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In Vivo Footprinting Analysis of Lrp Binding to the *ilvIH* Promoter Region of *Escherichia coli*

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An in vivo footprinting analysis of the *ilvIH* regulatory region of *Escherichia coli* showed that the transcription activator Lrp binds to six sites, scattered over 250 bp upstream of the transcriptional start point. When Lrp-mediated activation was impaired by the presence of exogenous leucine, only one promoter-distal site (site 2) was partially protected by Lrp binding. Equilibrium dialysis experiments showed the formation of an Lrp-leucine complex in vitro. These results suggest that leucine negatively affects *ilvIH* transcription because its interaction with Lrp reduces the efficiency of binding of the regulatory protein to the promoter region.

Lrp (leucine-responsive regulatory protein) is an *Escherichia coli* master regulator affecting the expression of at least 18 operons with known functions (6, 18) and many others with unknown functions, identified either by two-dimensional electrophoresis (9) or by the analysis of mutants generated by *plac*Mu transposition (16). Lrp can function as an activator or as a repressor, and in both cases leucine can be required for or can abolish the Lrp effect (1, 3, 4, 9–12, 14, 22). In some cases, the Lrp-dependent activation or repression is not affected by leucine (18).

The *lrp* gene has been cloned (2, 15, 21) and sequenced (2, 30), and the protein has been purified to near homogeneity (30). In solution, it forms a dimer of two identical subunits each with a predicted size of 18.8 kDa and amounts to about 0.1% of the total protein in an *E. coli* crude extract (30). A recent work by Platko and Calvo (20) suggests that Lrp has distinct domains for binding to DNA in a presumptive helix-turn-helix motif (30), for activating transcription, and for responding to leucine. The last feature suggests a possible direct Lrp-leucine interaction.

The *ilvIH* operon is one of the most studied operons regulated by Lrp (21, 23, 28, 29). It encodes one of the enzymes (acetohydroxy acid synthase) involved in the biosynthesis of branched-chain amino acids (7, 8). Several pieces of evidence suggest that Lrp interaction with the *ilvIH* promoter region is responsible for transcription activation: (i) the integrity of a 250-bp region upstream of the promoter is necessary for normal transcription (13); (ii) the level of *ilvIH* transcription is reduced more than 30-fold in a strain carrying a Tn*10* insertion in the *lrp* gene (21); (iii) Lrp binds in vitro to six sites within this region, with different binding affinities (28); and (iv) the level of *ilvIH* transcription is reduced in strains carrying mutations in five of the six Lrp binding sites (28). However, the mechanism of the Lrp function in vivo has not yet been determined.

ilvIH transcription is repressed 5- to 10-fold by addition of leucine to the growth medium (8, 27). Although it is thought that leucine and Lrp form a complex which is not able to activate *ilvIH* transcription, such interaction is so far only hypothetical.

The in vivo footprinting analysis reported here showed the occurrence, in cells grown in minimal medium, of Lrp binding to various nucleotides within the regulatory region, some of which belong to five of the six in vitro binding sites and others of which occur in a region not protected in vitro but required for *ilvIH* activation (28). In cells grown in the presence of leucine, only one promoter-distal binding site was involved in Lrp-DNA binding. Equilibrium dialysis experiments showed the formation of an Lrp-leucine complex in vitro. We suggest that lack of *ilvIH* activation in leucine-containing medium is due to a reduced affinity of an Lrp-leucine complex for the promoter region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this study were CV975 [*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI 1734], CV1008 [*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI 1734 *lrp-35*::Tn*10*], and CV976 [*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI 1734 *lrp-1*]. Cells were grown at 37°C in M9 minimal salts (25) supplemented with 5 μg of thiamine per ml, 50 μg of proline per ml, 50 μg of valine per ml, 25 μg of isoleucine per ml, and 0.2% glucose. For repressing conditions, 100 μg of leucine per ml was added to the growth medium.

