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# An altered-specificity mutation in a human POU domain demonstrates functional analogy between the POU-specific subdomain and phage λ repressor

(transcription factors/DNA–protein interactions/mutagenesis/DNA-binding proteins/macromolecular recognition)

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ABSTRACT **The POU motif, conserved among a family of eukaryotic transcription factors, contains two DNA-binding domains: an N-terminal POU-specific domain ($POU_S$) and a C-terminal homeodomain ($POU_{HD}$). Surprisingly, $POU_S$ is similar in structure to the helix–turn–helix domains of bacteriophage repressor and Cro proteins. Such similarity predicts a common mechanism of DNA recognition. To test this prediction, we have studied the DNA-binding properties of the human Oct-2 POU domain by combined application of chemical synthesis and site-directed mutagenesis. The $POU_S$ footprint of DNA contacts, identified by use of modified bases, is analogous to those of bacteriophage repressor–operator complexes. Moreover, a loss-of-contact substitution in the putative $POU_S$ recognition α-helix leads to relaxed specificity at one position in the DNA target site. The implied side chain–base contact is identical to that of bacteriophage repressor and Cro proteins. These results establish a functional analogy between the $POU_S$ and prokaryotic helix–turn–helix elements and suggest that their DNA specificities may be governed by a shared set of rules.**

The bipartite POU motif defines a conserved family of eukaryotic transcription factors broadly involved in tissue-specific gene expression and the specification of cell fate (1–7). The motif consists of two domains, an N-terminal POU-specific domain ($POU_S$) and a C-terminal homeodomain ($POU_{HD}$). Jointly required for DNA recognition, each domain contributes base-specific contacts (8–13). The structure of an isolated $POU_S$ fragment has recently been determined by NMR methods (14, 15) and found to be similar to the helix–turn–helix (HTH) domains of bacteriophage repressors (16–18). This similarity, which was not anticipated from sequence comparisons, predicts an analogous mechanism of DNA recognition. Such an analogy would have broad implications, as POU mutations are associated with developmental abnormalities in a variety of eukaryotes (7), including humans (19).

Here we investigate the DNA-binding properties of the human Oct-2 POU domain. Oct-2, a member of a family of octamer-binding factors (3, 20), participates in the regulation of immunoglobulin genes in human B cells (4–6). Specific DNA contacts were determined by incorporation of nucleoside analogs into the octamer binding site. The inferred "footprint" of contacted and noncontacted bases is found to be identical to that of bacteriophage repressor–operator complexes (16–18). Further, a specific contact between an adenine hydrogen-bond donor and the $POU_S$ HTH is established by analysis of a loss-of-contact substitution (21–23) in the putative $POU_S$ recognition α-helix. The implied side chain–base contact is inconsistent with the orientation of the HTH in a homeodomain–DNA complex (24) but is analogous to a conserved feature of bacteriophage repressor and Cro proteins (16–18). Together, our results demonstrate a shared prokaryotic and eukaryotic mechanism of DNA recognition.

## MATERIALS AND METHODS

**Synthetic Methods.** The protected phosphoramidites of 2′-deoxypurine (also designated nebularine) and $N^6$-methyl-2′-deoxyadenosine were prepared as described (25) and characterized by $^1H$ and $^{31}P$ NMR. 5-Methyl-2′-deoxycytidine was synthesized from thymidine as described (26) and converted to 5′-*O*-(4,4′-dimethyoxytrityl)-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite by standard methods. Oligonucleotides were synthesized by solid-phase phosphoramidite chemistry. The presence of modified bases was verified by GC/MS following acid hydrolysis and by analysis of limit dinucleotides following enzymatic digestion with nuclease P1 and bacterial alkaline phosphatase (Sigma). Oligonucleotides containing standard bases, 2′-deoxyuridine, and 5-bromo-2′-deoxyuridine were purchased from Oligos, Etc. (Guilford, CT).

**Site-Directed Mutagenesis.** The Oct-2 POU domain (nucleotides 646–1143) was cloned into phage M13mp18. Oligonucleotide-directed mutagenesis was accomplished by the phosphorothioate method as described by the vendor (Amersham). Mutants were identified by single-stranded DNA sequencing.

