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Identification and Characterization of Ksposi's sarcoma-associated herpesvirus ORF11 promoter

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SUMMARY

Open reading frame 11 (ORF11) of Kaposi's sarcoma-associated herpesvirus belongs to a herpesviral homologous protein family shared by some members of the gamma-herpesvirus subfamily. Little is known about this ORF11 homologous protein family. We have characterized an unknown open reading frame, ORF11, located adjacent and in the opposite orientation to a well-characterized viral IL-6 gene. Northern blot analysis reveals that ORF11 is expressed during the KSHV lytic cycle with delayed-early transcription kinetics. We have determined the 5' and 3' untranslated region of the unspliced ORF11 transcript and identified both the transcription start site and the transcription termination site. Core promoter region, representing ORF11 promoter activity, was mapped to a 159nt fragment 5' most proximal to the transcription start site. A functional TATA box was identified in the core promoter region. Interestingly, we found that ORF11 transcriptional activation is not responsive to Rta, the KSHV lytic switch protein. We also discovered that part of the ORF11 promoter region, the 209nt fragment upstream of the transcription start site, was repressed by phorbol esters. Our data help to understand transcription regulation of ORF11 and to elucidate roles of ORF11 in KSHV pathogenesis and life cycle.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), the eighth human herpesvirus (HHV-8) discovered (Chang *et al.*, 1994), is the causal agent of Kaposi's sarcoma (KS)(Aluigi *et al.*, 1996; Ambroziak *et al.*, 1995; Memar *et al.*, 1995; Rady *et al.*, 1995). KSHV infection is also closely related to two B-cell lymphoproliferative disorders, primary effusion lymphomas (PELs) and the plasmablastic form of multicentric Castleman's disease(Cesarman *et al.*, 1995; Cesarman *et al.*, 1996; Soulier *et al.*, 1995). KSHV belongs to the *Rhadinovirus* genera of the *Gammaherpesvirinea* subfamily (Chang *et al.*, 1994). Also in this subfamily are viruses like *Herpesvirus saimiri* (HVS), a primate homology of KSHV, and Epstein-Barr virus (EBV).

Like other characterized herpesviruses, the life cycle of KSHV includes latent replication and lytic replication. *In vivo* KSHV can attain a latent stage in human B cells and endothelial cells(Renne *et al.*, 1996; Staskus *et al.*, 1997). During latency, KSHV genomes exist as closed circular episomes. A minimal array of genes, major players of latency establishment and maintenance, was expressed, including LANA, viral cyclin (vCyc), viral FLICE inhibitory protein (vFLIP), Kaposin (K12) and vIRF3 (also called LANA2)(Lubyova & Pitha, 2000; Rivas *et al.*, 2001; Staskus *et al.*, 1997). Latently infected cells will survive. Latent genomes can become lytic upon proper stimulation with chemical agents or upon the expression of Rta (replication and transcription activator), the lytic switch protein of KSHV lytic replication(Renne *et al.*, 1996; Sun *et al.*, 1998). During lytic replication, there is massive viral gene expression and viral genes

70 are expressed in a cascade manner(Sarid *et al.*, 1998). New infectious progeny virions
71 will be produced in the lytic phase and lyticallly infected cells will be lysed
72 eventually(Renne et al., 1996).
73
74 All herpesviruses share a similar genome structure, a long unique region bounded by
75 terminal repeats, and an array of conserved genes, including genes encoding a large
76 number of enzymes involved in nucleic acid metabolism, DNA synthesis, and protein
77 modification, and those genes involved in DNA replication, and virion structure(Borchers
78 *et al.*, 1994). Some genes are only conserved among members of a herpesviral subfamily.
79 Some genes are unique to each individual herpesvirus. KSHV open reading frame 11
80 (ORF11) is a conserved gene shared by many members of gamma-herpesvirus
81 subfamily(Alba *et al.*, 2001).
82
83 KSHV ORF11 was initially identified in a single 13kb divergent locus from KS and
84 Bcbl-1 samples(Nicholas et al., 1997), next to a well-characterized viral IL-6 gene(Deng
85 *et al.*, 2002; Molden *et al.*, 1997; Moore *et al.*, 1996; Neipel *et al.*, 1997; Osborne *et al.*,
86 1999; Wan *et al.*, 1999). Little is known about ORF11 gene expression in KS or PEL
87 tumors. Earlier investigation using a tetracycline-inducible Rta expression in Bcbl-1 cells
88 suggests that ORF11 is a lytic viral gene(Nakamura et al., 2003). However, the
89 expression kinetics of ORF11 transcription is still not clear. Computational analysis of
90 ORF11 coding sequence by Davison AJ et al revealed a dUTPase related
91 domain(Davison & Stow, 2005), suggesting ORF11 encoded protein may function as a
92 dUTPase in KSHV infected cells. So far, no evidence of dUTPase activity of ORF11

protein has been observed both *in vitro* and *in vivo*. The presence of ORF11 protein in purified KSHV virions(Zhu *et al.*, 2005), even though it is still controversial(Bechtel *et al.*, 2005), and the association of ORF11 with tegument protein ORF45(Rozen *et al.*, 2008) strongly indicate that ORF11 is either involved in KSHV virion morphology, or involved in KSHV latency establishment. The observation of deregulated ORF11 transcription in BJAB cells infected with a vIL-6 deletion mutant(Chen & Lagunoff, 2007) indicates that ORF11 is not significantly involved in the regulation of other viral gene expression.

To better understand how ORF11 contributes to KSHV pathogenesis or viral life cycle, in this study we first characterized the expression kinetics of ORF11, mapped the core promoter region of the ORF11 gene, and briefly determined how ORF11 transcription is regulated in the tightly regulated KSHV lytic cascade. Our data suggest that ORF11 is a lytic viral gene with delayed-early kinetics. The ORF11 core promoter activity was defined and a functional TATA box was identified in the core promoter region. We further showed that ORF11 is not directly regulated by Rta, the lytic switch protein, indicating other viral or cellular factors are essentially involved in the regulation of ORF11 gene expression. These findings help to further elucidate authentic roles of ORF11 in KSHV life cycle and pathogenesis.

