708 (1988)]. The general agreement between these independent approaches, conducted at different sites, further supports the interpretation of our data.
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Repression of HIV-1 Transcription by a Cellular Protein

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A cellular DNA binding protein, LBP-1, sequentially interacts in a concentration-dependent manner with two sites that surround the transcriptional initiation site of the human immunodeficiency virus type 1 (HIV-1) promoter. Although sequences in the downstream site (site I) were found to enhance transcription, purified LBP-1 specifically repressed transcription in vitro by binding to the upstream site (site II), which overlaps the TATA element. The binding of human TATA binding factor (TFIID) to the promoter before LBP-1 blocked repression, suggesting that repression resulted from an inhibition of TFIID binding to the TATA element. Furthermore, mutations that eliminated binding to site II both prevented repression in vitro and increased HIV-1 transcription in stably transformed cells. These findings suggest that a cellular factor regulates HIV-1 transcription in a manner that is characteristic of bacterial repressors and that this factor could be important in HIV-1 latency.

TRANSCRIPTIONAL REGULATION OF HIV-1 appears to be intimately related to the onset of acquired immunodeficiency syndrome (AIDS). In addition to the HIV-1–encoded Tat protein (*1*), a number of heterologous viral proteins and extracellular stimuli activate HIV-1 gene expression through multiple cis-acting elements (*2*). However, little is known about negative regulation of HIV-1 transcription (*2*). A cellular protein, termed UBP-1 (*3, 4*) or LBP-1 (*5*), has been described that interacts with a long stretch of the HIV-1 promoter that includes a functional TATA element (*5, 6*) and a potential initiator element (*7*). Deoxyribonuclease I (DNase I) footprint experiments showed that the LBP-1 binding site on the HIV-1 promoter consists of a high-affinity site [site I; nucleotides (nt) −16 to +27] and a low-affinity site (site II; nt −38 to −16) (*4, 5*) (Fig. 1A). Because the TATA-binding transcription initiation factor TFIID has the potential to interact both with the TATA element and with sequences surrounding and downstream of the initiation site (*8, 9*), LBP-1 might also contribute to the regulation of HIV-1 transcription. Mutational analyses have shown that sequences immediately downstream of the initiation site are essential for LBP-1 binding and increase transcriptional efficiency in vitro (*5, 10*).

To directly examine the function of LBP-1, we purified the protein to near homogeneity from a nuclear extract of cultured human cells by chromatography on Bio-Rex 70 and DEAE-Sephacel and by three cycles of oligonucleotide-affinity chromatography (*4, 11*). Comparable binding activities and indistinguishable binding specificities were observed with nuclear extracts from HeLa and Jurkat T cells. On analysis by SDS–polyacrylamide gel electrophoresis and silver staining, the purified protein showed two closely spaced bands of ~64 and 68 kD (*4*) (Fig. 1B, lane 1).

At high molar ratios of factor to template (>14), LBP-1 covered both site I and site II, whereas at lower ratios (<4.5), interactions were observed only with site I (Fig. 1C). To analyze the effects of LBP-1 on transcriptional activity through interactions at these binding sites, we constructed clustered base-substituted mutants (IS1 to IS4) (Fig. 1A). In agreement with previous studies (*5, 12*), a mutant (IS4) with three discontinuous substitutions (nt −3 to −1, +8 to +10, and +16 to +18) in presumptive LBP-1 recognition sites (*3, 4, 5, 12*) completely lost the ability to bind LBP-1 (Fig. 1D, lane 10). Base changes in positions −37 to −32 (IS1 mutant) or −10 to −6 (IS3 mutant) eliminated the extended protection on site II, but mutations in positions −28 to −26 (IS2 mutant, which contains a disrupted TATA element) did not (Fig. 1D, lanes 4, 8, and 6, respectively). These data show that several specific sequences surrounding the HIV-1 TATA element jointly participate, along with site I, in the specific binding of LBP-1 to site II (*13*). Gel mobility shift analysis with DNA fragments extending from nt −42 to +2 and from −6 to +19 suggested that these regions have independent LBP-1 binding activity (*14*), although it remains possible that there are cooperative interactions between these two sites.