**Protein Expression and Purification.** The native Oct-2 POU domain and isolated homeodomain ($POU_{HD}$) were expressed in pGEX-2T (Pharmacia), purified, cleaved with thrombin, and fractionated as described (13). Mutant POU coding regions were recloned by PCR into pGEX-2T and resequenced. The mutant domains were isolated as described for the wild type; purity was >90% as assessed by SDS/PAGE.

**DNA-Binding Assays.** Gel retardation assays and quantitative titrations were done at 4°C under low-salt conditions (13). Shifted and unshifted counts were quantified with a PhosphorImager (Molecular Dynamics).

## HYPOTHESIS

The structure of Oct-1 $POU_S$ contains four α-helices (Fig. 1*A*) (14, 15); helices 2 and 3 comprise a putative HTH (shaded). A model of a $POU_S$–DNA complex has been proposed (14,

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Abbreviations: HTH, helix–turn–helix; $POU_S$, POU-specific subdomain; $POU_{HD}$, POU homeodomain.

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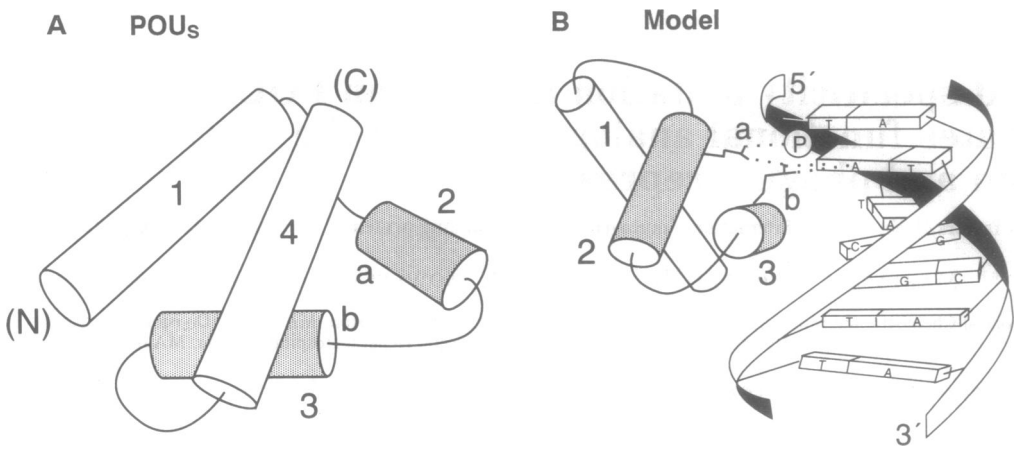


FIG. 1. (*A*) Cylinder model of Oct-1 $POU_S$ as determined by NMR (14, 15). The putative HTH element is shaded ($\alpha$-helices 2 and 3); among $POU_S$ sequences and phage repressor and Cro proteins glutamine is invariant at the N termini of shaded helices (HTH positions *a* and *b*). (*B*) Model of the Oct-1 $POU_S$-DNA complex (14, 15) based on the cocrystal structure of the N-terminal domain of $\lambda$ repressor (16). Glutamines *a* and *b* are proposed to participate in a network of hydrogen bonds (dotted lines) as observed in cocrystal structures of phage repressor and Cro proteins (16–18). The 5′ sequence of the octamer motif (5′-TATGCAAAT-3′; ref. 20) is represented.

15) based on the cocrystal structure of the DNA-binding domain of $\lambda$ repressor (16). This model (Fig. 1*B*) predicts a distinctive pattern of HTH–DNA interactions. The $\lambda$ repressor (exclusive of the N-terminal arm) contacts the bases underlined in the operator half-site (16) illustrated below.

```
   1  2  3  4  5  6  7  8  9
5′-T-A-T-C-A-C-C-G-C-3′
3′-A  T  A  G  T  G  G  C  G-5′
   1′ 2′ 3′ 4′ 5′ 6′ 7′ 8′ 9′
```

