Journal of Sciences, Islamic Republic of Iran 15(1): 21-26 (2004)
University of Tehran, ISSN 1016-1104

Varicella Zoster Virus (VZV) Origin-Dependent Plasmid Replication in the Presence of the Four Overlapping Cosmids Comprising the Complete Genome of VZV

M.M. Yaghoobi,[1] M. Sadeghi-Zadeh,[1,*] H. Naderi-Manesh,[2] and M.R. Noori-Daloii[3]

[1] *Department of Genetics, School of Basic Sciences, Tarbiat Modarres University, P.O. Box 14115-175, Tehran, Islamic Republic of Iran*
[2] *Department of Biophysics, School of Basic Sciences, Tarbiat Modarres University, P.O. Box 14115-175, Tehran, Islamic Republic of Iran*
[3] *Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran*

Abstract

The Varicella-Zoster Virus (VZV) genome contains both *cis*-acting and *trans*-acting elements, which are important in viral DNA replication. The *cis*-acting elements consist of two copies of o*ri*S, and the *trans*-acting elements are those genes whose products are required for virus DNA replication. It has been shown that each of the seven genes required for *ori*-dependent DNA synthesis of Herpes Simplex Virus type-I (HSV) has a homologue in the VZV genome. However these seven genes of VZV never supported replication of the plasmid containing VZV *ori*S, in the same assay. On the other hand, several laboratories have shown that co-transfected cosmid DNAs containing the overlapping VZV fragments can supply all of the necessary functions for the VZV virion development. In the present study we found that the four overlapping cosmids are as efficient as virion in supporting the *in vitro* replication of co-transfected plasmids containing o*ri*S. On the basis of these results, subcloning of each of the cosmids used in this system should allow one to identify all of the VZV genes required for DNA replication in cultured cells.

Keywords: VZV; HSV; Cosmid; o*ri*-Dependent replication

Introduction

Varicella-Zoster virus (VZV) is one of the human herpesviruses belonging to the alphaherpesvirinae subfamily of the herpesviridae [12]. VZV is a neurotropic herpes virus which causes chickenpox after primary infection and shingles upon reactivation of latent virus from dorsal root ganglia [1]. VZV has a

* *E-mail: sadeghma@modares.ac.ir*

linear double stranded DNA and its entire 125884-bp genome containing at least 71 open reading frames (ORF) had been sequenced [6]. (A new ORF, named ORF S/L has been reported recently) [9]. There is one origin of DNA replication (*ori*S) in VZV genome, located in the IRS/TRS region between ORFs 62 and 63 [15]. VZV replicates its DNA in the middle stage of infection and all proteins needed for DNA replication are encoded by its genome, so host cell proteins have no essential role in this phase [12]. Herpes viruses which have large linear DNA genome, are useful model systems for studying eukaryotic DNA replication and Herpes Simplex type-1 (HSV-1) is a well known herpesvirus for which the function of many gene products has been elucidated. Among them, the seven genes of HSV-1 required for its DNA replication have been isolated via the method described by Challberg [3,16]. Direct genetic studies of VZV, however, have lagged behind that of HSV-1 largely, because of the inability to produce high-titre cell-free virus particles. Moreover the VZV DNA has no infectivity and can not produce CPE (cytopathic effects) on the host cells [4]. Therefore, in spite of the fact that HSV-1 and VZV have similar genomic organizations, and most of the HSV-1 genes have homologues in the VZV genome, it is not certain which genes of VZV are needed for its DNA replication [5]. Although plasmids containing *ori*S of VZV can replicate in VZV infected cells, VZV DNA can not support replication of these plasmids and there is no report of *ori*-dependent replication in the presence of VZV isolated genes.