In vivo methylation. Bacterial strains were grown overnight in minimal medium. Cells were then diluted 1:100 and grown to mid-exponential phase. Methylation was performed by adding freshly diluted dimethyl sulfate (DMS) (Aldrich) to a final concentration of 0.1% and incubating for 3 min at 37°C with shaking. The methylation reaction was stopped by adding an equal volume of ice-cold saline phosphate buffer (150 mM NaCl, 40 mM K_2HPO_4, 22 mM KH_2PO_4; pH 7.2). Cells were harvested by centrifugation at 10,000 × *g* for 10 min and washed twice with saline phosphate buffer. Chromosomal DNA was purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Contaminating

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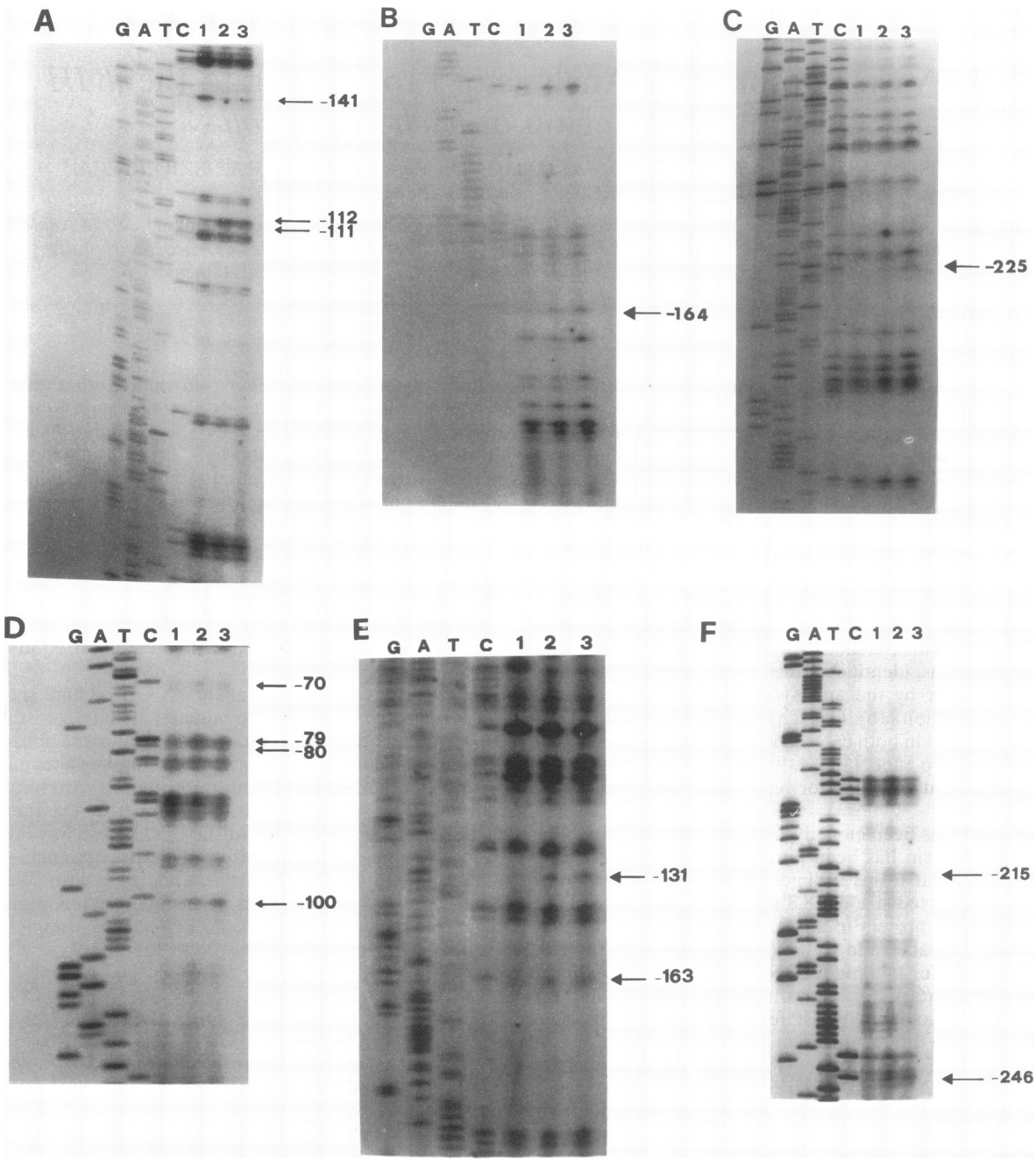