The binding specificity of LBP-1 suggests that this factor could regulate HIV-1 transcription, positively or negatively, by direct interplay with basic transcription factors such as TFIID. To test this possibility, we incubated specific mutant templates in a nuclear extract with increasing amounts of purified LBP-1 (Fig. 2A). Transcription from the wild-type template was specifically decreased (by a factor of 100 at the highest factor to template ratio), in good agreement with the quantitative footprint patterns (compare Fig. 1C and Fig. 2A). By contrast, only weak repression, if any, was observed for the mutant template IS3, which shows only site I binding. Similarly, no repression (but rather a slight activation) was observed for IS4, which lacks binding at both sites I and II (*15*). (Although the activation mechanism of the IS4 template by LBP-1 is not known, it could reflect the copurification with LBP-1 of one or more proteins that activate through downstream sequences.) The activity of the adenovirus major late promoter template (pMLcat) was not affected by LBP-1. Thus, it was possible that inhibition of TFIID interactions with the TATA element was a major determinant for the repression mediated by LBP-1. The recent cloning of the human TFIID cDNA (*16*) enabled us to test this possibility with a human TFIID protein that had been expressed in *Escherichia coli* and highly purified by conventional chromatography (*17*) (Fig. 1B, lane 2). Incubation of the templates with exogenous TFIID before the addition of LBP-1 blocked the repression (Fig. 2B, lane 3), whereas simultaneous addition of TFIID with LBP-1 did not (Fig. 2B, lane 4). Control reactions without LBP-1 showed no loss of HIV-1 transcription as a result of an initial incubation with TFIID (Fig. 2B, lanes 1 and 2). Because the binding of TFIID to TATA elements is relatively stable (*8, 18*), TFIID must have remained bound over the incubation period despite the challenge by LBP-1. The dominance of LBP-1 in the normal incubation protocol, or when added simultaneously with exogenous TFIID, is probably explained by the rather slow, temperature-dependent binding of TFIID, which contrasts with that of other site-specific binding proteins such as LBP-1 (*8, 18*).

To investigate the relevance of the in vitro repression activity of LBP-1 to in vivo function, we measured expression from a mutant

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HIV-1 template (IS3) in HeLa cells. Because the molar ratio of factor to template is critical for in vitro repression, a stable cell-transformation assay was used rather than a transient assay in which a number of plasmids are introduced in a single cell. We transfected plasmids containing the chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 promoters with pSV2NEO (*19*). One cell line carrying wild-type (H-WT) and two lines carrying IS3 (H-IS3-1 and H-IS3-2) promoters, each a mixture of ~100 colonies, were selected in the presence of G418. H-IS3-1 and H-IS3-2 expressed a higher amount (reproducibly three- to fivefold) of the transcript than H-WT when cytoplasmic RNA was assayed by primer extension (Fig. 3). However, RNA from the simian virus 40 (SV40) early promoter in pSV2NEO was expressed at the same level in all cell lines. The HIV-1 promoters in these cell lines were equally activated (approximately 100-fold) by transfecting a Tat-producing plasmid (*14*), suggesting no significant role of LBP-1 in trans-activation by Tat (*12*). These results indicate that binding site II contributes to down-regulation of HIV-1 transcription in vivo, possibly by interacting with LBP-1.

Our results show that LBP-1 can regulate HIV-1 transcription in a dominant negative fashion by interaction with the core promoter region and thereby block TFIID binding to the TATA element. Our data also suggest that the binding of TFIID before LBP-1 suffices for formation of a committed preinitiation complex that is resistant to repression. The fact that LBP-1 binding to site I alone did not cause repression is consistent with the following observations. Partially purified human TFIID or *E. coli*–expressed human TFIID binds to a relatively narrow region of the HIV-1 promoter centered around the TATA element (*14*) and on other promoters is sufficient for template commitment (*20*). In contrast, site II overlaps the TATA box and occupancy of this site at higher LBP-1 concentrations could