A powerful system circumventing the problem of obtaining cell-free virus has been developed in recent years. In this system transfection of cells with the four overlapping cosmid DNAs, comprising the entire VZV genome, results in infectious VZV particles and CPE are formed on the monolayer of cells. Therefore there is no requirement in obtaining VZV virion DNA for transfection [4]. To determine whether the four cosmids can provide all essential elements for *ori*-dependent replication, we used them in a manner similar to Challberg's method. In this study we assayed the ability of the four cosmids to support amplification of the plasmid containing VZV origin of DNA replication (*ori*S), in human cells, *in vitro*. As these cosmids can produce infectious VZV particles upon transfection, we expect them to support origin dependent plasmid amplification in appropriate cells. As the results showed, the plasmid has been replicated, and this will be a powerful system for isolation of VZV genes needed for *in vitro ori*-dependent replication just as it has been done for HSV-1.

Materials and Methods

Cells and Viruses

Human melanoma (Mewo) and human kidney fibroblast (293) cells were purchased from the American Type Culture Collection (ATCC) and were propagated in Dulbecco's minimal essential medium (DMEM, GIBCO BRL), supplemented with 5% fetal calf serum (GIBCO BRL). The cultures were incubated at 37°C in humidified incubator with 5% CO_2. The KOS strain of HSV-1 and Scott strain of VZV were used for positive control of plasmid replication.

Cosmids and Plasmids

VZV cosmids pVFsp4, pVSpe5, pVPme19 and pVSpe21 containing the entire genome of the Oka strain of VZV inserted into AscI site of cosmid superCos-1, were kindly provided by Dr. John Hay from New York State University [9], (Fig. 1).

Plasmid pEcoRI-E contains VZV origin of DNA replication (*ori*S). If this plasmid is digested with EcoRI enzyme, a 6 Kb fragment is produced encompassing pBR322 vector, which can be hybridized with pUC18 probe (Fig. 2). pBamHI-J is another plasmid containing VZV origin of DNA replication that upon digestion with EcoRI, release a 9 Kb fragment that can be detected by hybridization with pUC 18 [3].

Plasmid pMS62 encodes the VZV ORF62 protein, which is a potent transactivator of putative VZV genes, and enhances the infectivity of VZV cosmid DNAs [10,11]. The Challberg's system plasmids that contain the seven genes required for *in vitro ori*-dependent replication of HSV-1 are: pMC160, pCW15, pNN5, pNN2, pNN1, pK1-2 and pCW8 encoding *ori*-binding protein, polymerase accessory, primase, polymerase, DNA binding protein, transactivator and helicase respectively. Plasmid pSG25 contains HSV-1 origin of DNA replication (*ori*S), and like pEcoRI-E releases a 6 kb fragment after digestion with EcoRI enzyme. All these plasmids were kindly provided by Dr. M.D. Challberg [3,16].

Plasmid Amplification

The DH5α strain of *E. coli* was transformed by Hanahan's method [8] and bacterial plasmids isolated by Birnboim and Doly's method [2]. Plasmid structure was analyzed by digestion with different restriction enzymes including EcoRI, BamHI and Hind III. Large scale plasmid extraction (Maxi. Prep.) was carried out by affinity chromatography column (Qiagen).

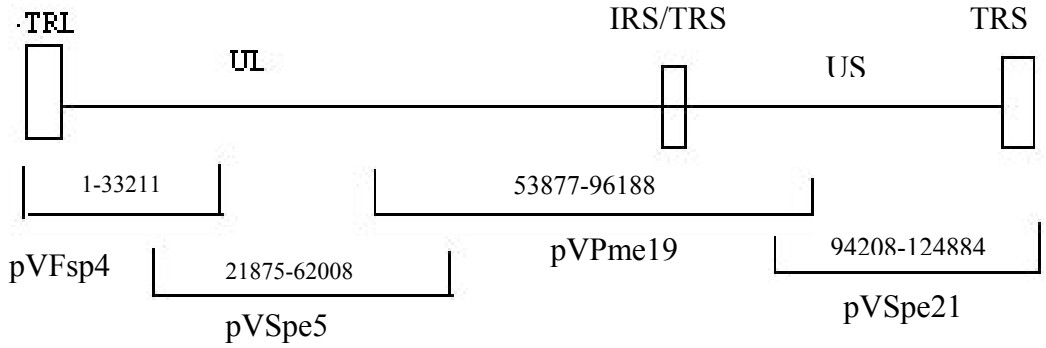

Figure 1. The schematic representation of the linear VZV genome and the four overlapping cosmids. The region of genome that each cosmid carry is indicated within.