MATERIALS AND METHODS

Cell culture. BCBL-1 cells were grown as previously described(Renne et al., 1996). BCBL-1 cells were carried in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine, and β-mercaptoethanol. Human Embryonic Kidney (HEK) 293 cells were grown at 37°C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

Construction of reporter plasmids and site-directed mutagenesis. Various length of putative ORF11 promoter region upstream of the ORF11 translational initiating ATG codon were PCR-amplified using primers listed in Table 1 and inserted into a promoterless and enhancerless reporter plasmid pGL3-basic (Promega) in a sense orientation between KpnI and XhoI sites. Mutation or deletion of the putative TATA box at -27 nt upstream transcriptional start site was performed with QuickChange site-directed mutagenesis kit following manufacturer's instructions (Stratagene). The mutation or deletion analysis was performed on pGL3-209 reporter plasmid using primers listed in Table 1. Resulting reporter plasmids containing mutated or deleted TATA box were confirmed by DNA sequencing.

5' and 3' rapid amplification of cDNA ends. 5' and 3' rapid amplication of cDNA ends (RACE) were performed according to the manufacturer's protocol. Briefly, total RNA from TPA-induced Bcbl-1 cells was reverse transcribed using the FirstChoice RLM-RACE kit (Ambion) with random decamers. The product was used as template in a PCR with one of the following ORF11-specific primers (5RACE-1:

CGCTGGGGCACGAAGGGAGACA, 5RACE-2:
CGTTTGCTGCTTGCGGTGCGTGT, 3RACE-1:
CCCTCTTCATCATCGCACCCAAGG, and 3RACE-2:
GTCACAGCCATCGTGTCAAACCACTGCT). 5' AND 3' RACE PCR products were cloned into pDrive cloning vector (Qiagen) and were sequenced with T7 promoter primer.

RNA extractions and Northern blots. Bcbl-1 cells treated with TPA (20ng/ml) for 0, 5, 12, 24, 36, 48 and 72 hours, cycloheximide (CHX, 100μg/ml) for 12 hours or Phosphonoacetic acid (PAA, 300μg/ml) for 30 hours were collected for total RNA isolation(Wang et al., 2001). Total RNA was extracted from Bcbl-1 cells using RNA-Bee (Tel-Test) according to the manufacturer's instructions. Poly(A)+ messenger RNA was purified with Oligotex direct mRNA kit, following the manufacturer's instruction (Qiagen). For northern blot hybridization, 1μg messenger RNA from each sample was separated in a 1% agarose gel containing 18% formaldehyde and transferred to a nylon membrane, which was then hybridized with ^{32}P-radiolabeled probes.

Transient transfection. Transient transfection of human embryonic kidney (HEK) 293 cells was performed using Mirus TransIT-293 transfection reagents following manufacturer's instruction (Mirus Bio) with slight modification. Briefly, HEK293 cells were transiently transfected in 6-well plates with cell at 70% confluence. 10μl TransIT-293 reagent was mixed with 200μl serum-free DMEM and incubated at room temperature for 15 minutes. 2μg each reporter plasmid was then mixed with diluted

TransIT-293 reagent and incubated at room temperature for 20 minutes for complex formation. Replace medium with minimal volume of serum-free DMEM. The TransIT-293 reagent/DNA complex was added dropwise to the cells. Gently rock the plate every 30 minutes. After three hours, replace medium with freshly made complete DMEM medium.

Reporter gene assays. HEK293 cells were transfected in a six-well dish using the Mirus TransIT-293 reagent (Mirus) with 2μg of reporter plasmid containing various length of putative ORF11 promoter region as well as 2ng of control Renilla luciferase expression vector pRL-SV40 (Promega) for transfection efficiency normalization. Luciferase assays were performed on cell lysate 24 hours after transfection with dual reporter luciferase kit (Promega) following manufacturer's instructions. Firefly luciferase expression in HEK293 cells was normalized to that of Renilla luciferase activity.

RESULTS

Identification of the ORF11 transcript

To identify the transcription start site of the ORF11 transcript, we performed 5' and 3' rapid amplification of cDNA ends (RACE). For 5' RACE analysis, poly(A)$^+$ messenger RNA was isolated from TPA-induced Bcbl-1 cells and treated with Calf Intestine Alkaline Phosphatase (CIP) and Tobacco Acid Pyrophosphatase (TAP), respectively. A

45 base RNA adapter was adapted to the decapped mRNA population carrying a 5'-monophosphate using T4 RNA ligase. The resulting mRNA population was used as the template for an initial reverse transcription step with random primers. A nested PCR with gene-specific primers (Fig. 1A) was performed to amplify the 5' end of the ORF11 transcript. For 3' RACE analysis, first cDNA strand was synthesized from poly(A)-selected RNA using a 3' RACE adapter. The 3' end of the ORF11 transcript was then amplified using the cDNA as template with a gene-specific primer (Fig. 1A) and a 3' RACE primer complementary to the adapter. Both 3' and 5' RACE PCR products (Fig. 1B) were cloned into pDrive (Qiagen) cloning vector and were sequenced with T7 promoter primer. After sequencing of 5' and 3' RACE fragments, the transcription start site of ORF11 gene was identified to be 160nt upstream of the translation initiation site and designated as +1 (Fig. 1C); the transcription termination site of ORF11 gene was identified to be 67nt downstream of translation termination TAG codon (Fig. 1D). Full-length ORF11 transcript including 5' and 3' untranslated regions (UTR) was PCR amplified and cloned into pBluescript II SK(+) phagemid. Sequencing of full-length ORF11 transcript reveals that ORF11 transcript is not spliced and the coding sequence is identical to genomic open reading frame (BC-1 position 15790-17013; Fig. 2).