Adenine-2 (boldface) is recognized by bidentate hydrogen bonds to N7 and 6-$NH_2$ from a glutamine side chain at position 1 of the recognition $\alpha$-helix (Fig. 2*A*). This major-groove interaction, specific for adenine, is conserved among bacteriophage HTH repressors (17, 18). Other base contacts (underlined above) include side-chain hydrogen bonds to the N7 and/or O6 functions of guanine-4′ and guanine-6′, a water-mediated hydrogen bond to the N4 of cytosine-4, and van der Waals interactions with the methyl groups of thymines at positions 1, 3, and 5′. Adenine-1′, thymine-2′, adenine-3′, and adenine-5 are not contacted. Analogous interactions are observed in cocrystal structures of other bacteriophage repressor and Cro proteins (17, 18). The pattern of contacted and noncontacted bases differs from that of the homeodomain (24) and thus provides a general "footprint" of the prokaryotic HTH motif. Of particular interest is the interaction between adenine 2 and glutamine: because glutamine is conserved at position 1 of the putative $POU_S$ HTH (1–7), an analogous adenine–glutamine contact is predicted (14, 15).

## RESULTS

**The $POU_S$ Footprint Is Identical to That of $\lambda$ Repressor.** Major-groove contacts between the Oct-2 POU domain and a consensus DNA site (the "octamer motif," 5′-TATGCAAAT-3′; ref. 20) were identified by use of base analogs. Because an isolated $POU_S$ fragment binds DNA very weakly (8, 11–13), its base contacts were investigated by comparison of the DNA-binding properties of the intact POU domain and isolated $POU_{HD}$ fragment. DNA modifications that interfere with binding of POU but not $POU_{HD}$ are assigned below as $POU_S$ binding sites. Use of modified bases extends the resolution of conventional footprinting methods (27, 28). It is important to caution, however, that in principle such modifications can indirectly perturb protein binding through transmitted changes in DNA structure.

Contacts to adenine 6-$NH_2$ were inferred by using an analog lacking 6-$NH_2$ (purine 2′-deoxynucleoside; Fig. 2*B*) (25). Introduction of this analog at position 2 reduced POU binding by a factor of at least 50 but had no effect on binding of $POU_{HD}$ (Fig. 3). Similar results were obtained with $N^6$-methyl-2′-deoxyadenosine (data not shown). The small effects of purine 2′-deoxynucleoside at positions 3′ and 1′ (Fig.

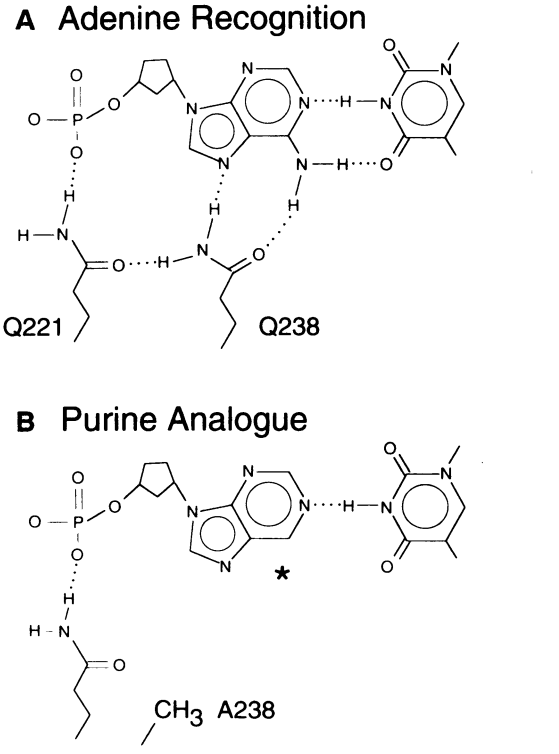


FIG. 2. (*A*) Glutamines *a* and *b* in phage repressor and Cro proteins form a network of hydrogen bonds, which effects recognition of adenine-2 (16–18); analogous residues in human Oct-2 are Q221 and Q238, respectively (4). In repressor cocrystal structures an additional contact occurs between the methylene chain of glutamine *b* and the thymine-1 methyl group (not shown). (*B*) Structure of 2′-deoxypurine (nebularine), which lacks the 6-$NH_2$ hydrogen-bond donor (asterisk) (25). Unlike native Oct-2, the Q238A mutant does not distinguish between adenine and purine at position 2 (see text).