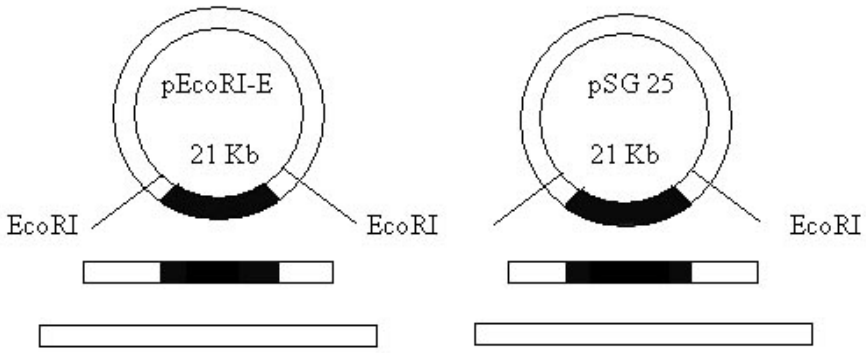

Figure 2. Structure of plasmids containing *ori*S of VZV (pEcoRI-E) and *ori*S of HSV-1 (pSG25), which upon digestion with EcoRI, release a 6 Kb fragment that can be hybridized with pUC18 probe. The filled boxes correspond to regions with homology to pUC 18.

Transfection

To verify whether the four cosmids, can produce CPE and plaque in human Cells, cosmids pVFsp4, pVSpe5, pVPme19 and pVSpe21 (each at 1 µg) and 1 µg of plasmid pMS62 were used to transfect 60 mm dishes containing Mewo cells using the calcium phosphate procedure [7]. Four days after transfection, the cells were seeded into 75-cm^2 flasks and monitored for CPE and plaque formation.

Analysis of Plasmid Replication

293 cells were plated in 60-mm dishes and incubated until they were nearly confluent. At this time the cells were transfected using the calcium phosphate technique. After 48 h, total DNA was extracted using the procedure described previously by Challberg [3] A quarter of the DNA obtained from a dish was digested with EcoRI and a quarter was digested with EcoRI+DpnI. The DNA was fractionated by 1% agarose gel electrophoresis and was transferred onto nitrocellulose by Southern's blot technique [14]. One microgram of linearized α^{32}P labelled pUC18 DNA was used as probe and after hybridization, DNA fragments were located by autoradiography for 5 to 24 h using Kodak XAR film and intensifying screen as described [13].

Results

All of the *in vitro* VZV origin-dependent DNA replication functions can be supplied by the four overlapping cosmids.

Digestion with DpnI enzyme serves as the basis for an assay of plasmid DNA replication. This unusual enzyme cuts DNA at the sequence G-^{m}A-T-C and it requires the methylation of the adenine moieties in both strands of DNA for cleavage to occur. The *dam* methylation system of *E. coli* is responsible for this methylation, thus pEcoRI-E (or pBamH-I-J) and pSG25

that were amplified in *dam*$^+$ strain of *E. coli* were cleaved into several fragments with DpnI, but plasmids replicated in mammalian cells are resistant to DpnI and could not be cleaved. (mammalian cells do not have the *dam* system). The digested DNA was fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with pUC18 for the presence of DpnI-resistant plasmids. If plasmid pEcoRI-E (or pSG25) is digested with EcoRI, a 6 Kb fragment is released that hybridizes with pUC18 probe. Thus if these plasmids are replicated, a band of 6 Kb size, resistant to DpnI, must be observed in Lane of DNA that has been digested with EcoRI+DpnI, whereas a band of 9 kb size will be observed upon replication of plasmid pBamHI-J.