FIG. 1. In vivo methylation of the *ilvIH* regulatory region top (A to C) and bottom (D to F) strands. Strain CV975 (*lrp*$^+$) was grown in the absence (lanes 1) and in the presence (lanes 2) of leucine; strain CV1008 (*lrp*) (lanes 3) was grown only in the absence of leucine. Lanes G, A, T, and C, nucleotide sequencing reactions for the strand being extended (Gs in the methylated strand correspond to Cs in the sequencing lane). Oligonucleotides utilized for primer extension in each panel were T1 (A), T2 (B and C), B2 (D), and B1 (E and F) (oligonucleotide sequences are shown in Materials and Methods). Nucleotide positions are indicated to the right of the gels.

RNA was removed by treatment with RNase A and T_1 followed by polyethylene glycol 6000 precipitation (25).

For in vitro methylation, plasmid DNA carrying the *ilvIH* regulatory sequence was extracted from untreated cells. Contaminating RNA was removed as described above. Methylation was performed by adding freshly diluted DMS (final concentration, 10 mM) to 1 μg of DNA in 100 μl of 30 mM Tris-HCl, pH 8. The DNA sample was incubated at 37°C for 5 min, and the methylation reaction was stopped by addition of 200 μl of 3 M ammonium acetate–1 M 2-mercaptoethanol–20 mM EDTA followed by ethanol precipitation.

Primer extension analysis. Breakage points of the modified DNAs were revealed by a primer extension method adapted from that of Brewer and coauthors (5) as follows. Chromosomal DNA was primer extended in a linear PCR using *Taq* polymerase. The primer pairs T1 (5′-CCAGACAACATCTC

CATGG-3′; nucleotides +48 to +30) and T2 (5′-CGATAA AATCCTCCATTAC-3′; nucleotides −99 to −117) and B1 (5′-CATCAGTGGATGGAAGAGC-3′; nucleotides −331 to −313) and B2 (5′-GAGAAATTGCTGTAAGTTG-3′; nucleotides −172 to −154), used to probe the top and the bottom strand, respectively, were synthesized with an Applied Biosystems DNA synthesizer. End labeling was performed with [γ-^{32}P]ATP and T4 polynucleotide kinase as described elsewhere (25). Primer extension reactions were carried out in a volume of 20 μl, containing 150 ng of DNA, 0.5 pmol of ^{32}P-end-labeled oligonucleotide, 2 μl of 10× *Taq* polymerase reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 20 mM $MgCl_2$, 0.2% [wt/vol] gelatin), and a final concentration of 200 μM each deoxynucleoside triphosphate. The DNA was denatured by incubating the samples at 95°C for 5 min, and then 1 U of *Taq* polymerase was added. A program of 40 cycles, each consisting of 1 min of denaturation at 94°C, 5 min of annealing at 63°C, and 2 min of chain elongation at 72°C, was used for the amplification procedure. Four microliters of formamide dye mixture (25) was added to the samples, and the extention products were separated by electrophoresis on 6% polyacrylamide–urea sequencing gels (25). The nucleotide sequence was determined by the dideoxy chain-termination method (26). The gels were fixed, dried, and autoradiographed on Kodak X-Omat AR films with an intensifying screen at −70°C for various lengths of time.