Kinetics of the ORF11 mRNA expression

To determine the expression kinetics of ORF11 we performed northern blot with Bcbl-1 cells. Total RNA was harvested at 5, 12, 24, 36, 48 and 72 hours post induction from

208 vehicle (ethanol) treated cells, or cells treated with 20ng/ml TPA. Poly(A)+ messenger
209 RNA was purified with Oligotex direct mRNA kit (QIAGEN). One microgram of
210 Poly(A)+ messenger RNA per sample was fractionated on a formaldehyde-agarose gel
211 and transferred to Hybond-N membrane (Amersham). Northern blot analysis was
212 performed with an ORF11 probe. After hybridization, ORF11 was observed in three
213 different transcripts, 1.5kb, 3.4kb and 5.9kb in size (Fig. 3A). The 1.5kb transcript, the
214 major transcript observed, is very close in size to the predicted ORF11 size of ORF11
215 transcript. Two minor transcripts, 3.4kb and 5.9kb in size, are very likely polycistronic
216 products. The presence of 1.5kb transcript at very low levels in vehicle (ethanol) treated
217 Bcbl-1 cells (Fig. 3A) is indicative of spontaneous lytic reactivation. After TPA
218 induction, the 1.5kb ORF11 major transcript was readily detected at 24 hours
219 postinduction, which is in accordance with previous published data(Nakamura *et al.*,
220 2003), indicating that the ORF11 gene is transcribed with delayed-early (DE) kinetics.
221 The signal peaked at 36 hours postinduction and remained high at 72 hours (Fig. 3B).
222 Northern blots with the vIL-6-specific probe and the K8.1-specific probe were included
223 (Fig.3B) to demonstrate KSHV genes transcribed with early (vIL-6) and late (K8.1)
224 kinetics(Sun *et al.*, 1999), respectively. Northern blot with GAPDH-specific probe in the
225 lower panel (Fig. 3B) was included to show equal loading of individual Poly(A)+ mRNA
226 samples. ORF11 transcripts were also observed to be very sensitive to 100ug/ml
227 cycleheximide (CHX) treatment, inhibitor of de novo protein synthesis, while 300ug/ml
228 PAA had no effects on ORF11 transcription, indicating ORF11 transcription is
229 independent of viral genome replication (L Chen, data not shown). ORF11 thus encodes a
230 lytic viral protein that is transcribed with delayed-early kinetics.

231

232

233 ***Determination of the ORF11 promoter activity***

234

235 The 5' flanking sequences upstream of ORF11 gene transcription start site (BC-1 position
236 15630) were analyzed to characterize promoter elements of ORF11. A putative TATA
237 box was identified 28nt upstream of ORF11 transcription start site. Several putative
238 transcription factor binding sites such as AP-1, Sp-1, Oct-1 and C-EBP were also
239 identified (Fig. 4). In order to access promoter activity of the 5' flanking region of
240 ORF11 gene, a set of pGL3-luciferase reporter plasmids were constructed by inserting
241 various lengths of fragments upstream of ORF11 translational initiating ATG codon (BC-
242 1 position 15790) into a promoter-less and enhancer-less pGL3-basic vector (Promega)
243 (Fig. 5A). The putative promoter-luciferase reporter plasmids pGL301739, pGL3-1111,
244 pGL3-808, pGL3-509, pGL3-209 and pGL3-21 were transiently transfected into
245 HEK293 cells. Putative promoter activity was accessed by firefly luciferase activity using
246 a dual luciferase reporter assay (Promega). As shown in Fig. 5B, maximal firefly
247 luciferase activity was observed 24 hours post transfection in pGL3-209 reporter plasmid
248 transfected HEK293 cells. In contrast, pGL3-1739, pGL3-1111, pGL3-808, pGL3-509
249 and pGL3-21 had firefly lucifrease activity 41.18%, 32.13%, 77.15%, 55.66% and
250 91.63% lower than that of pGL3-209, respectively. Interestingly, pGL3-808 had
251 relatively lower firefly luciferase activity than pGL3-1739, pGL3-1111, pGL3-509 and
252 pGL3-209, suggesting a potential inhibitory element in pGL3-808 that represses the
253 ORF11 promoter activity in HEK293 cells. To further map the core promoter region of

ORF11 gene, another set of pGL3-luciferase reporter plasmids were constructed (Fig. 6A) and transiently transfected into HSK293 cells. As shown in Fig. 6A, pGL3-159 was sufficient for transcriptional activation of the ORF11 gene. In contrast, pGL3-139, resulted from a further 20nt deletion at the 5' end, had firefly luciferase activity 91.9% lower than that of pGL3-159, suggesting the 20nt deleted (BC-1 position 15471-15491) is critical for ORF11 gene core promoter activity. These findings suggest that the 159nt fragment 5' most proximal to transcription start site carries core promoter activity and is critical for the transcription of the ORF11 gene.

A functional TATA box in the ORF11 gene promoter

The 5' 159nt proximal to transcription start site was identified to be the ORF11 core promoter. Computational analysis of the ORF11 core promoter sequence identified a putative TATA box 28nt upstream of ORF11 transcription start site (Fig. 4). To determine whether this putative TATA box contributes to the ORF11 promoter activity, the putative TATA box was mutated or deleted using pGL3-209 as template, with primers shown in table 1. As shown in Fig. 7, mutation of TATA box from TATATC to TATGTC (pGL3-209m) reduced firefly luciferase activity by 37.78%, whereas deletion TATA box (pGL3-209d) reduced firefly luciferase activity by 82.58%, suggesting ORF11 gene promoter contains a functional TATA box and this TATA box contributes to ORF11 gene promoter activity.

277

278 ***Effects of TPA induction and Rta transactivation on ORF11 gene promoter activity***