HSV-1 replication was considered as the positive control, at first, (Fig. 3), and digestion of DNA extracted from pSG25 transfected cells, by EcoRI and EcoRI+DpnI showed that pSG25 replication can not occur alone (Lane 3, Fig. 3). However pSG25 replication may occur if transfected cells superinfected by HSV-1 virion after 4 h (Lane 5, Fig. 3). pSG25 replication was also investigated in the presence of Challberg's plasmids pMC160, pCW15, pNN5, pNN2, pNN1, pK1-2, and pCW8 (Lane 7) or linear HSV-1 DNA (Lane 9), the results clearly showed that pSG25 is replicated in the presence of both plasmids in Challberg system and HSV-1 DNA.

Replication of pBamHI-J was then tested in the presence of VZV virion, VZV DNA and VZV plasmids. No band was observed in 9 kb size region of Lane 3, (Fig. 4) which indicates that the plasmid pBamHI-J can not replicate in the absence of viral enzymes (negative control). To determine if the plasmid pBamHI-J is active and can be replicated in the presence of viral enzymes, 293 cells were transfected with plasmid pBamHI-J. Then after 4 hrs transfected cells were infected with cells containing VZV virion for 24 h. Afterward extracted DNA was digested by EcoRI and EcoRI+DpnI, and a band with approximate size of 9 kb was observed in Lane 5, (Fig. 4) indicating that the plasmid replicated in the presence of VZV virion. Replication of pBamHI-J in the presence of linear VZV DNA was also examined. Results in Lane 7 showed that linear VZV DNA can not support replication of pBamHI-J. HSV-1 has seven genes required for its *in vitro* DNA replication and VZV has homologous to these genes in its genome. These ORFs are 6, 16, 28, 29, 51, 52, 55 and encode primase, polymerase accessory, polymerase, DNA binding protein, *ori*-binding protein, helicase-primase accessory, and helicase, respectively. The ORF 6 has been cloned in plasmid pKpnC, the ORF 16 in plasmid pKpnA, the ORFs 28 and 29 together in pBamEZ, and the ORFs 51, 52 and 55 have been cloned together in plasmid pKpnB [6]. The replication of pBamHI-J in the presence of these seven genes of VZV was studied. The results showed no replication of pBamHI-J (Lane 9, Fig. 4). Thus HSV-1 virion, HSV-1 DNA and HSV-1 plasmids, support HSV-1 *ori*S replication, but VZV *ori*S can replicate only in the presence of VZV virion.

Then VZV DNA cosmids were used in *ori*-dependent system after CPE and plaque formation tests (data not shown). The replication of pEcoRI-E in the presence of the four overlapping cosmids containing VZV genome: pVFsp4, pVspe5, pVpme19 and pVSpe21 was assayed by transfecting 293 cells with the four cosmids and plasmid pEcoRI-E plus plasmid pMS62 containing IE62 gene (IE62 is an Immediate Early gene of VZV and acts as a transactivator to turn on early genes, homologous to ICP4 gene of HSV-1). The results showed pEcoRI-E can replicate in the presence of the four cosmids in 293 cells (Fig. 5).