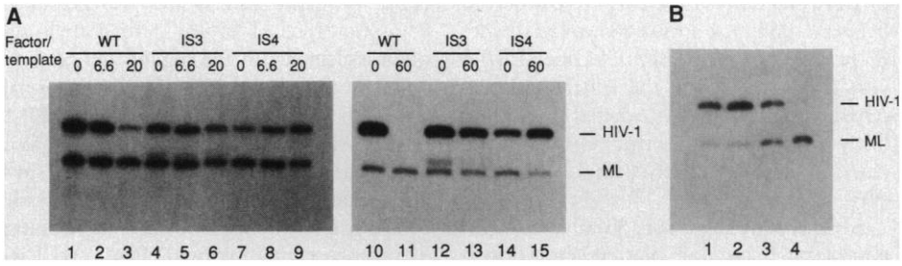

Fig. 2. HIV-1 transcription in vitro is specifically repressed by LBP-1 through site II. (**A**) In vitro transcription experiments with increasing amounts of LBP-1. Reactions (25 μl) were performed for 60 min at 30°C under standard conditions, with 8 μl of HeLa nuclear extract, 0 to 18 ng of LBP-1, 0.6 μg of poly(dI-dC), and 30 ng (lanes 1 to 9) or 10 ng (lanes 10 to 15) of both a circular HIV-1 plasmid (WT, IS3, or IS4) and the circular adenovirus major late plasmid (pMLcat). Lanes 1, 4, 7, 10, 12, and 14, no LBP-1; lanes 2, 5, and 8, 6 ng of LBP-1; and lanes 3, 6, 9, 11, 13, and 15, 18 ng of LBP-1. Molar ratios are given above the lanes. RNA synthesized in vitro was analyzed by primer extension (*29*) with 5′ end-labeled primers that hybridized to a sequence in the CAT gene. The accurately initiated transcripts from the HIV-1 (120 nt) and pMLcat (100 nt) (ML) templates are marked by lines. Bands shown were excised and radioactivity was measured in a liquid scintillation counter. The activities from the HIV-1 templates, which were normalized to that of pMLcat, are given as a percentage of the WT activity in the absence of LBP-1 as follows: lanes 1 to 3, 4 to 6, and 7 to 9 (100, 77, 25), (57, 53, 52), and (38, 42, 75), respectively; lanes 10 and 11, 12 and 13, and 14 and 15 (100, 1.0), (58, 38), and (39, 74), respectively. (**B**) Prevention of LBP-1–mediated repression by prior binding of TFIID. WT HIV-1 (10 ng) and pMLcat (10 ng) templates were incubated for 30 min at 30°C in 5 μl of 0.3× BC buffer containing 6 mM $MgCl_2$ and 0.2 μg of poly(dG-dC). *Escherichia coli*–produced human TFIID (50 ng) was added before (lanes 1 and 3) or after (lanes 2 and 4) the incubation. Transcription reactions were started by adding HeLa extract and poly(dI-dC) without (lanes 1 and 2) or with (lanes 3 and 4) 18 ng of LBP-1 and were performed under standard conditions for 30 min at 30°C.