279

280 Northern blot analysis of TPA treated Bcbl-1 cells showed that ORF11 transcript was
281 significantly induced to high level after TPA treatment (Fig. 3B) or after infection with a
282 recombinant adenovirus expressing KSHV Rta, a KSHV lytic transactivator. To
283 determine if there is KSHV Rta responsive elements (RRE) in the ORF11 promoter,
284 pGL3-lucifrease reporter plasmids carrying different truncations of the ORF11 promoter
285 region were co-transfected into HEK293 cells with various amount of pcDNA3.1-Rta
286 construct, or pcDNA3.1 empty vector as control. 24 hours after co-transfection, firefly
287 lucifrease activity was assessed. As representatively shown in Fig. 8A, pGL3-509-
288 luciferase reporter construct was not responsive to KSHV Rta lytic transactivation. Same
289 was observed in HEK293 cells co-transfected with pcDNA3.1-Rta and pGL3-1739,
290 pGL3-1111, pGL3-808, pGL3-209 or pGL3-159 reporter plasmid (L Chen, data not
291 shown), suggesting lack of RRE responsive elements in the ORF11 gene promoter. To
292 explore the mechanism of TPA induction of ORF11 gene transcription, pGL3-lucifrease
293 reporter plasmids carrying different truncations of the ORF11 promoter region were
294 transfected into HEK293 cells. Cells were treated with 20ng/ml TPA immediately after
295 transfection and firefly luciferase activity was assessed as described in materials and
296 methods. Cells transfected with pGL3-1739, pGL3-1111 or pGL3-808 luciferase reporter
297 constructs showed no response to TPA treatment (L Chen, data not shown). In contrast,
298 TPA treated cells transfected with pGL3-509 or pGL3-209 had significantly lower firefly
299 luciferase activity than vehicle (ethanol) treated cells transfected with corresponding

luciferase reporter plasmid. As representatively shown in Fig. 8B, in cells transfected with pGL3-509, TPA treatment reduced firefly luciferase activity by 78.39%, whereas no significant change was observed in pGL3-basic vector transfected HEK293 cells. In cells transfected with pGL3-209, TPA treatment reduced firefly luciferase activity by 73%. Interestingly, no significant difference was observed in pGL3-159 luciferase reporter plasmid transfected cells (L Chen, data not shown), suggesting negative regulation of promoter region (BC-1 position 15421-15471) by TPA. These findings suggest KSHV ORF11 gene promoter is not directly regulated by Rta transactivator and possibly negatively regulated by TPA.

DISCUSSION

Like all herpesviruses, KSHV has a latent life cycle and a lytic life cycle. During latent replication, only a handful of viral genes are expressed, such as LANA, vFLIP, vCyclin D, Kaposin and LANA2(Lubyova & Pitha, 2000; Rivas *et al.*, 2001; Staskus *et al.*, 1997). Upon induction with chemicals such as TPA and sodium butyrate or expression of KSHV Rta protein, a KSHV lytic transactivator, KSHV enters into lytic life cycle(Renne *et al.*, 1996; Sun *et al.*, 1998). During lytic replication, there is massive gene expression and viral gene transcription occurs in a cascade fashion. Lytic genes are further subdivided into three categories: immediate-early, early and late genes(Sarid *et al.*, 1998; Sun *et al.*, 1999). ORF11 is a viral lytic gene with unknown functions shared by some gammaherpesviruses, including KSHV, Epstein-Barr virus, saimiriine herpesvirus 2,

323 ateline herpesvirus 3, alcelaphine herpesvirus 1, Macaca mulatta rhadinovirus, equid
324 herpesvirus 2 and murine herpesvirus 68 (Alba *et al.*, 2001). However, kinetics of ORF11
325 gene transcription is largely unknown. In this report, we first characterized ORF11 as an
326 early gene with delayed-early kinetics, as ORF11 gene transcription is sensitive to CHX
327 treatment but resistant to PPA treatment. Unlike other characterized early genes such as
328 viral IL-6 that is readily detected at 5 hours post induction, ORF11 transcripts are readily
329 detected at 24 hours post induction, suggesting a delayed-early kinetics. Both of vIL-6
330 and ORF11 transcripts peak at 36 hours post induction, whereas K8.1, a characterized
331 lytic gene with late kinetics, peaks at 72 hours post induction. These findings demonstrate
332 that ORF11 is a lytic viral gene with delayed-early kinetics.
333
334 Three ORF11 transcripts were observed in this study, 1.5kb, 3.4kb and 5.9kb in size,
335 respectively. The size of the 1.5kb transcript is very close to that of predicted ORF11
336 transcript. Transcription start site of the 1.5kb ORF11 transcript is determined to be
337 genomic location 15630 (BC-1 position), 160nt upstream of ORF11 translation initiating
338 ATG codon. Transcription termination site of the 1.5kb ORF11 transcript is determined
339 to be genomic location 17080 (BC-1 position), 67nt downstream of ORF11 translation
340 termination TAG codon. Sequencing of the 1.5kb transcript did not reveal any splicing
341 event. The 3.4kb and 5.9kb transcripts were detected in very low abundance by northern
342 blot analysis. They are very likely polycistronic transcription products. The nature of
343 these polycistronic transcripts needs to be further investigated. It is possible that other
344 ORF11 transcripts do exist and could not be detected in this study due to very low
345 abundance.

How ORF11 gene expression is regulated is still not clear. Characterization of the ORF11 gene promoter is critical for a better understanding of ORF11 transcription regulation. In this study, we first identified a core promoter region, between genomic location 15471 and transcription start site, representing ORF11 promoter activity. Further deletion analysis suggests that genomic region 15471-15491 (BC-1 position) is critical for ORF11 core promoter activity. Computational analysis of genomic region 15471-15491 reveals three putative transcription factor binding sites: SRY, E2F and Oct-1(Grabe, 2002). It is very likely that these transcription factors, alone or synergically, contribute to regulation of ORF11 gene transcription. Some viral genes such as viral IL-6 are directly regulated by KSHV Rta that binds to the Rta responsive element in the viral promoter and activates gene expression (Deng *et al.*, 2007; Deng *et al.*, 2002; Song *et al.*, 2003). It is very likely that no such Rta responsive element existing in the ORF11 gene promoter, as cotransfection with Rta expression plasmid did not increase promoter activity. So it is unlikely that Rta binds to the ORF11 gene promoter directly and activates gene expression. However, we cannot rule out the possibility that a cellular factor required for Rta activation is absent in HEK293 cells. It is possible that ORF11 is indirectly regulated by Rta. For example, ORF11 is regulated by a cellular protein or a viral protein that is regulated by Rta expression. In previous study, ORF11 deregulation was observed in BJAB cells infected with vIL-6 deletion mutant(Chen & Lagunoff, 2007), suggesting possibly a role of vIL-6 in regulating ORF11 gene transcription. The striking observation that partial ORF11 gene promoter is repressed by phorbol esters suggests other mechanisms such as potential viral *cis*-elements may be involved in the regulation of

ORF11 gene transcription, as the repression was only observed in genomic region 15421-15471. Further characterization of potential *cis*-elements upstream or downstream of this genomic region will be critical for further elucidation of ORF11 gene regulation.

