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Detection of Mouse Skeletal Muscle-specific Product, Which Includes ZF5 Zinc Fingers and a VP16 Acidic Domain, by Reverse Transcriptase PCR

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ZF5, which we have cloned as a repressor on the mouse c-myc promoter, is a zinc finger protein containing Kruppel-type zinc finger and ZiN/POZ domains. In a reverse transcriptase PCR assay using mouse skeletal muscle RNA, we identified a 827 bp PCR product including the zinc finger domain of ZF5 and the acidic domain of VP16. The presence of the VP16 acidic domain induced the reduction of DNA-binding activity of the zinc finger domain. In addition, the inhibitory effect of the VP16 acidic domain was demonstrated on the human immunodeficiency virus (HIV) promoter, but there was no effect on the thymidine kinase (TK) promoter.

Previously we have cloned a cDNA encoding a C_2H_2-type zinc finger protein, ZF5(1). This protein contains five Kruppel-type zinc fingers (2) in the C-terminus and ZiN/POZ domain in the N-terminus. ZiN; (Zinc zinger N-terminal domain) (1) and POZ; (POxvirus and Zinc finger) (3). The ZiN/POZ domain defines a newly characterized protein-protein interaction interface. It is highly conserved throughout metazoan evolution and generally found at the N-terminus of either actin-binding or nuclear DNA-binding proteins. ZF5 binds to two GC-rich sites in the mouse c-myc promoter; one site is located between PRF (4) and YY1 (5) sites, while the second site is immediately 3′ of the YY1 site. It also binds to the herpes simplex virus (HSV) thymidine kinase (TK) and the human immunodeficiency virus (HIV) promoters. ZF5 was shown to be a repressor in a cotransfection assay using c-myc or TK promoter, however an activator on the HIV promoter. Over-expressed ZF5 acts as a growth-suppressor in mouse cells, but did not cause c-myc down regulation (6). Recently, M. Hagiwara and his colleague have isolated a cDNA encoding a human homologue of ZF5 using autoimmune sera from a patient with overlap syndrome (dermatomyositis and scleroderma) (unpublished data). This result suggests that ZF5 is linked to the autoimmune disease.

HSV is an important human pathogen and its large genome is a linear, double-stranded DNA molecule. This virus has a broad range of hosts and can infect most tissue-culture cell lines that have been derived from vertebrate species (7). The wide range of hosts suggests that the receptors for HSV on cell surface are widely distributed (8-12). VP16, first identified by Preston and colleague (13), is one of HSV-encoded polypeptides. One activating domain in VP16 is located within the C-terminus about 70 amino acids and is characterized by its acidity. Although VP16 is normally introduced into cells infecting virions, its transactivating function can also be observed by cotransfection assay with a plasmid that encodes VP16 (14).

In this report, we demonstrate that we could detect a fusion PCR product in the RT-PCR analysis using mouse skeletal muscle RNA. We also show that the function of this fusion protein was different from that of wild-type ZF5.

MATERIALS AND METHODS

Mice. Four weeks old B6C3F1 (female C57BL/6N × male C3H/He) mice. They were housed in our animal facility and fed under the condition of constant temperature (24°C) and regular 12 hr light/dark cycle.

Plasmids. Construction was by standard method; all plasmid structures were verified by appropriate restriction digestion and sequencing. For in vitro transcription and translation, each coding sequence was inserted into pBS-ATG (a gift of H. Singh) in frame with an initiating methionine and an N-terminal peptide. To make various GAL4-fusion constructs, we linked each ZF5 fragment to GAL4(1-147) expression vector. SMZF (Skeletal Muscle specific Zinc Finger domain) expression vector ZF5 Δm.

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Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared by sedimentation using guanidium thiocyanate and cecium chloride (15). The RT-PCR experiments were performed essentially as described by Montarras et al (16). 15μg of total RNA were reverse-transcribed in a 20μl reaction mixture containing 50mM Tris-HCl, pH8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 1mM dNTPs, 148.5 ng of random hexanucleotides, and 200 units of reverse transcriptase. After incubation at 42°C for 90 min, the reaction mixtures were boiled for 3 min. The 5μl of the reaction mixture was amplified using the primers for 20 cycles (95°C, 55°C, 72°C). Each reaction mixture was applied on 6% acrylamide gel in 1 × TBE (23 mM Tris, 23 mM borate, 0.5 mM EDTA).

primer (1): 5′-CATGAAGTTCGAGTACTTGC-3′
primer (2): 5′-CTCTAGCTACAGGCAATTGT-3′

Northern blot analysis. Total RNA was used in the assay. Following electrophoresis on 1% agarose/formaldehyde gel, RNA was transferred to Gene Screen Plus Nylon membrane (Du Pont). Probes were made from gel-purified fragments using the random priming method.

