Mutational Analysis of a Predicted Zinc-Binding Motif in the 26-Kilodalton Protein Encoded by the Vaccinia Virus A2L Gene: Correlation of Zinc Binding with Late Transcriptional Transactivation Activity

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Transient transfection assays indicated that A2L is one of three vaccinia virus intermediate genes that are required for the transcriptional transactivation of viral late genes. We have expressed the A2L open reading frame in *Escherichia coli* and shown by blotting experiments that the 26-kDa protein binds zinc, a property predicted by the presence of a $CX_2CX_{13}CX_2C$ zinc finger motif. The specificity for zinc binding was demonstrated by competition with other metals. The role of the sequence motif in zinc binding was established by analysis of a series of mutations, including truncations and conservative single amino acid substitutions. Mutations that reduced zinc binding in vitro prevented the ability of A2L to transactivate late genes in vivo.

Vaccinia virus and other members of the poxvirus family provide unique systems for combined biochemical and genetic investigations of transcriptional regulatory mechanisms (reviewed in reference 18). Unlike other DNA viruses, poxviruses replicate exclusively in the cytoplasm of infected cells and encode a multisubunit DNA-dependent RNA polymerase that resembles the host enzyme, stage-specific transcription factors, mRNA capping and methylating enzymes, and a poly(A) polymerase. All of the proteins needed for the synthesis and posttranscriptional modification of early mRNA are packaged within the infectious virus particle. Upon entry into the cytoplasm, early gene expression initiates a cascade of events that lead to DNA replication and successive expression of intermediate and late genes (3, 14, 25). Late gene expression results in the production and assembly of virion proteins, including factors that are specific for viral early transcription (1, 5, 11).

The virus-encoded proteins required for early gene transcription include the multisubunit eukaryotic-like RNA polymerase (reviewed in reference 19), an RNA polymerase-associated protein of 94 kDa (1), and vaccinia virus early transcription factor (6). For intermediate gene transcription, RNA polymerase, capping enzyme, and two partially purified factors are needed (12, 21, 23, 24). Transfection studies led to the identification of three intermediate genes, A1L, A2L, and G8R, that are required for activation of late gene expression (14). The products of two of the open reading frames (ORFs), G8R (26) and A1L (15), have in vitro transcription factor activity and were named vaccinia virus late transcription factors 1 and 2 (VLTF-1 and VLTF-2), respectively. The third transactivator gene, A2L, has not yet been shown to have in vitro transcription factor activity. However, examination of the A2L ORF revealed an amino acid sequence, $CX_2CX_{13}CX_2C$, that resembles the C_2C_2-type zinc finger motif found in some transcription factors (8) such as the one located within the transactivator region of

the adenovirus E1A protein (9). Typical zinc finger motifs are not present within the A1L and G8R ORFs, although the former has a $CX_2CX_{23}CX_3C$ sequence with an uncommon spacing between the cysteines of the second pair. We report here that both the A1L and A2L gene products have a specific affinity for zinc. The role of the A2L zinc finger motif in zinc binding and transactivation activity was investigated.

MATERIALS AND METHODS

Protein expression. The A1L, A2L, and G8R ORFs were inserted into bacterial plasmids under the control of a bacteriophage T7 promoter. Polymerase chain reaction (PCR) was used to insert an *Nde*I site at the ATG initiation codon of the A1L ORF and a *Bam*HI site after the stop codon so as to allow ligation at the *Nde*I and *Bam*HI sites of the pET-3C vector (22). PCR was used to add a *Sal*I site at the start of the A2L and G8R ORFs and *Bam*HI sites after the stop codons to allow insertion at the *Sal*I and *Bam*HI sites of the pETsec1a vector. *Escherichia coli* BL21(DE3) was used as the host, and expression was routinely induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Construction of the vector expressing the G8R ORF was described previously (26); the protein product was used to generate antiserum that reacted with authentic G8R protein made in vaccinia virus-infected cells.

