and N′, any base pair; M, cytosine or adenosine; ■-, bases protected from DMS modification by arginine; ◀-, nucleotides that when modified interfere with binding to an arginine affinity column; □-, a base that becomes accessible to modification on arginine binding; ◁-, bases with an apparently shifted pK_a; ●-, phosphates that when alkylated with ENU interfere with both guanosine and arginine binding; and ○-, alkylated phosphates that interfere only with guanosine binding. Two conserved arginine triplets are in bold face. (**C**) Chemical probing of the binding site by alkylation and reverse transcriptase (RVT) mapping. PRE, modified but unfractionated RNA, and NP, no ligand. Bases protected from modification by arginine (■-) and those that when modified interfere with binding to the column (◀-) are indicated. C40 □- becomes more accessible to modification on arginine binding. Because RVT does not detect DMS methylation of guanosine, G bands (*) are background transcriptase stops (-DMS lane).

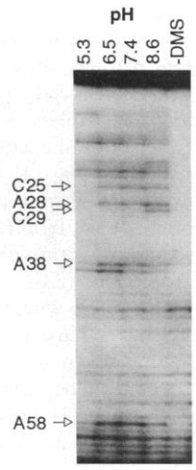


Fig. 5. Electrophoretic detection of shifted base pK_a's. See text for explanation.

tive when arginine binds, presumably as a result of conformational change. These four effects are saturated by 5 mM arginine and not seen with 200 mM lysine, reaffirming that they reflect ligand association. Methyl guanidinium produces the same DMS reactivities (*13*), so these four bases are implicated in guanidinium binding.

Denatured RNA (*12*) was also modified, refolded, and passed through an arginine column. Modifications of bases that contact arginine or are important for folding hinder RNA binding to the column and will be selectively recovered in the flow-through and will be reduced in fractions that bind and elute with arginine (Fig. 4C). These criteria identify all arginine-protected bases in native RNA. In addition, several other nucleotides appear. These (C42, C43, and C61) are weakly modified under native conditions and therefore cannot be detected by protection of native RNA.

We could detect differential use of the joint site by its two ligands. The joint motif has stabilizing contacts with the guanine C-6 O and possibly N-7 (Fig. 3), which have no counterparts in arginine. Accordingly, arginine and guanosine binding sites should overlap but not be identical. A minor motif from the guanosine selection has no measurable affinity for arginine. For the major joint motif, *N*-nitroso-*N*-ethylurea (ENU) was used in a modification-interference assay to identify alkylated phosphates that hinder binding to the arginine or guanosine columns (Fig. 4A) (*13*). Although alkylation of two contiguous top loop phosphates interferes with both affinity ligands, modification of two adjacent phosphates affects only guanosine binding.

The internal loops of both guanosine and joint RNAs contain many C and A nucleotides (Figs. 2 and 4). A C-A (N-1–protonated) base pair is known in DNA (*14*), and a p*K*-shifted A has been reported in an RNA tertiary structure (*15*). Unattributed high p*K*s in RNA are reported in the literature (*16*). To detect such transitions, we determined the pH dependence of joint RNA affinity for the guanosine column. Lowered pH weakened column affinity; little binding was detectable at pH 5.3, and the apparent half-titration point of affinity was ~6.0 (*13*, *17*). Titratable groups in RNA with a pK_a closest to 6.0 are 5′ terminal phosphate (pK_a 7.7), N-1 of adenosine (pK_a 3.5), and N-3 of cytosine (pK_a 4.2). Terminal phosphate, even if shifted down in pK_a, seems an unlikely candidate for this pK_a; boundary experiments showed that no particular terminus is crucial to binding. Thus, the active structure probably has at least one highly shifted base pK_a, and protonation of C or A nucleotides disrupts binding. Other C-A interactions are not eliminated (*18*).

We have devised a way (Fig. 5) (*19*) to measure the pK_a's of C and A within a complex RNA structure. Because the N-1 atom of A and N-3 atom of C are both protonated and methylated by DMS, a base becomes unreactive toward DMS near its pK_a. This event is readily detected with RVT, which stops before alkylated C and A. There are several nucleotides in the joint RNA that reproducibly become unreactive between pH 6.5 and 5.3 (no ligand; Figs. 4A and 5): C25, A28, A38, and A58. These pK_a shifts are dependent on higher order RNA structure. They are not observed when modification occurs under denaturing conditions (*12*); instead, we observed the expected A and C pK_a's throughout the sequence (*13*). Titration of a shifted base, or of a structural unit containing a shifted pK_a, could account for the effect of pH on affinity.