Cloning and sequencing. Amplified DNA from RT-PCR product was subcloned into TA-cloning vector (Invitrogen CORP.) and sequenced by 373A DNA Sequencing System (Applied Biosystems).

Electrophoretic mobility shift assay (EMSA). ZF5(132-449), ZF5(272-449) and ZF5 Δm proteins were made by in vitro transcription and translation methods using ^{35}S-Methionine. A 30 bp double stranded nucleotide, containing ZF5-binding consensus sequence, was used as a probe. Binding reaction was performed in 15μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, 100μM $ZnSO_4$) containing 2-4 μg of poly(dA-dT).poly(dA-dT) and 0.1 ng of the labeled probe. Binding mixtures were incubated at 22°C for 16 min prior to loading onto 4.5% acrylamide gel in 0.25 × TBE.

Culture, transfection, and CAT assay. NIH3T3 cells were cultured in MEM supplemented with 10% FCS. For CAT assay, NIH3T3 cells were cultured at a density of 2.5 × 10^4 cells/ml the day before transfection. Each plasmid was added into 0.3 ml of 0.25 M $CaCl_2$ and next 0.3 ml 2 × HEPES buffer (274 mM NaCl, 10 mM KCl, 2% dextrose, 42 mM HEPES, pH 7.13). Each precipitate was incubated at 30°C for 30 min before being added to the cells. After incubation for 18 hr at 37°C, medium was changed. Cells were harvested 40 hr after transfection and assayed for CAT activity as described by C. Gorman (17). The amount of cell extract used for CAT assay was adjusted based on the amount of beta-galactosidase from a co-transfected pEF-beta galactosidase gene.

SDS-PAGE. 2 μl of in vitro translated proteins was separated in 10% SDS-acrylamide gel. After electrophoresis, the gel was incubated in the commercial enhancing reagent and exposed to film.

RESULTS

Skeletal Muscle-specific RT-PCR Product

To examine the expression pattern of ZF5 mRNA in the mouse tissues, we performed the reverse transcriptase polymerase chain reaction (RT-PCR) analysis. In Fig. 1A, we described the function domains of ZF5 and the location of primers used in this experiment; primers locate 5′ and 3′ side of zinc finger domain. Fig. 1B shows the results of RT-PCR analysis on various mouse tissues. The expected PCR product (567bp) was detected in all tissues, although the intensity of bands vary among tissues. Unexpectedly, we detected an extra-PCR product (827bp) in the skeletal muscle. We also found a same band (827 bp) in liver,

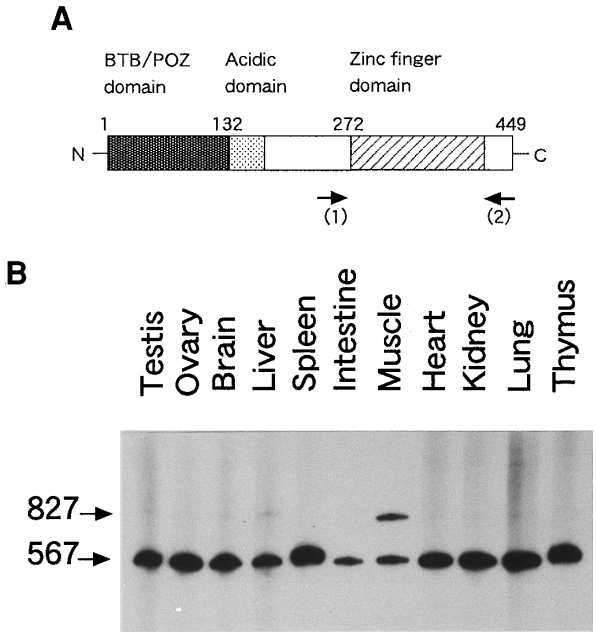

FIG. 1. Functional domains in ZF5 and RT-PCR analysis. (A) Schematic diagram of wild-type ZF5. (1) and (2) are locations of used primers. (B) RT-PCR analysis of mouse tissue RNA using (1) and (2) primers.

but the intensity of this band was very weak. Then, we conclude that this strong 827bp product is specific to the skeletal muscle.