Zinc binding. Recombinant *E. coli* cells were collected by centrifugation, lysed in Laemmli sample buffer (16), heated at 100°C for 5 min, and applied to 0.1% sodium dodecyl sulfate (SDS)–12.5 or 20% polyacrylamide gels. After electrophoresis, the separated proteins were either stained with silver (Bio-Rad) or transferred to an Immobilon-P polyvinylidene membrane (Millipore). The membrane was washed three times for 1-h periods in 100 mM Tris-HCl (pH 6.8)–50 mM NaCl–10 mM dithiothreitol (4). The blots were probed with 10 μCi of $^{65}ZnCl_2$ per lane in 100 mM Tris-HCl (pH 6.8)–50 mM NaCl for 20 min. The membranes were then washed four times over a 1-h period in 100 mM Tris-HCl (pH 6.8)–50 mM NaCl–1 mM dithiothreitol and then exposed to Kodak XAR film.

Mutagenesis. Truncations were made in the A2L ORF by

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using PCR to introduce a stop codon and a *Bam*HI restriction enzyme site. The 3' oligonucleotide primers for mutant 26D1 were complementary to nucleotides 540 to 558, and those for 26D3 were complementary to nucleotides 117 to 135. Point mutations were made in the zinc finger motif by using oligonucleotide primers that changed the cysteine codons UGU and UGC to serine codons AGU and AGC, respectively. The amplified DNAs were cloned into the pETsec1a vector.

Transient expression analysis. Monolayers of human 293 cells (approximately 1.4×10^6) in 12-well Costar tissue culture trays were infected with 10 PFU of either the WR strain of vaccinia virus or recombinant vaccinia virus vTF7-3 (10) per cell. After 30 min, the unadsorbed virus inoculum was removed from each well and 2 ml of minimal essential medium containing 5% fetal calf serum and 40 μg of cytosine arabinoside (AraC) per ml was added. The cells were then transfected with 6 μg of plasmid in a calcium phosphate precipitate prepared by adding the DNA in 250 mM $CaCl_2$ to 2 mM Na_2HPO_4–280 mM NaCl–40 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 7.05) and allowing the precipitate to form for 20 min (7). After 4 h at 37°C, the medium was replaced with 2 ml of fresh AraC-containing medium, and incubation was continued for an additional 12 h. The monolayers were then washed in phosphate-buffered saline (pH 7.0), the cells were frozen and thawed, and debris was removed by low-speed centrifugation. All transfections were done in duplicate, and the lysates were tested for β-galactosidase activity (17) by using a microplate reader (2).

RESULTS

Zinc binding of the products of the A1L and A2L ORFs. The A1L, A2L, and G8R ORFs, encoding late transactivator proteins of 17, 26, and 30 kDa, respectively, were inserted into *E. coli* bacteriophage T7 promoter-regulated pET-3C (for A1L) or pETsec1a (for A2L and G8R) expression vector. In the latter vector, expressed proteins have a 22-amino-acid N-terminal segment derived from the *E. coli* *ompT* protein. Nevertheless, we refer to each protein according to the size predicted by the vaccinia virus ORF. Each recombinant viral protein was made, although the 17- and 26-kDa proteins accumulated to higher levels than the 30-kDa protein did (Fig. 1). We also noted that expression of A2L was not as tightly repressed in the absence of IPTG as was expression of the others.

The proteins from a gel identical to the one in Fig. 1 were transferred to a polyvinylidene membrane, allowed to renature, and probed with ^{65}Zn. An autoradiograph (Fig. 2) indicated intense zinc binding to the 17- and 26-kDa proteins encoded by the A1L and A2L ORFs, respectively, but not to the 30-kDa protein encoded by G8R, which was expressed at a lower level. Consistent with these results, the A1L ORF has the sequence $CX_2CX_{23}CX_3C$, which resembles a zinc-binding motif except for the spacing between the cysteines of the second pair, and the A2L ORF has a typical $CX_2CX_{13}CX_2C$ motif. By contrast, the G8R ORF has no putative zinc-binding sequence.