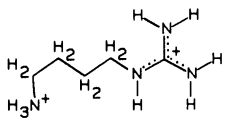
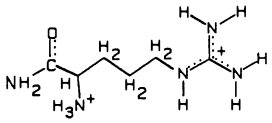
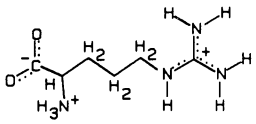
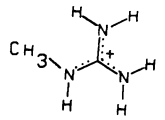
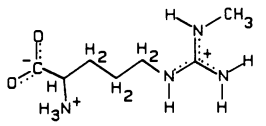
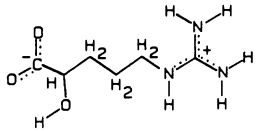
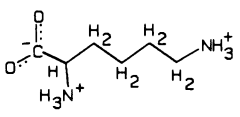
The pK_a of C29 appears shifted upward almost 4 pK units, but unlike the others, this occurred in only four of six titrations.

Another possible pK_a should be considered; 7-methylguanosine (^{7m}G) has a pK_a near 7 (*20*) and is undetected when alkylation is assayed with RVT. Titration of this alkylated group might cause conformational change, changing other base reactivities during subsequent alkylation. Though ^{7m}G might cause an apparent pK_a shift (C29?), our observations cannot all be attributed to it. The pK_a of 6.0 for column affinity was measured without DMS.

Specific binding and potential acid-base catalytic elements are here combined in an RNA small-molecule binding site. Two bases with apparent shifted $pK_a \sim 6$ were also protected from DMS modification by arginine (A38 and A58); thus, they are associated with the binding site. Groups with pK_a's near neutrality are maximally effective general acid-base catalysts at biological pH. Though no catalysis occurs here, this suggests that ribozymes can employ efficient general base (and perhaps acid) catalysis.

Evolution of ribozymes requires that a primordial catalyst mutate to new specificities. As described in this report, the modification of, at minimum, one nucleotide of an RNA guanosine site (Fig. 2, B and C) increases a low unselected affinity for the amino acid arginine more than 15-fold (Fig. 3). Accordingly, this RNA binding site is sufficiently stable to tolerate substitution, and nucleotide substitution can generate a new specificity.

Table 1. Joint site K_d for some arginine analogs. The K_d was determined from chromatographic mobility on an arginine affinity column with eluants containing the compound in solution (Fig. 3). The K_d lower limits are based on experiments that showed no enhancement of elution.

Compound	Structure	K_d (mM)
Agmatine		2.0 ± 0.5
L-Argininamide		2.3 ± 0.6
L-Arginine		4.1 ± 0.4
D-		4.7 ± 0.2
Methyl guanidinium		4.5 ± 0.9
N^G-monomethyl-arginine		77 ± 1
L-2-Hydroxy-5-guanidino valeric acid		>100
L-Lysine		>100

Alternate joint motifs (Fig. 4, A and B) make a complementary point; as for proteins (*21*), even apparently unrelated RNA sequences can fold to perform the same function. The bottom strand of the parental internal loop has essential functions, evidenced by modification and interference experiments (Fig. 4). Loop sequence is also conserved under reselection and therefore required for activity. Reselection with only 13 randomized nucleotides is unlikely to produce a joint site not observed in the original selection on 25 nucleotides. Thus, though the alternate lower loop is unlike the parental in sequence and size (seven instead of five nucleotides, Fig. 4B), it probably folds to give an isomorphic binding site because it collaborates equally well with the rest of the site in both arginine and guanosine binding.

Two arginine-coding triplets occur within the joint site loops, and both triplets include functional nucleotides. On the top loop CGC is conserved in all 31 isolates from the initial selection, and on the bottom loop AGA is conserved in all 13 isolates of this motif from the reselection. Both sequences contact guanidinium or are required for its site (Fig. 4). These data support RNA–amino acid interaction as the basis of the genetic code. At least seven distinguishable ways to fold a specific RNA site for arginine exist (Fig. 3) (*2*, *5*, *22*); four of these sites contain five phylogenetically conserved sequences corresponding to arginine triplets. Evidence for contact between arginine and the triplets exists for two sites (Fig. 4) (*23*). Conceivably, the evolution of translation chose among such amino acid binding sites, and functional sequences within chosen sites became modern codons (*23*).

Despite a selection protocol tailored to it, the *Tetrahymena* helix-like motif is not among seven guanosine sites we have recovered. The absence of a *Tetrahymena* derivative suggests that its tertiary structure may require more sequence space. However, seven different guanosine binding sites did satisfy our selection. Therefore, it seems unlikely that chemical determinism (a paucity of possible functional structures) limits the diversity of guanosine binding sites in modern RNAs, though this was suggested (*24*) for the spliceosome [compare with (*25*)]. Instead, evolution of a variety of different binding sites seems likely.