To confirm the expression of the fusion protein mRNA, we performed Northern blot analysis using both acidic domain of VP16 and zinc finger domain of ZF5 as a probe (Fig. 2). We chose brain as a representative source of RNA except skeletal muscle, since it showed both no expression of 827 bp band and strong expression of 567 bp band in RT-PCR analysis. When Northern blots were hybridized with the acidic domain of VP16, two specific weak bands were detected in both brain and skeletal muscle. There was no difference of the intencity between brain and skeletal muscle. In contrast, when we used ZF5 zinc fingers as a probe, we could detect clear difference of band between brain and skeletal muscle. This difference is consistent with that shown in RT-PCR analysis.

The entire 827bp PCR product was sequenced (Fig. 3A). The 567 bp fragment of the 5′ side is identical to the zinc finger domain of ZF5 and an additional 260bp fragment was inserted in the 3′ side of zinc finger domain. Subsequently, this 827bp fragment was named SMZF (Skeletal Muscle specific Zinc Finger domain). There are two small non-specific fragments in both sides of 260 bp fragment, 20 bp at the 5′ side and 6 bp at the 3′ side. The amino acid sequence deduced from this 234bp contains 77 amino acids. The location of stop codon in the 260bp is different from that in wild type ZF5. As shown in Fig. 3B, the nucleotide sequence of

234bp is highly homologous to that of the acidic domain of VP16 (96.6% identity). Especially, the 5′-side 98 bp fragment is 100% identical. There is also significant homology in amino acid sequences between the 234bp fragment and that of VP16 (82.1% identity) (Fig. 3C). In the acidic domain of VP16 (between amino acids 5 and 75), 21/71 (29.6%) of the residues are either glutamic or aspartic acid residues. Whereas, SMZF has 18/70 (25.7%) of same residues. These results suggest that SMZF protein may be biochemically active in the transcriptional regulation.

Function of SMZF Protein

To test the DNA binding ability of SMZF, we performed electrophoretic mobility shift assay (EMSA) using the monomer ZF5 binding sequence as a probe. As shown in Fig. 4A, three different ZF5 expression vectors were constructed as effectors; ZF5(132-449), ZF5(272-449) and SMZF expression vector ZF5 Δm. An amount of the synthesized proteins was tested. As shown in Fig. 4B, each construct was expressed almost in an equal amount. The result of binding assay is shown in Fig. 4C. A strong DNA-protein complex was observed using ZF5(132-449) (lane 2) or ZF5(272-449) (lane 3), but weak complex using ZF5 Δm (lane 4). It seems that the decreasing of DNA-ZF5 Δm complex didn't result from the longer amino acid structure of

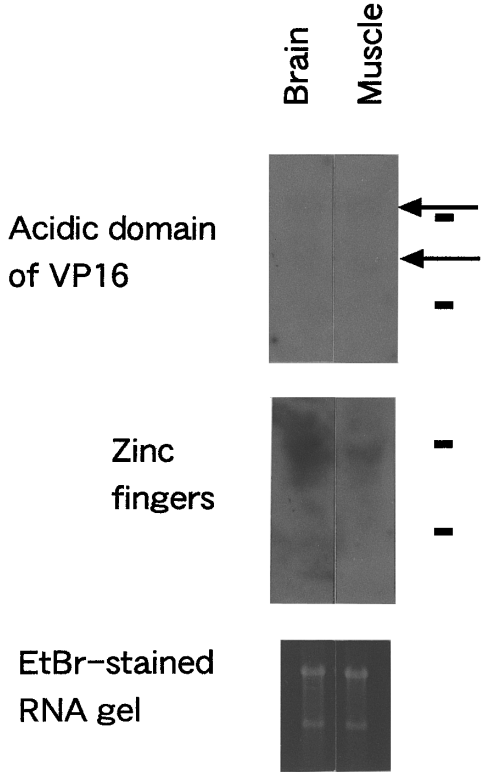

FIG. 2. Northern blot analysis. Total RNA (20 μg) was used in each lane. Acidic domain of VP16 and zinc fingers (ZF5 zinc fingers coding region) were used as probes. The ethidium bromide-stained gels are shown at the bottom of each gel. The arrows indicate specific bands.