We decided to further characterize the metal-binding properties of the 26-kDa protein. Competition metal-binding studies were done to determine the specificity of the 26-kDa protein for zinc. Identical electrophoretic lanes containing the lysates of *E. coli* expressing the 26-kDa protein were blotted to a membrane, which was cut into strips and probed with 10 μCi of ^{65}Zn^{2+} in the presence or absence of
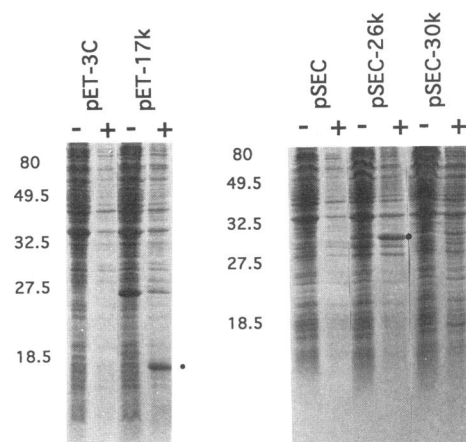

FIG. 1. Expression of vaccinia virus late gene transactivators in *E. coli*. The A1L, A2L, and G8R ORFs encoding 17-, 26-, and 30-kDa proteins, respectively, were cloned in pET-3C or pET-sec1a (pSEC) vectors and expressed in *E. coli* in the absence (−) or presence (+) of IPTG. Equivalent amounts of lysates were analyzed by electrophoresis on a 0.1% SDS–12.5% polyacrylamide gel, which was subsequently silver stained. The difference in the amounts of protein in the + and − IPTG lanes is due to the cessation of bacterial growth after induction. The dots indicate positions of the overexpressed proteins. Sizes (in kilodaltons) of marker proteins are indicated on the left.

unlabeled competitor metal ions. The membranes were exposed to X-ray film, and the amount of ^{65}Zn^{2+} bound to the 26-kDa protein was determined by densitometry. At 10 mM, only Zn^{2+} completely prevented ^{65}Zn^{2+} binding (Fig. 3). The competitor efficiency was Zn^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+}. Similar competition experiments with the *E. coli*-expressed A1L 17-kDa protein also were consistent with specific zinc binding (data not shown).

Effect of C-terminal truncations on zinc binding. The zinc finger motif of the A2L ORF is located in the N-terminal region between amino acids 6 and 26. To determine whether this region of the 26-kDa protein is capable of binding zinc,
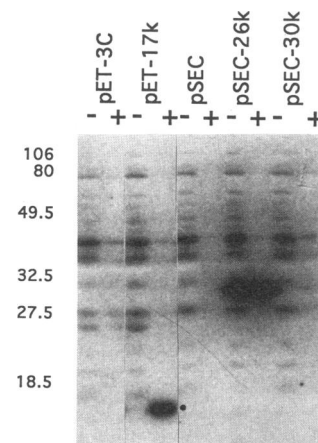

FIG. 2. Zinc binding to immobilized viral proteins. The proteins separated as for Fig. 1 were transferred to an Immobilon-P membrane and probed with ^{65}Zn. An autoradiograph is shown. The dots represent positions of the overexpressed viral proteins determined from the silver-stained gels. Sizes (in kilodaltons) of marker proteins are indicated on the left.
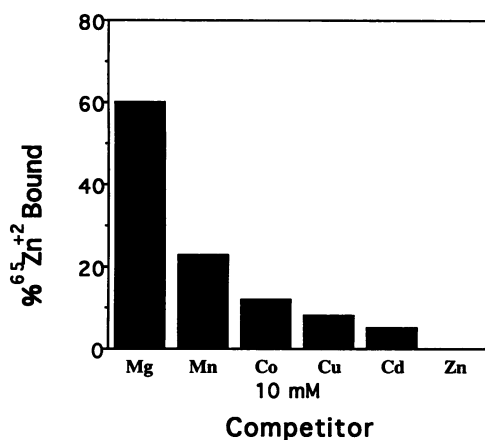
FIG. 3. Specificity of zinc binding. An *E. coli* lysate containing overexpressed 26-kDa protein was applied to a polyacrylamide gel as for Fig. 1. After electrophoresis, the proteins were transferred to a membrane as for Fig. 2. Individual strips were incubated with ^{65}Zn in the presence of 10 mM ZnCl$_2$, MgCl$_2$, MnCl$_2$, CoCl$_2$, CuCl$_2$, CdCl$_2$, or no metal. The membranes were then washed and exposed to X-ray film. The radioactivity was quantified by densitometry; the zinc bound in the presence of competitor was divided by the zinc bound in the absence of competitor, and the result was multiplied by 100 to determine the percent ^{65}Zn bound.