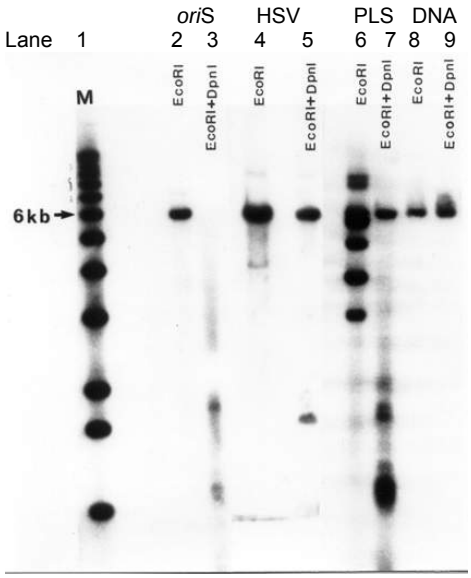

Figure 3. HSV-1 Origin-dependent plasmid replication supported by HSV-1 virion, HSV-1 DNA and Challberg's system plasmids. From Left side to right: DNA size marker (Lane 1). 293 cells were transfected with 2 µg of pSG25 alone and DNA from the transfected cells was digested with EcoRI (Lane 2) and EcoRI+DpnI (Lane 3) (Negative control). Then these cells were transfected with 2 µg of pSG25 alone but 4 h later infected with 0.4-1 PFU/ml of HSV-1. DNA from the cells was digested with EcoRI (Lane 4) and EcoRI+DpnI (Lane 5) (Positive control). Lanes 6 and 7: 293 cells were transfected with 1 µg each of pMC160, pCW15, pNN5, pNN2, pNN1, pK1-2, pCW8 and pSG25. Lane 6: EcoRI digested DNA, Lane 7: EcoRI+DpnI digested DNA. Lanes 8 and 9: 293 cells were transfected with 2 µg of pSG25 and 5 µg of linear HSV-1 DNA. Lane 8: EcoRI digested DNA, Lane 9: EcoRI+DpnI digested DNA. (PLS=Plasmids)

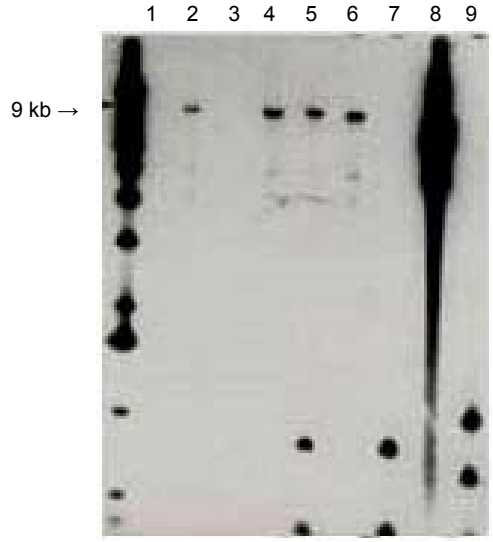

Figure 4. VZV origin-dependent plasmid replication results. From left side to right: DNA size marker (Lane 1), 293 cells were transfected with 2 µg of pBamHI-J alone and DNA from the transfected cells was digested with EcoRI (Lane2) and EcoRI + DpnI (Lane 3) (Negative Control). Then the cells were transfected with 2µg of pBamHI-J and superinfected with VZV virion, the DNA from the transfected cells was digested with EcoRI (Lane 4) and EcoRI + DpnI (Lane 5). Lanes 6 and 7: 293 cells were transfected with 2 µg of pBamHI-J and 5 µg of VZV DNA . Lane 6: EcoRI-Digested DNA, Lane 7: EcoRI+DpnI digested DNA. Lanes 8 and 9: 293 cells transfected with 2 µg of pBamHI-J plus the seven plasmids containing ORFs 6, 16, 28, 29, 51, 52 and 55 of VZV (each at 2 µg). Lane 8: EcoRI-digested DNA, lane 9: EcoRI+DpnI digested DNA.