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493 **FIGURE LEGENDS**

494

495 Table 1. Primers used for amplification of different promoter fragments upstream of

496 ORF11 translational initiating ATG codon, construction of luciferase reporter plasmids,

497 and for mutation or deletion of TATA box of ORF11 promoter.

498

499 Fig. 1. Delineation of the full length ORF11 transcript. A) Schematic representation of

500 ORF11 coding region (indicated by open bar), location of 5' adapter, 3' adapter

501 (indicated by dotted line) and RT-PCR primers (indicated by arrows) designed for 5' and

502 3' RACE. B) Agarose gel analysis of 5' and 3' RACE products. Lane 5RACE-1 and

503 5RACE-2 yielded products of 337 and 405 base pairs, respectively. Lane 3RACE-1 and

504 3RACE-2 yielded products of 215 and 363 base pairs, respectively. C) Sequence of 5'

505 UTR of the ORF11 1.5kb transcript. Translation initiating ATG codon is underlined. D)

506 Sequence of 3' UTR of the ORF11 1.5kb transcript. Stop codon TAG and

507 polyadenylation signal are underlined.

508

509 Fig. 2. Schematic representation of gene structure of ORF11. The transcription start site

510 at 15630 and TATA box at 15602 identified in this study are indicated. ORF11 coding

511 region is indicated by open bar.

512

513 Fig. 3. Expression kinetics of ORF11. Total RNA was harvested at 5, 12, 24, 36, 48 and

514 72 hours post induction from vehicle (ethanol) treated cells, or cells treated with 20ng/ml

515 TPA. Poly(A)+ messenger RNA was purified with Oligotex direct mRNA kit (QIAGEN).

516 One microgram of Poly(A)+ messenger RNA per sample was fractionated on a

formaldehyde-agarose gel and transferred to Hybond-N membrane (Amersham). Northern blot analysis was performed with an ORF11-specific probe. A) Northern blot analysis reveals three ORF11 transcripts, a major 1.5kb transcript and two minor transcripts of 3.4kb and 5.9kb, respectively. B) North blot analysis with ORF11-specific probe reveals kinetics of ORF11 expression (upper panel). Northern blot analysis with vIL-6 probe and K8.1 probe to demonstrate expression kinetics of an early and a late gene, respectively (Middle panel), Northern blot analysis with GAPDH probe to demonstrate loading control (lower panel).

Fig. 4. Putative binding sites of transcription factors in the 5' flanking nucleotide sequences of ORF11 gene. The transcription start site was determined by 5' RACE and marked as +1 (15630nt in BC-1 position). Consensus motifs of putative binding sites of transcription factors in the genomic region 15121nt and 15792nt (BC-1 position) were analyzed, underlined and marked. Numbers indicate position upstream (-) or downstream (+) of characterized transcription start site. A putative TATA box at -28nt upstream of transcription start site and the translation initiating ATG codon are underlined.

Fig. 5. ORF11 gene promoter activity in HEK293 cells. A) A schematic representation of different pGL3-luciferase reporter plasmids used to map ORF11 gene promoter activity. The transcription start site is marked as +1. Numbers indicate position upstream (-) or downstream (+) of characterized transcription start site. Constructs were named based on the 5' end nucleotide position in relative to transcription start site. B) The above luciferase reporter constructs were transiently transfected into HEK293 cells with Mirus

TransIT-293 transfection reagents in a six-well dish using 1ug of each reporter plasmid and 2ng of pRL-SV40. Twenty-four hours post transfection, firefly luciferase and renilla luciferase activity in each cell lysate was determined by a dual luciferase reporter assay system. Firefly luciferase activity in each sample was normalized to that of renilla luciferase activity. Average of fold activation of three independent experiments was shown in this figure.

Fig. 6. ORF11 gene core promoter activity in HEK293 cells. A) Schematic representation of ORF11 promoter construct pGL3-509 and various deletion truncates used to map ORF11 core promoter activity. Putative binding sites of transcription factors are indicated on the promoter segment. Transcription start site was marked as +1. Numbers indicate position upstream (-) or downstream (+) of characterized transcription start site. B) The above luciferase reporter constructs were transiently transfected into HEK293 cells with Mirus TransIT-293 transfection reagents in a six-well dish using 1ug of each reporter plasmid and 2ng of pRL-SV40. The promoter activity was determined and calculated as described in Fig. 5.

Fig. 7. Effects of mutation or deletion of the putative TATA box on ORF11 promoter activity. pGL3-209 was used as a template to mutate (pGL3-209m) or delete (pGL3-209d) putative TATA box -28nt upstream of transcription start site using primers as shown in table 1. The promoter activity was determined and calculated as described in Fig. 5. Average of three independent experiments are shown in this figure.

Fig. 8. Effects of Rta or TPA induction on ORF11 promoter activity in HEK293 cells. A) Effect of Rta expression on ORF11 promoter activity. 100ng of each luciferase reporter construct and 2ng pRL-SV40 was co-transfected into HEK293 cells with various amount of Rta expression plasmid, pcDNA3.1-Rta. Twenty-four hours post transfection, promoter activity was determined as described in Fig. 5. All reporter constructs were tested and only data from pGL3-509 were representatively shown in this figure. B) Effect of TPA induction on ORF11 promoter activity. 1ug each luciferase reporter construct an 2ng pRL-SV40 were transfected into HEK293 cells. Immediately after transfection, cells were treated with 20ng/ml TPA or vehicle (ethanol). Twenty-four hours post induction, ORF11 promoter activity was determined as described in Fig. 5. Average of three independent experiments are shown in this figure. All reporter constructs were tested and only data from pGL3-509 were representatively shown in this figure.