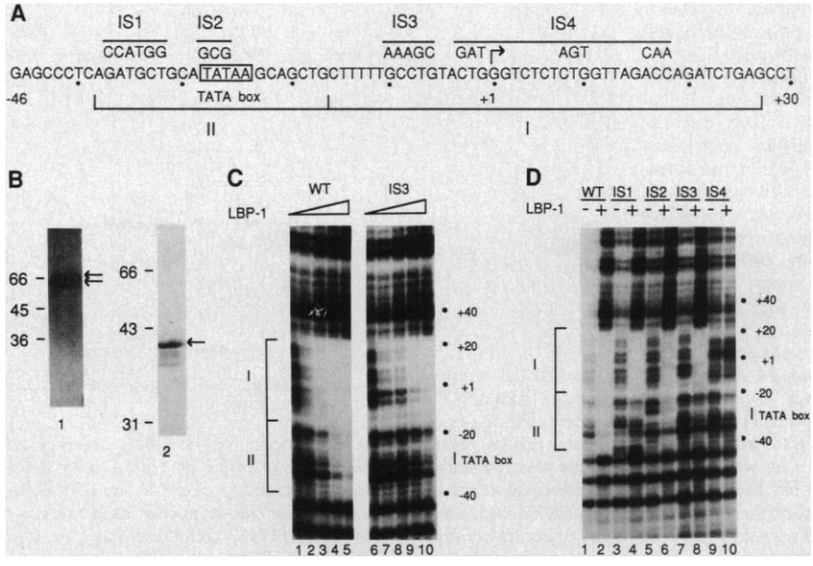

Fig. 1. Binding analyses of purified LBP-1 on the HIV-1 promoter by DNase I footprint analysis. (**A**) Sequences of the substitution mutants (IS1 to IS4) are shown above that of the wild-type HIV-1 promoter. We constructed the mutants from a plasmid containing the wild-type HIV-1 long terminal repeat sequence (nt −167 to +58), a derivative of p-167 (*28*), by replacing nt −39 (Dde I) to +21 (Bgl II) with synthetic oligonucleotides. Mutations were confirmed by the dideoxynucleotide sequencing method. The TATA element is boxed and the sequences protected by LBP-1 (sites I and II) are underlined. (**B**) Purified LBP-1 and *Escherichia coli*–expressed human TFIID proteins. Protein samples were separated by SDS–12% polyacrylamide gel electrophoresis and visualized by staining with silver for LBP-1 (lane 1) or with Coomassie brilliant blue for TFIID (lane 2). Samples (10 μl) of a third-cycle affinity chromatography fraction of LBP-1 (~3 ng/μl) or a purified fraction of TFIID (~100 ng/μl) were applied to the gels. LBP-1 and TFIID are indicated by arrows. The positions of molecular mass markers (sizes shown in kilodaltons) are indicated. (**C**) Dose-dependent change in LBP-1 binding patterns. Probes were prepared by 5′ end labeling at the Xho I site (nt −167) and by cutting the Eco RI site in the CAT gene. The DNA probe (8 fmol) was incubated with LBP-1 for 30 min at 25°C in a 50-μl reaction mixture containing 20 mM tris-HCl (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 8% glycerol, 0.04% NP40, bovine serum albumin (200 μg/ml), and poly(dA-dT) (20 μg/ml). The mixtures contained 0 ng (lanes 1 and 6), 1 ng (lanes 2 and 7), 3 ng (lanes 3 and 8), 9 ng (lanes 4 and 9), or 27 ng (lanes 5 and 10) of LBP-1. Corresponding molar ratios of LBP-1 (as monomer) to DNA probe were approximately 0, 1.5, 4.5, 14, and 40, respectively. After the binding reaction, samples were adjusted to 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$, and DNA was digested for 20 to 40 s at room temperature by addition of 8 ng of DNase I. The DNA samples were extracted with phenol-chloroform, precipitated with ethanol, and applied to a 6% polyacrylamide sequencing gel. The boundaries of the protected region are indicated. WT, wild-type probe. (**D**) LBP-1 footprint patterns with substitution mutants IS1 to IS4. Reactions were performed in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 15 ng of LBP-1 as described in (C).

interfere directly with TFIID binding (Fig. 4). A positive role for the site I sequence has been suggested (*5, 10*), as observed here for IS4 (compare Fig. 2A, lanes 1, 4, and 7) and for 3′ deletion mutants to position −4 (which show 15% of wild-type promoter activity) (*14*). However, in the presence of partially purified basic transcription factors, no clear differences in activity were observed between wild-type and IS4 templates either in the absence or presence of purified LBP-1 (*14*) (Fig. 4A). Thus, the enhancement in transcriptional activity mediated by site I is likely to depend on other factors acting alone or in cooperation with LBP-1 (Fig. 4B).

A eukaryotic repressor, SV40 T antigen, also occupies multiple binding sites, downstream and upstream of the transcriptional initiation site, in a concentration-dependent manner (*21, 22*). Binding of T antigen to the downstream site of the SV40 early promoter inhibits transcription, presumably by sterically blocking the interaction of RNA polymerase II in the region. This contrasts with the lack of repression by LBP-1 occupation of site I (located at a corresponding position) in the HIV-1 promoter. The molar ratios of factor to template required for in vitro repression by LBP-1 and T antigen are similar (*22*). It has been recently suggested that repression by elimination of the binding of basic factors might be a general mechanism of transcriptional regulation in eukaryotic cells (*23*).

Somewhat conflicting results have been described with regard to the in vivo roles of site I in basal transcriptional activity and in trans-activation by Tat (*5, 12*). This discordance probably reflects the presence of a complex array of multiple functional sequence elements in the region immediately downstream of the HIV-1 transcriptional initiation site, including not only the LBP-1 binding sites and the TAR element but also a potential initiator element (*7, 24*). A simple mutation could affect simultaneously both positive and negative control, resulting in a complex superimposed phenotype. Dissection of the functions of a single factor would be possible only with the use of the purified factor in vitro or by overexpressing the gene encoding the factor.

In the context of virus-infected cells, the most important questions raised by our findings concern how the in vivo binding activity of LBP-1 is regulated and the stage of HIV-1 replication that might be suppressed. It has been suggested that T cell activation via the transcription factor NF-κB plays a critical role in the manifestation of AIDS after a latent period (*25*), although this is still controversial (*26*). Thus, it will be necessary to assess the infectivity of the specified virus mutants under various conditions. The binding activity of LBP-1 appears to be ubiquitous and not to be altered by HIV-1 infection (*27*). However, in light of studies of other DNA binding proteins, the possibility remains that the binding activity of LBP-1 is physiologically regulated. Further study of this protein may provide better insights into the control of HIV-1 gene expression.