Equilibrium binding measurements. Binding measurements were conducted by the equilibrium dialysis technique with the EMD 101 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) by using Serva (Visking Dialysis Tubing) membranes with an 8,000-molecular-weight cutoff. The membranes were routinely boiled for 5 min in 5% (wt/vol) sodium bicarbonate containing 50 mM EDTA, extensively rinsed with distilled water, and stored at 4°C in 0.01 M potassium phosphate (pH 7.5). The membranes were rinsed with B buffer (20 mM Tris-acetate [pH 7.9], 0.1 mM dithiothreitol, 4 mM magnesium acetate, 50 mM NaCl, and 0.1 mM EDTA). Experiments were performed in a shaking water bath at 37°C with a total volume of 65 μl for each dialysis chamber. Equilibrium of L-[4,5-^{3}H]leucine (Amersham) versus buffer was attained within 3 h, but 4 h was routinely allowed for each experiment. The final Lrp concentration in the dialysis chambers was 0.74 μM, while the leucine concentration ranged from 0.5 to 70 μM in the different chambers, with the same specific activity of 2.43 × 10^5 cpm/nmol. As a control, RNA polymerase (Promega) was utilized at a final concentration of 0.26 μM in the dialysis chambers, while leucine concentrations were as described above.

A graphic analysis of the Lrp-leucine binding was employed, utilizing the linear regression method of Marquardt (17).

RESULTS AND DISCUSSION

In order to study Lrp-DNA interactions occurring under different growth conditions, an in vivo footprinting analysis of the *ilvIH* regulatory region was performed. Cells of the *E. coli* wild-type strain CV975 and of the isogenic strain CV1008, which lacks Lrp because of a Tn*10* insertion in the *lrp* gene (21), were grown in the absence (Fig. 1, lanes 1 and 3 in each panel) and in the presence (Fig. 1, lane 2 in each panel) of leucine. The analysis was focused on G residues protected by Lrp from DMS attack.

When cells were grown in minimal medium, protection of G residues of the top strand was detected at positions −111 and −112 (Fig. 1A), −164 (Fig. 1B), and −225 (Fig. 1C) in the wild-type strain (lanes 1), while no protection was observed in strain CV1008 (lanes 3). The G residue marked at position −141 appeared to be hypermethylated (Fig. 1A) in the wild-type strain compared with that in the *lrp* null mutant. Enhanced DMS reactivity of G residues has been observed previously as a consequence of protein-DNA interaction (19). Additional hypermethylated G residues (not marked in the figure) included residues −108, −109, −117, and −150 on the top strand (Fig. 1A) and −87, −88, and −90 on the bottom strand (Fig. 1D). The analysis of the bottom strand showed that the G residues at positions −70, −79, −80, and −100 (Fig. 1D), −131 and −163 (Fig. 1E), and −215 and −246 (Fig. 1F) were protected in the wild-type strain (lanes 1) and not in the *lrp* null mutant (lanes 3). In addition to cleavages at G residues, some cleavages at A residues were also observed.

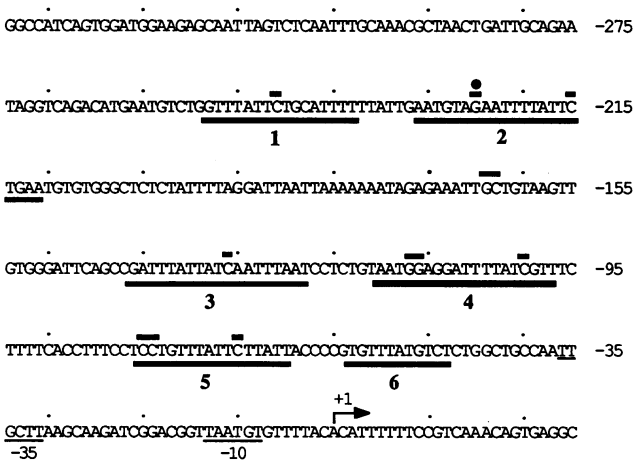

FIG. 2. Mapping of G residues protected in vivo from DMS attack in strain CV975 (*lrp*$^+$). Protection in the absence and in the presence of leucine (bars and circles, respectively) is indicated. Marked Gs and Cs correspond to guanine residues protected on the top and on the bottom strand, respectively. The six Lrp binding sites identified by in vitro footprinting (28) are shown.