Table 1

Primer[a]	Primer sequence
Primers used for the construction of pGL3 reporter plasmids	
PF-21	5`-AGTGGTACCAC CCCGGTAAGGCA-3`
PF-59	5`- AGTGGTACCGGACACAATA GTGGG-3`
PF-89	5`- AGTGGTACCCCCACAGACA CATCCT-3`
PF-139	5`- AGTGGTACCTACTCCTTCC GGGCAA-3`
PF-159	5`- AGTGGTACCGTGAAACAAA GTTTGT-3`
PF-209	5`- AGTGGTACCGCATGGTGCCAACGCC-3`
PF-509	5`- AGTGGTACCGTGTGACAACGTGGAA-3`
PF-808	5`- AGTGGTACCCATCGGCATTTGGGTA-3`
PF-1111	5`- AGTGGTACCATGCAGACAGAGGCAAC-3`
PR	5`- TGCTCGA*GGATCC*ATGTGCTCGGACAGTCACG -3`
Primers used for mutation analysis[b]	
mF-209	5`- GG CAATGGCTTG CTATGTCCAC CCCGGTAAGGCAGCCAGCC -3`
mR-209	5`- GGCTGGCTG CCTTACCGGGGTGGACATAGCAAGCCATTGCC -3`
Primers used for deletion analysis[b]	
dF-209	5`- GGGCGT GG CAATGGCTTG C*TCCAC CCCGGTAAGG CAGCC -3`
dR-209	5`- GGCTG CCTTACCGGGGTGGA*GCAAGCCATTGCC ACGCCC-3`

a PF: Forward primer; PR: Reverse primer; The number indicates the start number of oligonucleotide upstream of the transcription start site of ORF11 gene. The underlined nucleotides are the restriction enzyme sites.

b The nucleotides underlined in primer mF-209 is the mutated TATA box from TATATC to TATGTC; The * in primer dF-209 indicates the deletion position of TATA box. Primer mR-209 and dR209 are reverse complementary sequence of primer mF-209 and dF-209.

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Fig. 1A

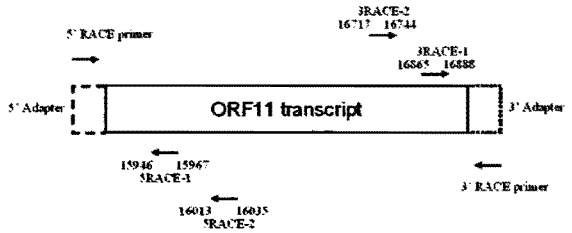

Fig. 1B

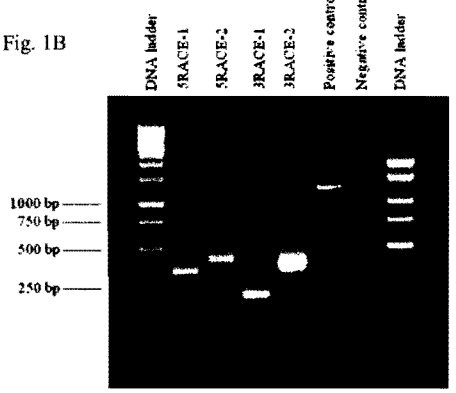

Fig. 1C

15630

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GGGTCTGTACACCACCTTGCCACGTAGCGATTAGGGCCGA

CCGCCACGAGGAACCCATGCAATCGTGACTGTCCGAGCA

CATATG

15792

Fig. 1D

17011

TAGGTGTCCGGTTCCCACCCACACATTTGTCTTTATTGCTTT

stop codon

CAAATAAAACGGTGTTCTCTCAACCTCC

polyadenylation signal 17050

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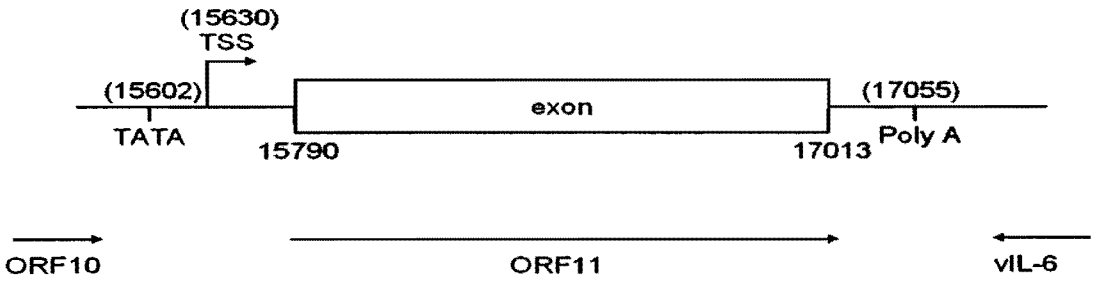
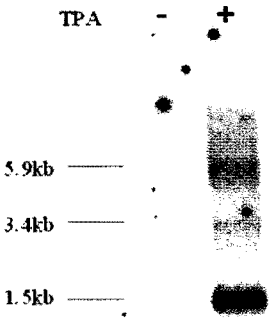
Fig. 2


(15630)
TSS
(15602)
TATA
exon
15790
17013
(17055)
Poly A
ORF10
ORF11
vIL-6

677

Fig. 3A



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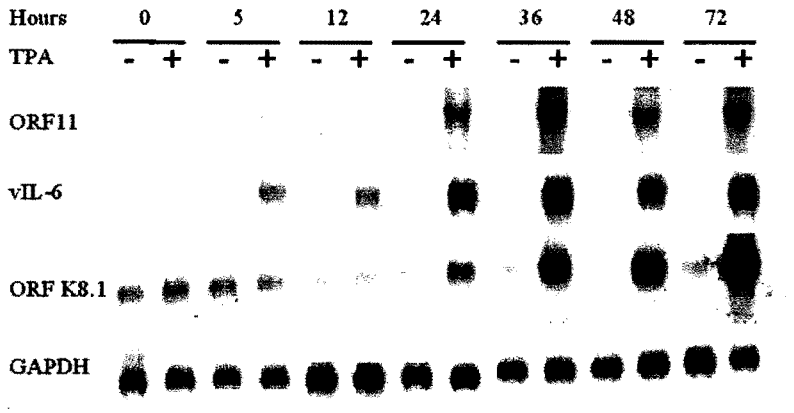