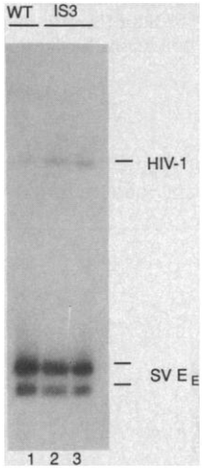

Fig. 3. Enhanced expression of IS3 in stably transformed cells. Stable cell lines were obtained as follows. Ten micrograms of HIV-1 WT or IS3 DNA together with 0.5 μg of pSV2NEO (*18*) were transfected into HeLa cells (10^6) by calcium phosphate precipitation (*30*). Transformed cells were grown in Dulbecco's modified essential medium containing 10% fetal bovine serum and G418 (400 μg/ml) (Gibco Labs.). Approximately 100 colonies that appeared 14 days later were pooled and cultured further. Cytoplasmic RNA (50 μg) from one WT cell-pool (H-WT) and two different IS3 cell-pools (H-IS3-1 and H-IS3-2) was analyzed by primer extension with two primers; one was the primer used for the in vitro transcription experiments, and the other hybridized with a leader sequence in the SV40 early gene. Products of accurately initiated transcripts from the HIV-1 promoter or the SV40 early promoter (SV E_E) are indicated at the right. Densitometric scanning revealed that the relative amounts of IS3 transcripts to the WT transcripts, normalized to that of SV40, were 3.4 (lane 2) and 3.2 (lane 3). The values were 4.5 (lane 2) and 4.1 (lane 3) when further normalized to the copy numbers of the integrated HIV-1 plasmids in these cell-pools by a dot blot analysis (*14*).

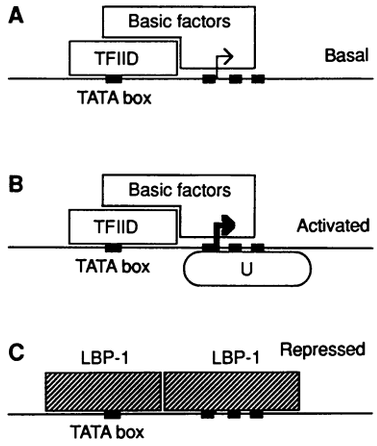

Fig. 4. A model for the differential regulation of HIV-1 transcription by LBP-1. (**A**) Basal level transcription independent of the downstream sequences. The transcriptional machinery is represented by TFIID and other basic transcription factors, which include RNA polymerase II. Boundaries of protection by TFIID and other basic factors are based on the results of footprint analyses (*8, 9, 14, 31*). (**B**) Transcriptional activation through site I by an unidentified trans-acting factor (U). LBP-1 binding to site I alone does not affect transcription significantly. (**C**) At high molar ratios, LBP-1 occupies site II as well as site I and represses transcription dominantly over upstream positive factors.

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A Nonconservative Serine to Cysteine Mutation in the Sulfate-Binding Protein, a Transport Receptor

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Serine 130 is one of seven residues that form a total of seven hydrogen bonds with the sulfate completely sequestered deep in the cleft between the two lobes of the bilobate sulfate-binding protein from *Salmonella typhimurium*. This residue has been replaced with Cys, Ala, and Gly by site-directed mutagenesis in an *Escherichia coli* expression system. Replacement with the isosteric Cys caused a 3200-fold decrease in the sulfate-binding activity relative to the wild-type activity, whereas replacement with Ala and Gly resulted in only 100- and 15-fold decreases, respectively. The effect of the Cys substitution is attributed largely to steric effect, whereas the Gly substitution more nearly reflects the loss of one hydrogen bond to the bound sulfate with a strength of only 1.6 kilocalories per mole.

BECAUSE OXYGEN AND SULFUR possess similar chemical properties and belong to the 6A chemical group of the periodic elements, interconversion of serine and cysteine is considered to be the most conservative between any pair of amino acid residues in proteins. Indeed it is this consideration that motivated investigators to undertake the conversion of serine-type proteinases (such as trypsin and subtilisin) to cysteine-type proteinases (such as papain) as far back as 25 years ago by chemical means (*1*, *2*) and more recently by recombinant technology (*3*).