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7. A 100-μl guanosine affinity column (Fig. 1) contained 15 mM guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS) linked through sulfur to Affi-Gel 102 agarose (Bio-Rad) derivatized with bromoacetyl *N*-hydroxysuccinimide (*26*). The eluant contained 375 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 40 mM tris-HCl (pH 7.8) at 4°C. The initial RNA pool consisted of 72-nucleotide oligomer transcripts containing 25 central randomized positions, encoded within flanking PCR primer sequences containing a T7 promoter. Approximately 20 μg of this RNA in water (~5×10^{14} molecules, transcribed from 5×10^{13} molecules) was heated at 65°C for 5 min, the salt concentration was adjusted, and the RNA cooled to 4°C over 10 min. Then 25 μl of RNA solution was loaded. After washing out weakly bound RNAs, we eluted bound RNA with 20 mM GMP in column buffer. Cycle 5 is shown (Fig. 1); the initial cycle was less stringent to minimize possible loss of poorly represented transcripts. Pooled RNA was purified on glass beads (BIO 101) and complementary DNA (cDNA) was synthesized, amplified by PCR, and transcribed into RNA for the next round of selection as described (*3*). After cycle 3, RNA was depleted of column-binding sequences by passage through acetylated agarose, and only the first 70 to 80% of RNA was applied to the subsequent guanosine column. Arginine affinity chromatography was similar, but utilized 19.7 mM arginyl-cysteine dipeptide linked through the sulfydryl to a thiopropyl Sepharose 6B (Pharmacia) matrix (*5*).
8. After five selection-amplification cycles, GMP-eluted RNA was refractionated on the guanosine column and washed successively with 20 mM CMP and AMP before GMP elution. The GMP-eluted pool was reverse transcribed, amplified, and cloned in pUC19 as for the joint RNA. Late cycles of enrichment in both selections showed minor low-affinity species eluting from the guanosine column (Fig. 1), which were not removed with subsequent purification. These are an alternative RNA conformation; when the low-affinity peak was pooled, de- and renatured, and again run through the column, it again yielded both high- and low-affinity peaks. In addition, increased Mg^{2+} (to 20 mM) plus 0.5 mM spermidine converted most low-affinity molecules from pure transcripts to a high-affinity form. However, this buffer did not alter elution of the initial random pool. For all subsequent work on the guanosine column, spermidine and the higher concentration of Mg^{2+} were used. However, they decreased affinity for the arginine column and were not used for arginine selections.
9. Analogs were initially screened for elution of RNA from the guanosine column. All were checked at 2 mM except for 8-mercaptoguanosine which was checked at a concentration of 1.1 mM (dictated by solubility). The K_d's were determined by competitive affinity chromatography (*5, 10*). We estimate that K_d's greater than 20 mM would not have been detected. All nucleotide ultraviolet spectra matched literature data.
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12. RNA was modified with DMS under native conditions (4°C for 2 hours in 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM Hepes, pH 7.7) in the presence of 5 mM arginine, 200 mM lysine, or no amino acid. RNA was also modified with DMS under denaturing conditions (90°C for 1 min in 1 mM EDTA and 50 mM Hepes, pH 7.7), then refolded as above. For chromatographic selection among modified RNAs, an arginine column was used as in the initial selection. The column was washed with three successive 300-μl volumes of buffer, then eluted with 25 mM L-arginine. Modified RNAs were precipitated and reverse transcribed with a three- to sixfold molar excess of ^{32}P-kinased primer (*29*).
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15. This experiment was inspired by unpublished work of P. Legault and A. Pardi, who used nuclear magnetic resonance to identify an adenosine with shifted pK_a in an internal loop of the leadzyme.
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Gene for Familial Psoriasis Susceptibility Mapped to the Distal End of Human Chromosome 17q

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A gene involved in psoriasis susceptibility was localized to the distal region of human chromosome 17q as a result of a genome-wide linkage analysis with polymorphic microsatellites and eight multiply affected psoriasis kindreds. In the family which showed the strongest evidence for linkage, the recombination fraction between a psoriasis susceptibility locus and *D17S784* was 0.04 with a maximum two-point lod score of 5.33. There was also evidence for genetic heterogeneity and although none of the linked families showed any association with *HLA-Cw6*, two unlinked families showed weak levels of association. This study demonstrates that in some families, psoriasis susceptibility is due to variation at a single major genetic locus other than the human lymphocyte antigen locus.

Psoriasis is a chronic inflammatory dermatosis that affects ~2% of the population. It is characterized by hyperproliferation of epidermal cells and inflammation resulting from infiltration of activated T helper cells and mononuclear cells and release of pro-inflammatory cytokines (*1, 2*). It may also be associated with arthritis and can be present as a severely inflammatory dermatosis in patients with acquired immunodeficiency syndrome (AIDS) (*3*). An understanding of the pathogenesis of psoriasis remains an important challenge in dermatologic research.

Associations between psoriasis and certain human lymphocyte antigen (HLA) alleles have been described, supporting the hypothesis that psoriasis is a T cell–mediated, autoimmune disorder (*4*). The presence of the *HLA-Cw6* allele may predispose to psoriasis because there is a strong association between age of onset, family history, and the presence of *HLA-Cw6, B-13* and *B-w57* (*5*), and the relative risk of *HLA-Cw6* carriers developing psoriasis is 20 (*6*). An "association" between a disease and HLA indicates that certain HLA alleles are more frequent in patients than in controls. Loci are "linked" when they do not assort independently at meiosis.

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