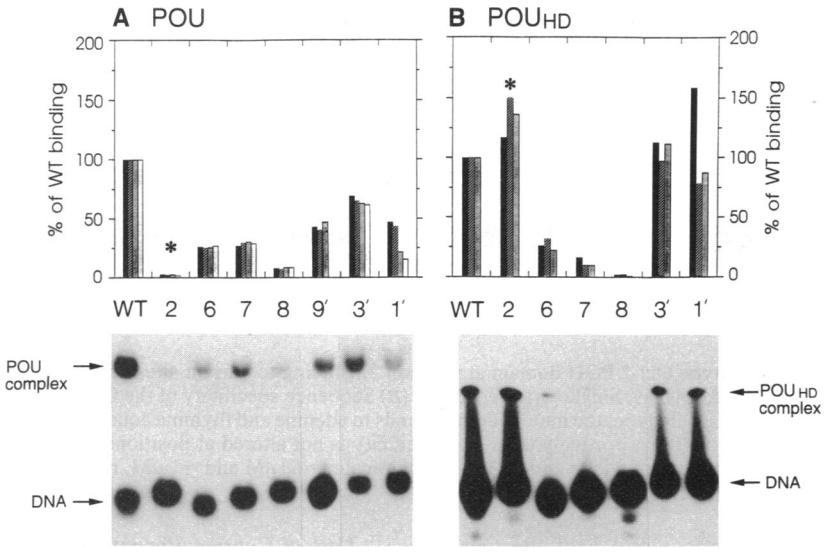


FIG. 3. Binding of the Oct-2 POU domain (*A*) and isolated $POU_{HD}$ (*B*) to variant DNA sites containing purine 2′-deoxynucleoside instead of adenosine. (*Upper*) Percent wild-type (WT) binding for each of three repetitions of the experiment. Asterisks indicate differential sensitivity of POU and $POU_{HD}$ to modification of adenine-2. (*Lower*) Representative autoradiograms from gel retardation assays, in each case a wild-type control is shown in the left-most lane. Concentrations of POU and $POU_{HD}$ were 60 nM and 3 μM, respectively.

3) were not observed with $N^6$-methyl-2′-deoxyadenosine and hence can be ascribed to perturbations in DNA structure. Possible guanine contacts were inferred from N7 methylation interference (9–11). Possible sites of interaction with thymidine were identified by using 2′-deoxyuridine and 5-bromo-2′-deoxyuridine (Fig. 4*A*). In each case protein binding was reduced by deoxyuridine substitution and restored by bromodeoxyuridine (27, 28). Sites of interaction with cytidine were likewise identified by using 5-methyl-2′-deoxycytidine (Fig. 4*B*). The resulting footprint of major-groove contacts (underlined) is analogous to that expected on the basis of the cocrystal structure of the λ repressor DNA-binding domain (exclusive of its N-terminal arm) (16).

```
   1  2  3  4  5  6  7  8  9
5′-T-A-T-G-C-A-A-A-T-3′
3′-A  T  A  C  G  T  T  T  A-5′
   1′ 2′ 3′ 4′ 5′ 6′ 7′ 8′ 9′
```

The position of the required adenine 6-$NH_2$ (boldface) is as predicted by analogy to λ repressor. Base pairs 7–9 are contacted only by $POU_{HD}$, which, like the λ arm, provides additional affinity and specificity (8–13).

**HTH Mutations in $POU_S$ and λ Repressor Have Analogous Effects.** In cocrystal structures of bacteriophage repressor and Cro proteins, the carboxamide of glutamine *b* (position 1 of α-helix 3) is coplanar with adenine-2 and contacts N7 and 6-$NH_2$, whereas the carboxamide of glutamine *a* (position 1 of helix 2) contacts the 5′-phosphate (Figs. 1*B* and 2*A*). The two residues are linked by side-chain hydrogen bonds (16). Each side chain–DNA interaction contributes to specificity (defined as the ratio of specific to nonspecific binding) (29). In the λ repressor point mutations of glutamine *a* and glutamine *b* to serine reduce specific binding by factors of 2286 and 557, respectively, without significantly affecting affinity for nonoperator DNA (29). Since the λ repressor binds to an operator site as a dimer, the monomer-specific decrement in specific binding would be approximately the square root of these values—i.e., 50 and 20, respectively. Why substitution of a phosphate contact (glutamine *a*) is more deleterious than that of a base contact (glutamine *b*) is not clear in either system but may reflect perturbation of the side chain–side chain interaction.