As summarized in Fig. 2, the G residues protected in vivo fell into five of the six regions identified in vitro (28). The promoter-proximal binding site identified in vitro (site 6) did not interact with Lrp in vivo. On the other hand, a genetic analysis by Wang and Calvo (28) shows that mutations within site 6 do not affect transcription, thus suggesting that in vitro binding at this site is not functional.

In vivo-protected G residues at positions −164 on the top strand and −163 on the bottom strand did not fall into any of the binding sites defined by in vitro experiments (Fig. 2). In this regard, it is interesting that mutations in a nearby region (from −161 to −156) do affect *ilvIH* expression, since they reduce the level of transcription to 45% of the wild-type value (28). A role for this region in the activating process is thus clearly established.

The analysis of in vivo Lrp footprints on the *ilvIH* regulatory region in cells grown in the presence of 100 μg of leucine per ml showed that the G residue at position −225 (Fig. 1C, lane 2) was protected in the wild-type strain and not in the *lrp* null mutant (Fig. 1C, lane 3). Although in the presence of leucine some in vivo interaction of Lrp with the *ilvIH* regulatory region may still occur, binding to sites 1, 3, 4, and 5 is impaired.

Two lines of evidence suggest that interaction of Lrp with promoter-proximal sites is strictly necessary for activation of transcription: (i) mutations in sites 3, 4, and 5 abolish transcription, while mutations in sites 1 and 2 and in the region

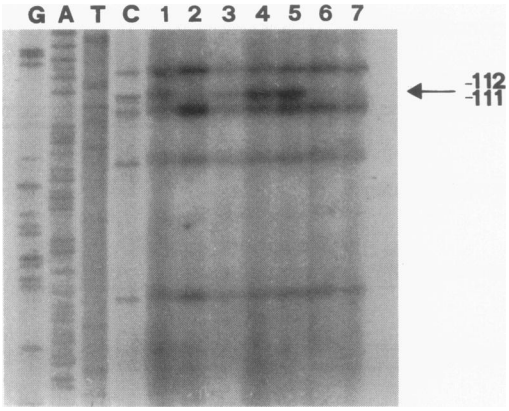

FIG. 3. In vivo methylation of the *ilvIH* regulatory region. Lane 1, in vitro methylation of the *ilvIH* regulatory region; lanes 2 and 3, CV975 (*lrp*$^+$); lanes 4 and 5, CV1008 (*lrp*); lanes 6 and 7, CV976 (*lrp-1*). Cells were grown in the absence (lanes 2, 4, and 6) and in the presence (lanes 3, 5, and 7) of 100 μg of leucine per ml. Lanes G, A, T, and C, nucleotide sequencing reactions.

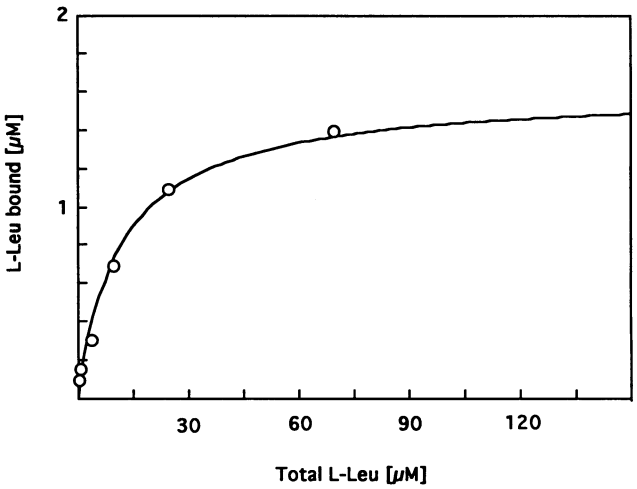

FIG. 4. Binding of L-leucine by the Lrp protein as determined by equilibrium dialysis. The ligand concentration at the beginning of the dialysis experiment ranged from 0.5 to 70 mM. The cells contained 0.74 μM Lrp.