FIG. 5. Zinc binding by truncated proteins. The plasmid vector itself (pSEC) or the vector containing the full-length (pSEC-26k) or truncated (pSEC-26D1 and pSEC-26D3) forms of A2L ORFs were expressed in *E. coli* and analyzed by electrophoresis on a 0.1% SDS–20% polyacrylamide gel. Portions of the gel were silver stained (A), blotted and probed with antibody to the 26-kDa protein (B), or blotted and probed with ^{65}Zn (C). The dots indicate the positions of the full-length and truncated 26-kDa proteins.

C-terminally truncated forms of the ORF were made by PCR. In deletion mutants 26D1 and 26D3, the sequences coding for amino acids 186 through 224 and 46 to 224, respectively, were removed (Fig. 4). The truncated ORFs were inserted into the same pETsec1a vector used for the full-length ORF and thus contained 22 additional N-terminal amino acids. Synthesis of the proteins was induced in *E. coli*, and lysates were analyzed by electrophoresis on a 0.1% SDS–20% polyacrylamide gel. Despite their smaller size, the truncated proteins were readily visualized by silver staining (Fig. 5A) and Western blotting (immunoblotting) (Fig. 5B). Both truncated proteins retained the ability to bind ^{65}Zn (Fig. 5C). Therefore, the first 45 amino acids, which contain the zinc finger motif (amino acids 6 to 26; Fig. 4), are sufficient for the binding of the 26-kDa protein to zinc in vitro. Slowly migrating immunoreactive bands in the pSEC-26k and pSEC-26D1 lanes (Fig. 5) could represent SDS-resistant dimers. If so, the amounts are low since they could not be seen as unique bands in the silver-stained gels or as radioactive bands in the zinc blots.

Effects of single amino acid substitutions on zinc binding. To establish that the zinc binding was due to the zinc finger motif, point mutations were made in the full-length A2L

ORF. Each cysteine of the motif was individually changed to serine by replacing the T, the first nucleotide of the cysteine codon, with an A (Fig. 6). The PCR products containing the desired point mutations were then inserted into the pETsec1a vector and expressed in *E. coli*. The approximate amount of the wild-type or mutant 26-kDa protein in each lysate was determined by SDS-polyacrylamide gel electrophoresis. Volumes of each lysate calculated to give similar amounts of 26-kDa protein were then applied to an SDS-polyacrylamide gel, which was analyzed by silver staining (Fig. 7A). Blots were probed with antibody to the 26-kDa protein (Fig. 7B) and ^{65}Zn (Fig. 7C). No zinc binding was detected when cysteine 23 or 26 was changed to a serine, and binding was reduced when a serine replaced cysteine 6 or 9. Similar results were obtained when the point mutations were made in the C-terminally truncated 26D3 mutant (data not shown). Thus, the zinc finger motif, located between amino acids 6 and 26 of the A2L ORF, appears to be both necessary and sufficient for the binding of the 26-kDa protein to zinc.

Effects of single amino acid substitutions in the zinc-binding motif on transactivation. Previous experiments had shown that the A1L, A2L, and G8R ORFs transactivated expression of a late promoter-regulated reporter gene in cells infected with vaccinia virus in the presence of AraC, an inhibitor of viral DNA replication. Further experiments were carried out to determine whether point mutations in the zinc
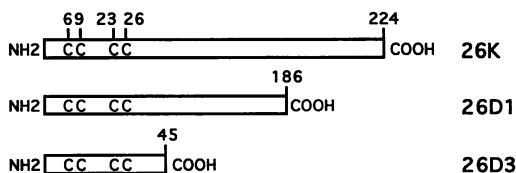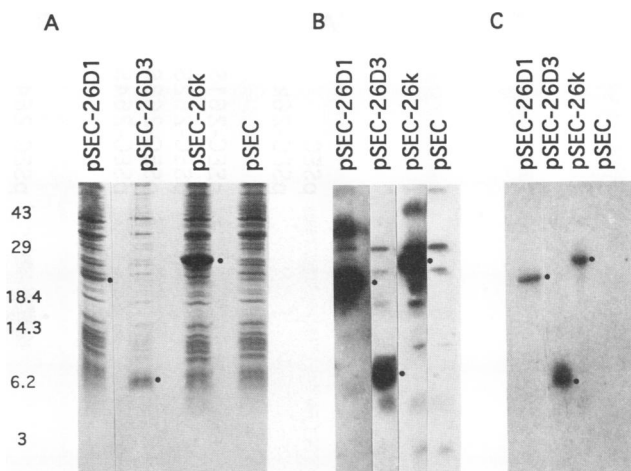