Among $POU_S$ sequences glutamine is invariant at positions *a* and *b* of the putative HTH (1–8). As in crystal structures of bacteriophage repressor and Cro proteins (16–18), the two side chains in the NMR structure of Oct-1 $POU_S$ are solvent-exposed and in close proximity (14, 15). To investigate their importance in DNA recognition, glutamine → alanine substi-

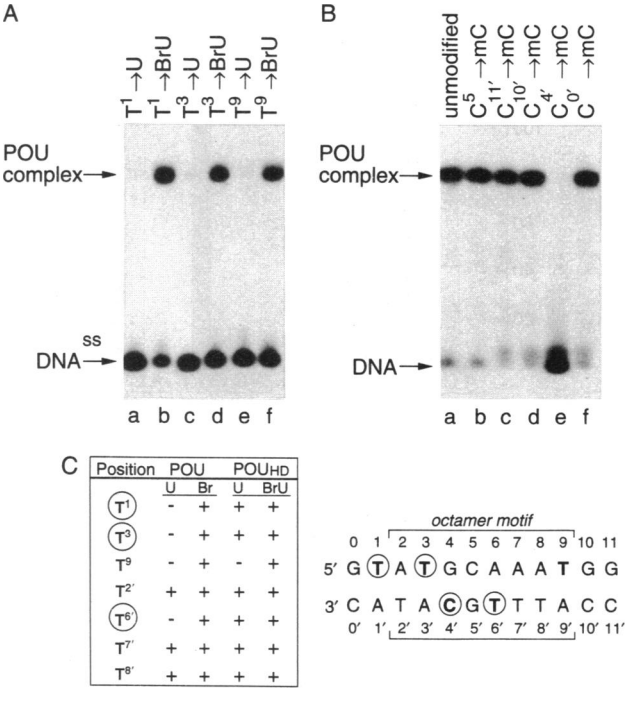


FIG. 4. (*A*) Autoradiograms from gel retardation assay of POU binding to variant octamer sites containing 2′-deoxyuridine (U) or 5-bromo-2′-deoxyuridine (BrU) substituted for thymidine (T) at indicated positions. Base numbering is given in *C Right*. Protein concentration was 60 nM. ss, Single-stranded DNA. (*B*) Autoradiogram from gel retardation assay of POU binding to variant octamer sites containing 5-methyl-2′-deoxycytosine (mC). No mC modification affects $POU_{HD}$ binding (data not shown). The protein concentration was ca. 500 nM. (*C*) (*Left*) Summary of effects of U and BrU substitutions on POU and $POU_{HD}$ fragments; +, no effect; −, reduced binding. Thymines at positions 1, 3, and 6′ (circled) are thus identified as $POU_S$ contacts. (*Right*) Summary of $POU_S$–pyrimidine contacts (circled) in the major groove of the octamer motif; thymine-9 (boldface) is identified as a $POU_{HD}$ contact, as expected (24).

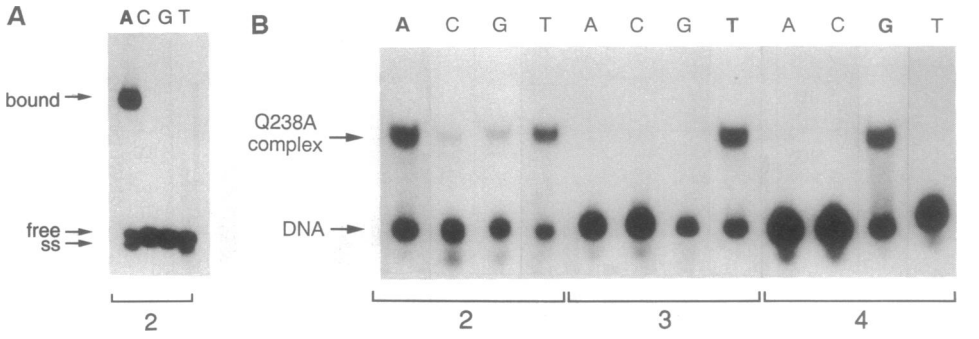


FIG. 5. (*A*) Specificity of the wild-type Oct-2 POU domain at position 2. Binding to variant sites containing thymine, guanine, or cytosine is reduced by a factor ≥50 (see also Fig. 6*A*). ss, Single-stranded DNA. (*B*) Sequence specificity of the Q238A mutant POU domain at positions 2 (*Left*), 3 (*Center*), and 4 (*Right*). Unlike wild type, the mutant domain binds to adenine and thymine equally well at position 2; binding to guanine or cytosine is reduced by factors of 4 or 10, respectively (Fig. 6). Specificity is not altered at positions 1, 3, or 4. The wild-type base at each site is shown in boldface. The concentrations of native and Q238A POU domain (≈60 nM and ≈1 μM, respectively) were chosen so that ≈50% of the labeled DNA molecules were shifted in each case.