from −161 to −156 allow transcription at 50 to 70% of the wild-type level (28) and (ii) a stereospecific alignment between the promoter and the Lrp promoter-proximal binding site is required for activation of transcription (24), suggesting that Lrp-RNA polymerase interaction is necessary for initiation of transcription. Thus, the results presented here, together with the studies mentioned above, lead to the hypothesis that interaction of leucine with Lrp may cause a conformational change of the protein and may in turn reduce its affinity for the promoter region. Lrp binding to the downstream sites would then be impaired, probably because these sites have an affinity binding constant lower than that of the upstream sites (28). Although the Lrp-leucine complex would still be able to bind to the high-affinity upstream site 2, the interaction would not be functional for activation, because of the dramatic decrease of Lrp-DNA binding efficiency at the downstream sites. In this regard, it is interesting to cite a comparative study of Lrp binding to the *gltBDF* and *ilvIH* operons which correlates the binding affinity constants (*K*) of Lrp with the sensitivity of the operons to repression by leucine (10). Ernsting et al. report *K* values of 6.9 nM for the Lrp-*ilvIH* activation complex and 2.0 nM for Lrp-*gltBDF* and suggest that this difference may reflect the greater sensitivity of the *ilvIH* promoter to repression by leucine. They also reported mobility shift assay results indicating that Lrp binding to *ilvIH* promoter-distal sites is maintained in the presence of leucine (10).

An analysis of the region from −70 to −126 (top strand) was also performed in cells of strain CV976 (carrying the *lrp-1* mutation, which renders *ilvIH* transcription insensitive to leucine) (21), grown in the absence and presence of leucine. Protection of G residues at positions −111 and −112 was observed under both growth conditions (Fig. 3, lanes 6 and 7), while no protection was detected in the *lrp* strain CV1008 (lanes 4 and 5). This result suggests that the mutated protein is unable to interact with leucine and therefore can bind to the promoter region and activate transcription regardless of the presence of the ligand in the growth medium.

Direct interaction of Lrp with leucine has not been shown yet. Mutations located in the part of the *lrp* gene giving rise to the C-terminal one-third of the protein abolish the negative effect of leucine on *ilvIH* transcription (20). This observation leads to the hypothesis that leucine could affect Lrp either directly or through another factor(s). To determine whether a physical interaction between leucine and Lrp occurs in vitro, equilibrium dialysis experiments were performed (see Materials and Methods for details). A binding curve for leucine concentrations, ranging from 0.5 to 70 μM, is shown in Fig. 4. The calculated values of the maximum concentration of leucine bound and the dissociation constant were 1.60×10^{-6} and 1.22×10^{-5}, respectively. The extrapolated value of about 2 mol ($n = 2.16$) of L-leucine bound per mol of Lrp dimer was obtained. In a control experiment with RNA polymerase instead of Lrp, no difference in the concentration of leucine in the two chambers was detected, showing the specificity of leucine binding to Lrp. The formation in vitro of an Lrp-leucine complex suggested a direct action of the ligand on Lrp in vivo.

Protection of one G residue belonging to binding site 2 was found in the presence of exogenous leucine. Previous studies have shown that Lrp has a high binding affinity for site 2 and that the presence of leucine does not completely abolish binding of Lrp to upstream sites in vitro (10, 28). Leucine would then cause a dramatic decrease of Lrp binding efficiency at sites for which the regulatory protein has a low affinity. This would result in hindrance of transcription of the *ilvIH* operon, since partial Lrp binding at site 2 is not sufficient to trigger activation of transcription initiation.

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