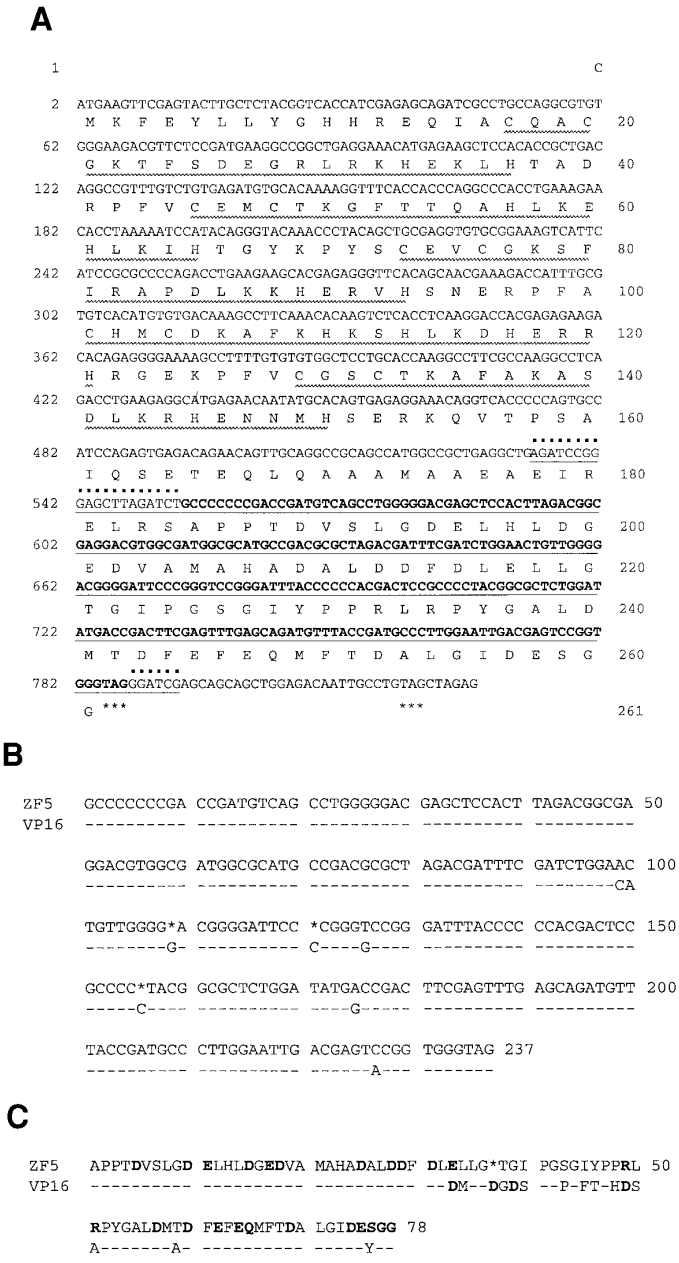

FIG. 3. Nucleotide sequence of the 827 bp PCR product and the predicted amino acid sequence. (A) The predicted amino acid sequence is shown in the single-letter code under the nucleotide sequence. Additional muscle specific sequence is indicated by continuous underlining and the region, that is homologous to VP16 acidic domain, is indicated by bold. The region non-homologous to VP16 is indicated by broken lining. The zinc fingers are indicated by wavy underlining. Stop codons are marked with asterisks. (B) Comparison of the nucleotide sequence between additional muscle specific sequence and the acidic domain of VP16. Underlining and asterisks indicate identical and missing residues, respectively. (C) Comparison of the amino acid sequence between additional muscle specific sequence and the acidic domain of VP16. Others are same with (B).