FIG. 4. Diagram of mutated A2L ORFs. Truncated versions of the A2L ORF were inserted into the pETsec1a vector. The full-length protein is designated 26K; the C-terminal deletions are designated 26D1 and 26D3. Numbers above the bars represent amino acid positions. Cysteine residues are indicated by C's within the bars.

		6	9		23	26
26K	RL	C SG	C	RHNGIVSEQGYEY	C IF	C ES
261S		S	C		C	C
262S		C	S		C	C
263S		C	C		S	C
264S		C	C		C	S

FIG. 6. Representation of single amino acid changes made in the A2L zinc finger. Amino acids 4 to 28 of the A2L ORF are shown, and cysteines are numbered. Cysteine-to-serine mutations are indicated to the right of the names of the mutants.
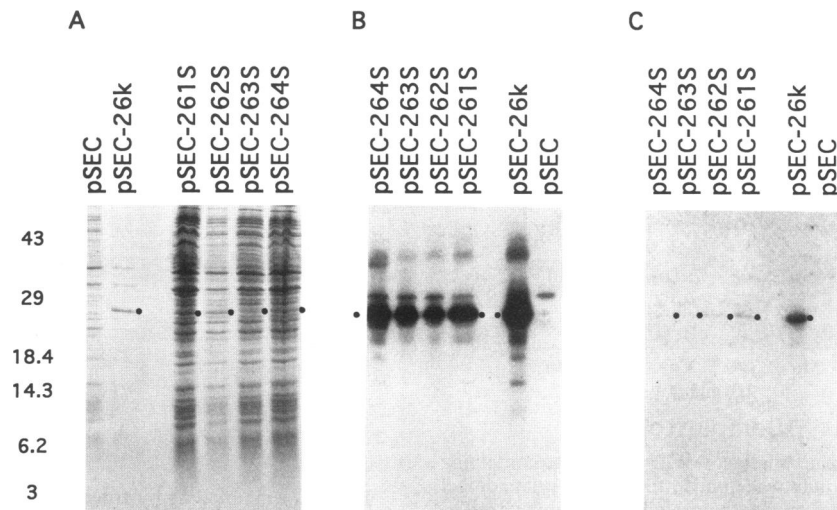
FIG. 7. Effects of single amino acid substitutions on zinc binding. Mutated ORFs (Fig. 6) were inserted into the pETsecla vector and expressed in *E. coli*. Lysates were subjected to electrophoresis on 0.1% SDS–12.5% polyacrylamide gels. (A) Silver stain; (B) Western blot with antibody to the 26-kDa protein; (C) ^{65}Zn blot. The dots indicate positions of the 26-kDa proteins. Sizes (in kilodaltons) of marker proteins are indicated on the left.

finger motif affect transactivation by the A2L ORF. We found by Western blotting that the 26-kDa protein was made in AraC-treated human 293 cells that were infected with vaccinia virus vTF7-3 (which expresses the bacteriophage T7 RNA polymerase) and transfected with any of the plasmids containing the wild-type or point-mutated forms of the A2L ORF (Fig. 8A). Moreover, pSEC26k (wild type) was capable of transactivating expression of β-galactosidase when plasmids containing the late promoter-regulated *lacZ* gene and the ORFs of the two other transactivators, A1L
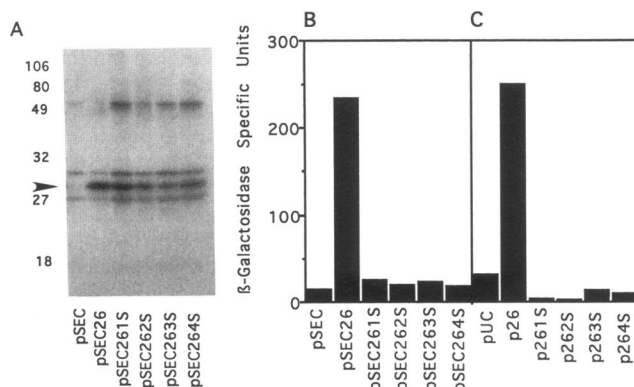