tutions were introduced at positions *a* and *b* of the Oct-2 domain (Q221A and Q238A, respectively); control substitutions were introduced into nonconserved positions in α-helix 1 (Q205A and Q212A). Whereas the control substitutions do not affect specific DNA binding, each substitution in the putative HTH reduces specific DNA binding (data not shown). The substitution at position *b* (Q238A) reduces binding of the POU monomer by a factor of ≈20, in accord with the estimated monomer-specific effect of the glutamine *b* → serine substitution in λ repressor. The substitution at position *a* (Q221A) reduces octamer binding more severely, by a factor of at least 100.

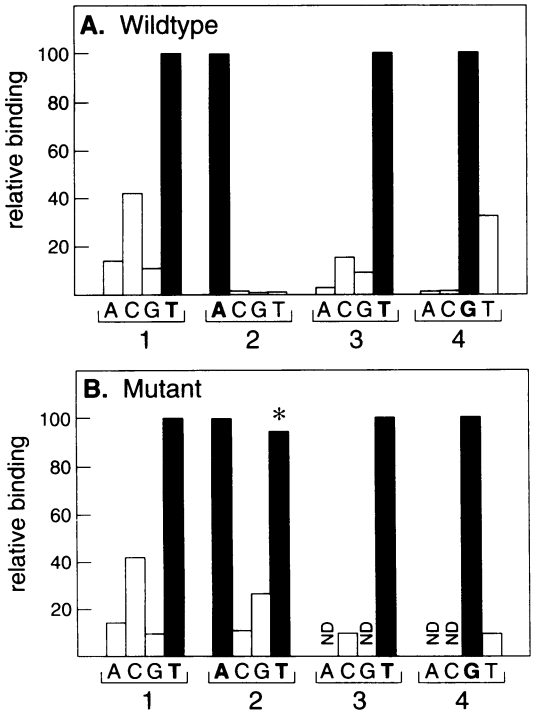


FIG. 6. (*A*) Specificity (% wild-type binding) of wild-type POU domain at positions 1–4 (5′-TATGCAAAT-3′). The wild-type base at each site is shown in boldface. (*B*) Sequence specificity of the Q238A mutant POU domain at positions 1–4. Asterisk indicates relaxation of specificity at position 2. In each panel, relative binding is defined as the fraction of variant DNA probe shifted relative to the native probe under conditions in which 50% of the native probe is shifted (Fig. 5). (Relative binding of wild-type base at each position is 100% by definition.) ND in *B* indicates relative binding of <5% that has not been determined more precisely.

**Loss-of-Contact Phenotype Demonstrates Analogous HTH Contact.** Specificity of the λ repressor at adenine-2 is stringent; substitution of thymine, cytosine, or guanine in a consensus half-site reduces repressor binding by a factor of at least 10 (30). The crystal structure of a wild-type complex implies that base-pair discrimination is accomplished by a network of hydrogen bonds involving glutamines *a* and *b* as described above (Figs. 1*B* and 2*A*). Although this inference has not been tested by mutagenesis of λ repressor, a homologous glutamine *b* → alanine substitution in phage 434 repressor alters the specificity of the protein at the corresponding site of the 434 operator (31).