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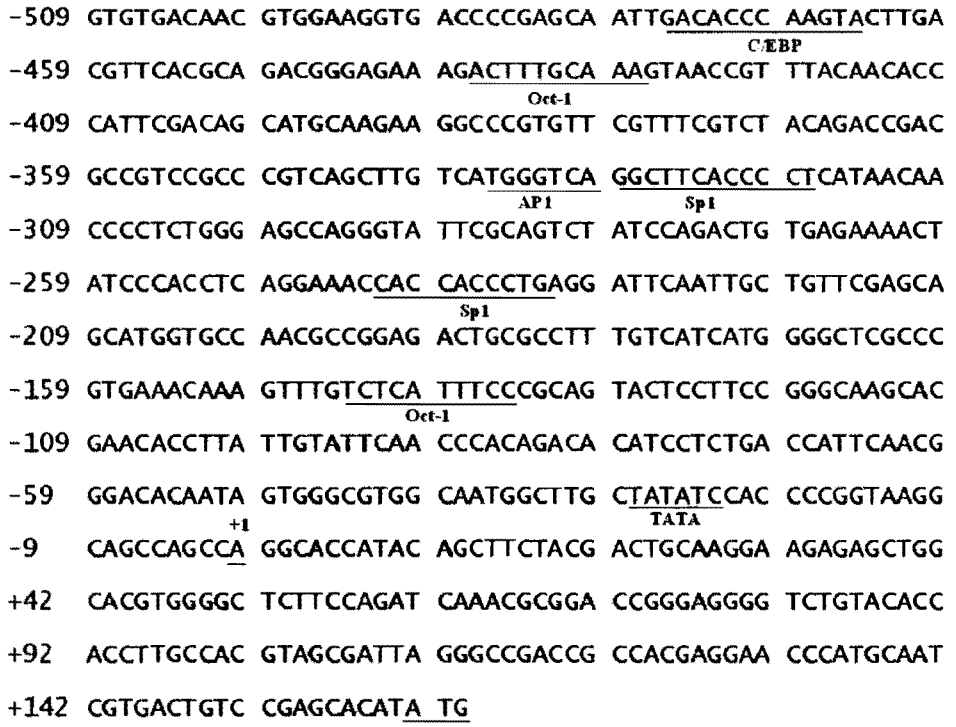
Fig. 4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C/EBP
-459 CGTTCACGCA GACGGGAGAA AGACTTTGCA AAGTAACCGT TTACAACACC
Oct-1
-409 CATTCGACAG CATGCAAGAA GGCCCGTGTT CGTTTCGTCT ACAGACCGAC
-359 GCCGTCCGCC CGTCAGCTTG TCATGGGTCA GGCTTCACCC CTCATAACAA
AP1
Sp1
-309 CCCCTCTGGG AGCCAGGGTA TTCGCAGTCT ATCCAGACTG TGAGAAAACT
-259 ATCCCACCTC AGGAAACCAC CACCCTGAGG ATTCAATTGC TGTTCGAGCA
Sp1
-209 GCATGGTGCC AACGCCGGAG ACTGCGCCTT TGTCATCATG GGGCTCGCCC
-159 GTGAAACAAA GTTTGTCTCA TTTCCCGCAG TACTCCTTCC GGGCAAGCAC
Oct-1
-109 GAACACCTTA TTGTATTCAA CCCACAGACA CATCCTCTGA CCATTCAACG
-59 GGACACAATA GTGGGCGTGG CAATGGCTTG CTATATCCAC CCCGGTAAGG
TATA
+1
-9 CAGCCAGCCA GGCACCATAC AGCTTCTACG ACTGCAAGGA AGAGAGCTGG
+42 CACGTGGGGC TCTTCCAGAT CAAACGCGGA CCGGGAGGGG TCTGTACACC
+92 ACCTTGCCAC GTAGCGATTA GGGCCGACCG CCACGAGGAA CCCATGCAAT
+142 CGTGACTGTC CGAGCACATA TG

Fig. 5A

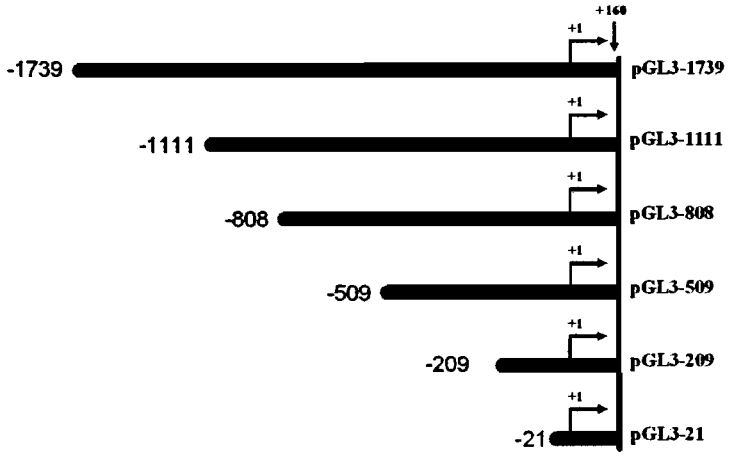
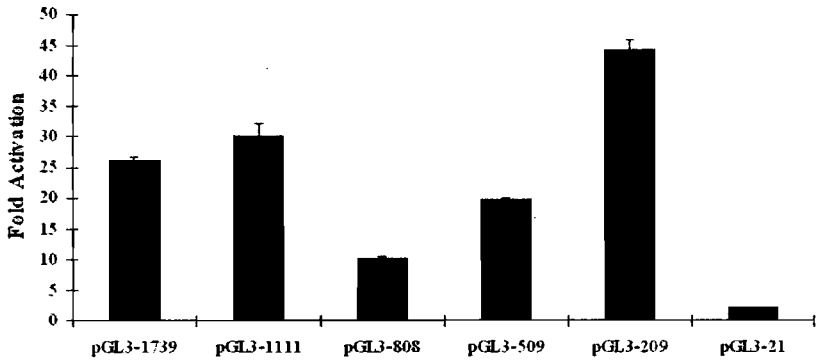

+160
+1
-1739
pGL3-1739
-1111
pGL3-1111
-808
pGL3-808
-509
pGL3-509
-209
pGL3-209
-21
pGL3-21