This consideration prompted us to investigate the effect of a Ser to Cys mutation at the oxydianion-binding site of the sulfate-binding protein (SBP). The sulfate-binding protein belongs to a large class of proteins found in the periplasmic space of Gram-negative bacteria. These monomeric proteins, collectively called "binding proteins," serve as initial high-affinity receptors for the active transport systems for various carbohydrates, amino acids, oligopeptides, and oxyanions (*4*). The SBP from *S. typhimurium* and *E. coli* has identical 310 amino residues, giving a molecular weight of ~34,700 daltons (*5*, *6*), and one high-affinity ligand-binding site (*7*, *8*). The ligand-binding site is exquisitely designed to bind fully ionized tetrahedral oxydianions (such as sulfate, selenate, and chromate) but not the weak acid phosphate (*7*, *8*).

The crystal structure of the liganded form of the *S. typhimurium* SBP that has been well refined at 2.0 Å resolution (*9*) and at 1.7 Å resolution (*10*) has revealed the atomic interactions between the protein and sulfate. The sulfate dianion, which is completely desolvated and sequestered ~7.5 Å deep in the cleft between the two lobes of the bilobate SBP, is held in place by seven hydrogen bonds formed with five NH groups of peptide units, one NH of Trp^{192} side chain, and one OH of Ser^{130} (Fig. 1). The sequence of Ser^{130} OH, Gly^{131} NH, and Gly^{132} NH forms a tripod for docking the O1, O2, and O3 sulfate oxygens, respectively. All of the hydrogen-bond donor groups to the sulfate are also buried. Serine 130, which precedes the first turn of a helix, is semi-enclosed in the wall of one domain facing the cleft.

The parameters of the hydrogen bond (OH···O) between Ser^{130} γ-OH and the sulfate oxygen derived from the 1.7 Å refined structure (*10*) are 2.65 Å for the O···O distance and 159.5° for the angle. The hydrogen-bond distance is the shortest of the seven hydrogen bonds (overall mean distance = 2.78 Å) between SBP and sulfate (*9*, *10*). As can be seen in Fig. 1, Ser^{130} γ-OH is involved in cooperative hydrogen bonding by further accepting an NH from the third peptide unit (Ala^{133}) of the first turn of helix IV (N···O distance = 2.95 Å).

We have recently cloned and sequenced the gene for the SBP and have developed a suitable expression system in *E. coli* for the overproduction of mutant proteins (*6*). Besides identical amino acid content, the *E. coli* SBP is extremely similar in antigenecity, ligand specificity, and equilibrium and kinetics of ligand binding to the *S. typhimurium* SBP (*6*). There are only 18 residue differences between the two SBPs, and based on the structure of the *S. typhimurium* SBP-sulfate complex, these are located at or near the protein surface, nowhere within a radius of 19 Å to the bound sulfate (*6*). Most importantly, these residue changes do not affect the ligand-binding activity of the *E. coli* SBP. Indeed, all of the residues important to sulfate binding are absolutely pre-

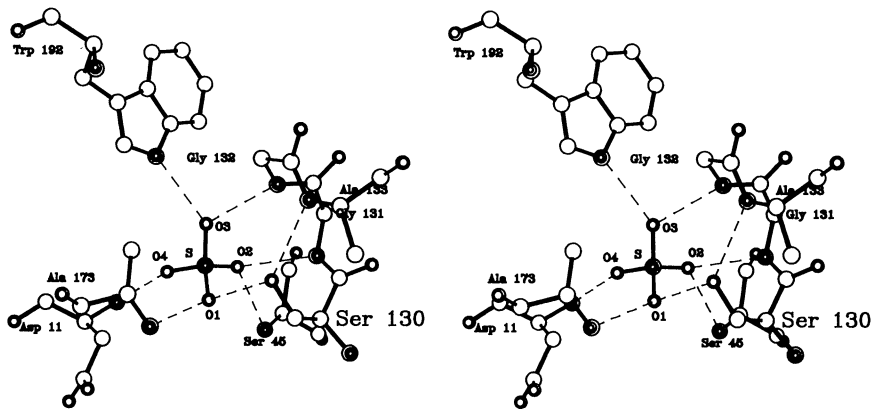

Fig. 1. Stereoscopic view of the hydrogen-bonding interactions between SBP and sulfate. Carbons are depicted as single circles, oxygens as double circles, and nitrogens as triple circles. [These interactions are those observed in the 1.7 Å crystallographic refinement of the *S. typhimurium* SBP-sulfate complex structure (*10*).]

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