Specificity of the Oct-2 POU domain at adenine-2 is also stringent: substitution of thymine, cytosine, or guanine reduces POU binding by a factor of at least 50 (Figs. 5*A* and 6*A*). Strikingly, substitution of glutamine *b* (Q238A) relaxes specificity at position 2 (loss-of-contact substitution; refs. 21–23): the mutant POU domain recognizes all four bases with relative affinities A = T > G > C (Figs. 5*B* and 6*B*). Specificity is not altered at other octamer positions. Selective removal of adenine-2 6-$NH_2$ (which strongly destabilizes the native complex; Fig. 3) has no effect on binding of the Q238A mutant (Fig. 7); sensitivity of binding to T → U substitutions is unchanged. These observations strongly suggest that in the native complex the side chain of glutamine *b* contacts ade-

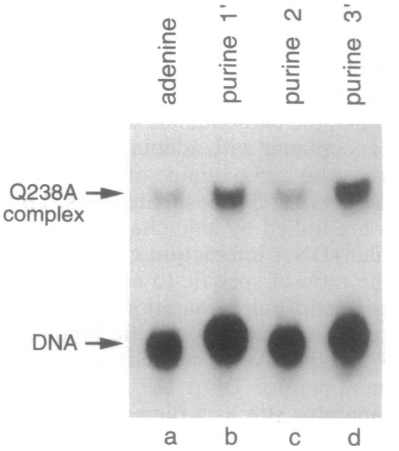


FIG. 7. 2′-Deoxypurine (nebularine) at positions 1′, 2, and 3′ (see text or Fig. 4*C* for numbering scheme) does not inhibit binding of Q238A mutant domain (lanes b–d); binding to the consensus octamer site is shown in lane a. Although the specific activity of the labeled probes differs from lane to lane, the percent shifted is similar in each case. Each lane contained ≈300 nM protein and 1 nM $^{32}$P-labeled duplex dodecanucleotide.

nine-2 as in prokaryotic systems (16–18). Although indirect mechanisms cannot formally be excluded, *in vitro* "complementation" between the mutant domain and 2'-deoxypurine analog implies that adenine-2 6-$NH_2$ donates a hydrogen bond to the glutamine carboxamide as predicted by analogy (14, 15). These data do not address whether the roles of glutamine *a* in $POU_S$ and phage HTH domains are also analogous.

The properties of the Q238A POU domain differ in one respect from the analogous analog of phage 434 repressor. The latter exhibits a new specificity: only thymine is recognized at the corresponding operator position, and with near-native affinity; such recognition (T > > A, G, or C) is ascribed to a favorable van der Waals interaction between the alanine and thymidine methyl groups (31). Why the analogous alanine substitution in $POU_S$ confers relaxed specificity (T = A > G > C) with lower affinity is not clear.

## CONCLUDING REMARKS

The POU motif defines a class of transcription factors of central importance in metazoan development (1–7). Bipartite DNA recognition (8–13) is effected by distinct HTH scaffolds, $POU_S$ and $POU_{HD}$, which differ in structure and presumed mode of DNA binding (14, 15, 24). The present study demonstrates a functional analogy between $POU_S$ and bacteriophage repressors (16–18), as well as a fundamental difference between homeodomain (24) and $POU_S$ DNA-binding mechanisms.

The Oct-2 $POU_S$ footprint, obtained by systematic study of modified DNA sites, is as predicted by the cocrystal structure of the DNA-binding domain of λ repressor and related proteins (16–18). This correspondence implies that the two HTH elements are similarly oriented in the major groove. Additional evidence is provided by the observation of relaxed specificity induced by a substitution in the putative $POU_S$ recogniton α-helix. An analogous genetic strategy to map HTH interactions has previously been applied to the *lac* repressor (22), *Escherichia coli* cAMP-receptor protein (CAP) (21, 32), phage 434 repressor (31), and the λ repressor and Cro proteins (23, 33). In each case the results are in accord with the results of x-ray crystallography (16, 17, 34) or two-dimensional NMR spectroscopy (35).

Whether $POU_S$ and bacteriophage HTH domains are products of convergent or divergent evolution is not apparent. Convergence would imply that the two families represent independent solutions to the shared structural problem of DNA recognition. This view is supported by the absence of a clear sequence taxonomy and of intermediate structural motifs in eukaryotes (such as dimeric $POU_S$ proteins as autonomous motifs). Independent evolution of a common DNA-binding mechanism would suggest that the range of possible mechanisms is limited; i.e., an α-helix may dock in the major groove of B-DNA in characteristic ways just as certain side chain–base interactions (such as bidentate hydrogen bonds between glutamine and adenine) are chemically preferred (36). In either case the present study raises the intriguing possibility that $POU_S$ and prokaryotic HTH elements effect sequence specificity by a common set of rules.

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