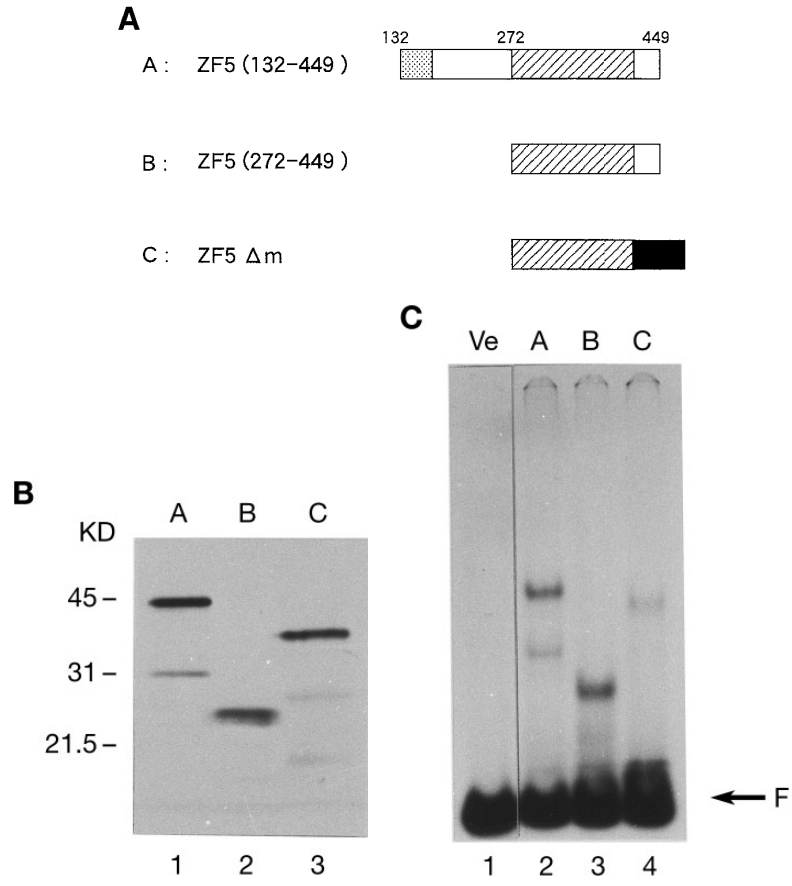

FIG. 4. Binding assay of in vitro translated proteins to the ZF5 binding consensus sequence. (A) Schematic diagram of the in vitro translated proteins. A: ZF5(132-449), B: ZF5(272-449), C: ZF5Δm. ZF5 Δm is SMZF expression vector.0(B) SDS-PAGE analysis of the in vitro translated proteins. (C) EMSA with in vitro translated proteins. Ve: Vector. F: Free probe.

ZF5Δm protein compared with that of ZF5(272-449), because ZF5(132-449) is much longer than ZF5 Δm (see lanes 1 and 3 in Fig. 4B). We conclude that the acidic domain of VP16 reduced the DNA binding activity of the zinc finger domain of ZF5.

In order to elucidate the effect of SMZF protein on the transcriptional regulation, we carried out the chloramphenicol acetyl transferase (CAT) assay using TK or HIV promoters as a reporter. We have already tested that both TK and HIV promoters have a binding site of ZF5 zinc fingers (1). We constructed two different GAL4-ZF5 expression vectors as effectors; GAL4-ZF5(272-449) and GAL4-ZF5 Δm (Fig. 5A). GAL4(1-147) has an DNA binding activity, but no transacting function (18). We used this vector as an only expression vector. Cotransfection into NIH3T3 cells of GAL4-ZF5(272-449) resulted in a very significant increase of CAT activity from HIV-CAT (lane 5). On the other hand, GAL4-ZF5 Δm showed a little increase of same CAT activity (lane 6). When tested with TK-CAT reporter, there was no significant difference between the CAT activity of GAL4-ZF5(272-449) and that of GAL4-ZF5 Δm (lanes 2 and 3). These results indicate that SMZF protein decreased the CAT activity of wild-type zinc finger on the HIV promoter, but had no effect on the TK promoter.

DISCUSSION

We detected the extra-PCR product in the zinc finger domain of ZF5 using mouse skeletal muscle RNA. The entire amino acid sequence revealed the zinc finger domain of ZF5 and an additional 260 bp fragment which is strongly homologous to the acidic domain of VP16. The binding activity of SMZF protein on the target element was weaker than that of the original zinc fingers. In CAT assay, the original zinc fingers activated the HIV promoter significantly, but SMZF protein did not. When tested with TK promoter, there was no difference in the CAT activity between the original zinc fingers and SMZF protein.

Herpes virus can become latent in their host. The latent state is characterized by the virus remaining in its host cells. The virus genomes are retained in daughter cells following successive rounds of cell division, even though their genomes are not always integrated into the host chromosome (19-21). When Southern blots