Fig. 5B


Fold Activation
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40
35
30
25
20
15
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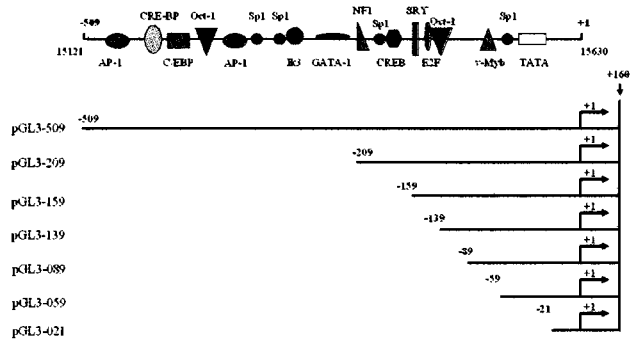
5
0
pGL3-1739
pGL3-1111
pGL3-808
pGL3-509
pGL3-209
pGL3-21

Fig. 6A


-509
CRE-BP
Oct-1
Sp1
Sp1
NF1
Sp1
SRY
Oct-1
Sp1
+1
15121
AP-1
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AP-1
IK3
GATA-1
CREB
E2F
v-Myb
TATA
15630
+160
pGL3-509
pGL3-209
pGL3-159
pGL3-139
pGL3-089
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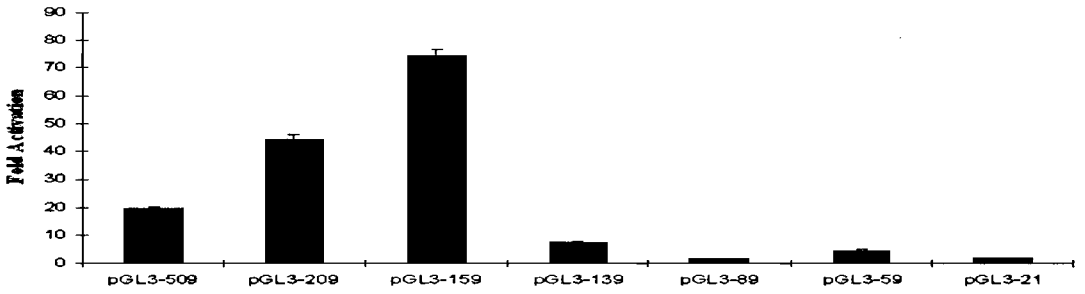
pGL3-021
-509
-209
-159
-139
-89
-59
-21
+1

Fig. 6B


Fold Activation
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50
40
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20
10
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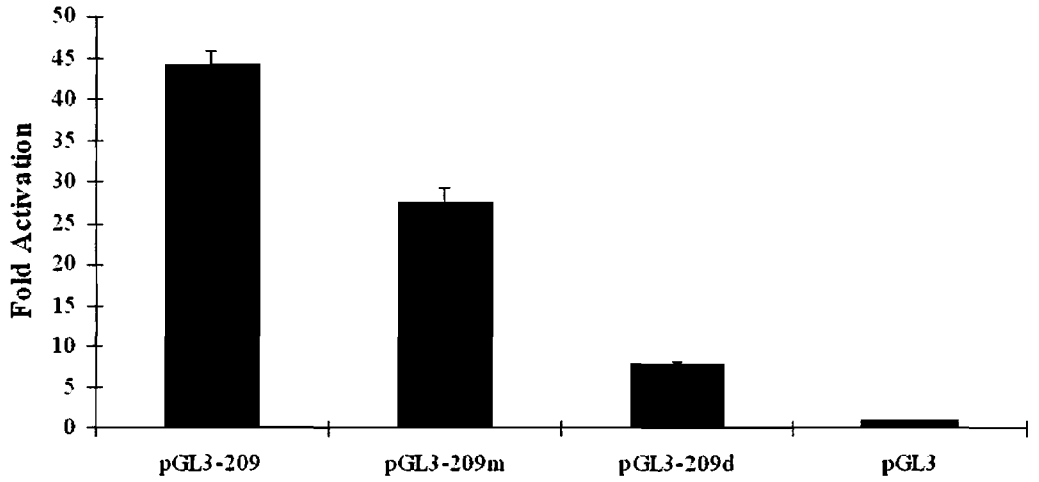
pGL3-89
pGL3-59
pGL3-21

Fig. 7


Fold Activation
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45
40
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10
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pGL3-209m
pGL3-209d
pGL3

Fig. 8A

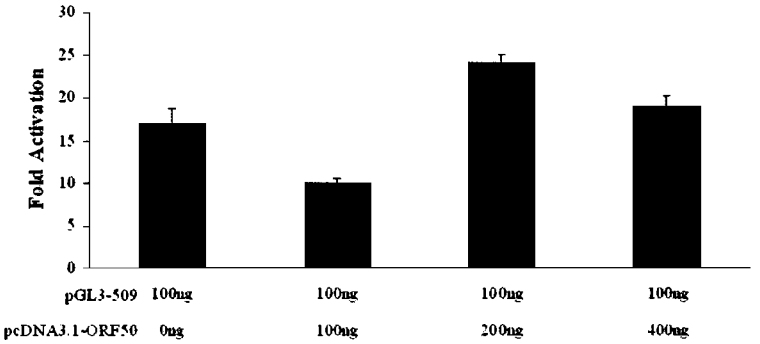
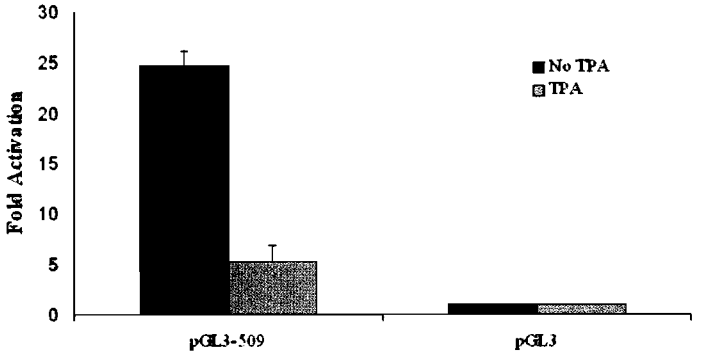


Fig. 8B



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