FIG. 8. Effects of single amino acid substitutions in the zinc-binding motif on transactivation. Human 293 cells were treated with AraC to prevent viral DNA replication, infected with vTF7-3 (a recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase), and cotransfected with p11Xβ (a reporter plasmid that has the *lacZ* gene under control of the vaccinia virus P11 late promoter), plasmids containing the A1L and G8R ORFs regulated by their natural promoters, and either pSEC, pSEC26, pSEC261S, pSEC262S, pSEC263S, or pSEC264S (Fig. 6). Expression of the 26-kDa proteins was determined by Western blotting (A), and expression of β-galactosidase was measured (B). The position of the 26-kDa protein is indicated by an arrowhead. Panel C is similar to panel B except that the cells were infected with wild-type vaccinia virus and the natural vaccinia virus promoter was used to regulate the wild-type and mutated A2L ORFs.

and G8R, were cotransfected (Fig. 8B). Under the same conditions, the plasmids containing point mutations in the zinc finger motif were inactive (Fig. 8B).

The 26-kDa proteins expressed from the pSEC vectors contained 22-amino-acid leader sequences. To confirm the foregoing results with the natural promoter and the authentic-length protein, the A2L ORFs containing point mutations were individually inserted into a pUC plasmid under the control of the A2L promoter. The experiment was then carried out as described above except that the AraC-treated cells were infected with wild-type vaccinia virus. The level of expression of the 26-kDa protein was too low to be detected by Western blotting. Nevertheless, the plasmid containing the authentic zinc finger motif was able to transactivate β-galactosidase expression to the same level as the more highly expressed product of the pSEC vector did (Fig. 7C), evidently because other factors were more limiting. Significantly, all of the plasmids with point mutations in the zinc finger motif were inactive (Fig. 8C).

DISCUSSION

A large number of eukaryotic transcription factors have been shown to possess zinc finger motifs of either the C_2H_2 or C_2C_2 type (8, 20). We previously determined that the A2L ORF of vaccinia virus encodes a transcriptional transactivator and noted the presence of a single C_2C_2-type motif (14). Here, we present evidence that the product of the A2L ORF expressed in *E. coli* is capable of binding zinc in vitro. Other metals did not compete well with zinc, indicating that the binding is specific. The motif $CX_2CX_{13}CX_2C$ is located near the N terminus of the protein between amino acids 6 and 26. As predicted, C-terminally truncated forms of the protein, including one retaining only the first 45 amino acids, retained zinc-binding activity. Single cysteine-to-serine substitutions in the motif abolished or decreased zinc binding by both full-length and truncated proteins.

Transfection assays were carried out to determine whether the zinc-binding motif is necessary for transcriptional transactivation activity. Conveniently, the same bacteriophage T7 promoter-regulated vectors used for expres-

sion of recombinant 26-kDa fusion proteins in *E. coli* also provided high-level expression in mammalian cells that were infected with a recombinant vaccinia virus encoding the T7 RNA polymerase (10). Moreover, the vector encoding a protein with an unmutated zinc-binding motif had good transactivator activity. We found that changing individual cysteines to serines in the zinc-binding motif completely abrogated transactivation. Similar results were obtained with use of the mutated A2L ORF expressed from its natural promoter. Because of the conservative nature of the substitutions, these results strongly suggest that zinc binding is required for transactivation. The zinc-binding motif is not sufficient for transactivation activity, however, since two C-terminally truncated forms (positions 1 to 186 and 1 to 45) with intact zinc fingers were inactive (data not shown).

With some transcription factors, zinc- and DNA-binding domains have been shown to coincide with each other (13). Additional studies are needed to determine whether the A2L product binds DNA and whether such binding is required to fulfill its role in transactivating late gene expression.

ACKNOWLEDGMENTS

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