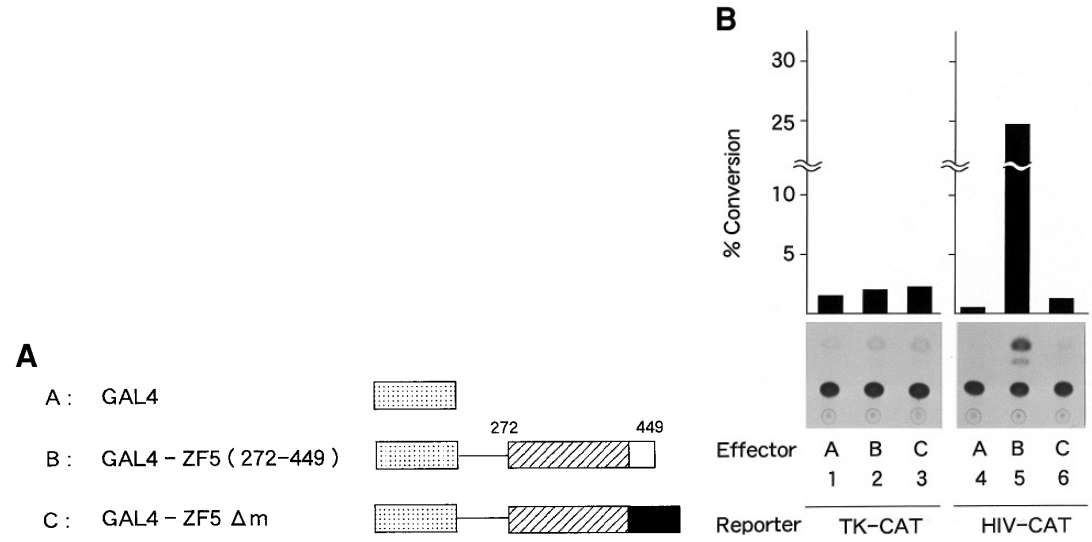

FIG. 5. Assays of transcriptional activity using GAL4-fusion proteins. (A) Schematic diagram of GAL4-fusion proteins as effectors. A: GAL4(1-147), B: GAL4 ZF5(272-449), C: GAL4-ZF5 Δm. ZF5 Δm is SMZF expression vector. (B) CAT assay. Each bar represents the average of two independent experiments.

of genomic DNA from mouse tissues were hybridized with a probe corresponding to acidic domain of VP16, a specific band was not detected (data not shown). This result indicates that the herpes virus genomes were not integrated in the host genomes. As shown in Fig. 2, expression of this fusion mRNA was not different between brain and skeletal muscle. This result is not consistent with the result of RT-PCR analysis. To answer to this paradox, we favor the view that RT-PCR analysis may be more sensitive in the ratios of the amount of mRNA than Northern blot analysis. In the skeletal muscle, the intensity of 827 bp PCR product is almost same with that of 567 bp product. However, when Northern blots were hybridized with either the acidic domain of VP16 or zinc fingers probes, there was different intensity between them. We can consider the possibility that the zinc finger probe was hybridized not only with ZF5 zinc fingers but also with other zinc fingers because there are many zinc finger proteins homologous to ZF5 zinc fingers (22). On the other hand, acidic domain of VP16 may not be cross-hybridized with other genes. Then, why is this fusion RNA expressed only in the skeletal muscle? This is a very interesting phenomenon. One potential explanation for this observation is that the transcribed SMZF mRNA might be stable in the skeletal muscle compared with other organs. Recently, increasing attention has been directed to the regulatory mechanism of skeletal muscle specific gene expression (23). Several evidences indicate that MyoD family and other factors play important role in qualitative and quantitative aspects of myogenesis and muscle specific gene expression (24-26).

We showed that in the zinc fingers, the presence of the VP16 acidic domain is associated with a significant reduction in DNA-binding affinity and also that this effect does not appear dependent on the size of the protein. S. McKnight and his colleague (27) demonstrate that the acidic domain of the VP16 achieves activation specificity via protein-protein interaction. The simplest interpretation of our data is that in the multimeric complex, the protein-protein interactions mediated by the acidic domain effectively reduced the interaction of the associated DNA-binding domain with DNA. The ability of ZF5 as an transcription repressor has been demonstrated in our previous reports (1) and this function is related to the N-terminal region, especially POZ domain (data not shown). Therefore, as shown in Fig. 5B, GAL4-ZF5 Δm has no effect on the TK-CAT activity (see lanes 2 and 3). On the other hand, the activation ability of ZF5 requires the presence of zinc finger domain. We therefore conclude that the reduction of DNA-binding affinity, which is related to the presence of VP16 acidic domain, generates the decreasing of the HIV-CAT activity. Now, we don't know what kind of abnormality in the muscle cells occurs by ZF protein. However, further study may help to establish the role of SMZF protein in the muscle cells.

Finally, our results reveal that HSV can modulate the function of ZF5 protein. This means that HSV mRNA can be inserted into important gene mRNA and play an essential role in the pathogenic changes of the infected cells. In addition, it is interesting for us to analyse the role of SMZF protein in the HSV infection.

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