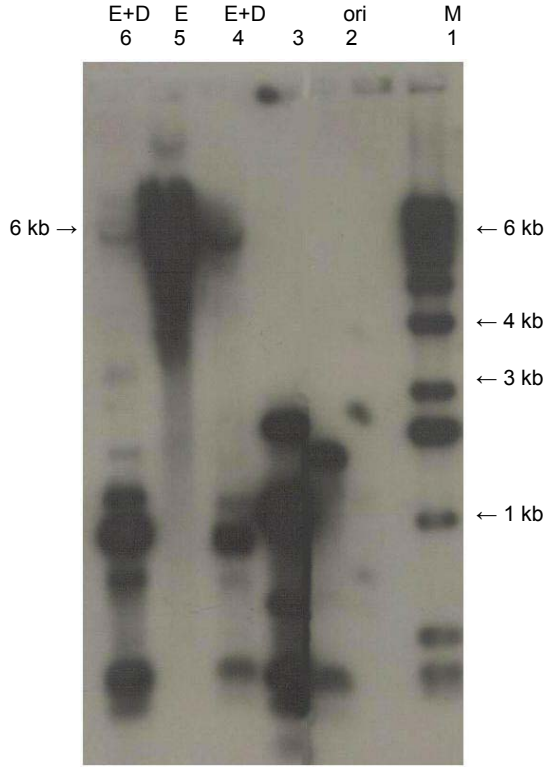

Figure 5. VZV origin-dependent plasmid replication supported by the four overlapping cosmids comprising VZV genome. From right side to left: DNA size marker (Lane 1). 293 cells were transfected with the pEcoRI-E alone and the DNA digested with DpnI, (Lane 2). 293 cells were transfected with the Challberg's system plasmids except pNN2, as negative control (Lane 3). 293 cells were transfected with the four cosmids pVFsp4, pVSpe5, pVPme19, and pVSpe21 (each at 2 µg) and plasmids pEcoRI-E+pMS62 (each at 3 µg). DNA from the transfected cells was digested with EcoRI+DpnI (Lanes 4 and 6) and EcoRI (Lane 5).

Discussion

The molecular biology of Varicella-Zoster Virus (VZV) has received little attention compared to other members of herpesviruses, particularly Herpes Simplex Virus type 1 (HSV-1), because of the difficulty in obtaining cell-free VZV virion. Therefore, the genetic and biochemical studies of this virus, has been considerably hindered, and as mentioned earlier, it is not known which genes of the VZV are essential for its DNA replication. To investigate viral genes involved in its DNA replication, we turned towards an *in vitro* replication system described previously [3]. In this system, host cells are co-transfected by cloned viral genes and a viral origin of DNA replication (*ori*), thereby circumventing the requirement for synchronous infection with cell-free virion. Since the complete nucleotide sequence of the HSV-1 and VZV genome are available, and the HSV-1 genes required for *in vitro ori*-dependent replication are known, it appears that a similar system could be developed for VZV, thus permitting identification of genes involved in the replication of this virus.

The initial experiment described in the first section of results indicated, however, that obtaining a functional replication system for VZV would be more problematic than HSV-1, because co-transfection with intact VZV DNA (which must carry the complete set of viral "replication genes") failed to support detectable replication from the VZV *ori*S (Fig. 4, Lane 7). On the other hand, although VZV origin-dependent plasmid replication has been functional in VZV infected cells, there has been no positive result of origin-dependent replication using isolated VZV replication genes (Fig. 4 Lane 9). Attention was therefore turned toward the issue of whether transcriptional activation was effective in the VZV system. So the open reading frames of the known immediate early (IE) transcriptional activator of VZV

(ORFs 4, 61, 62 and 63) were separated from their cognate promoter and cloned downstream of the strong IE promoter of human cytomegalovirus(HCMV). In spite of the fact that these proteins could be functionally competent in the control of transcription from promoters of the immediate early, early and late classes of VZV genes, addition of the recombinant plasmids to the VZV *in vitro* system, did not provide detectable level of replication. (Sadeghi-Zadeh M., unpublished data). Taking an alternative approach, the HCMV IE promoter was then used to drive the expression of VZV genes homologous to those seven genes of HSV-1 found necessary to activate *ori*-dependent replication. The outcome was again negative and there was no detectable stimulation of VZV *ori* replication (Sadeghi-Zadeh M., unpublished data).

Since 1993, several laboratories generated VZV virion from four cosmids that contain overlapping fragments of the complete VZV genome, derived from the Oka strain (V-Oka) [4]. The initial objective was to induce site-directed mutagenesis in special ORFs to determine whether they are required for replication *in vitro*. Because of the cell-associated nature of VZV, the cosmid mutagenesis approach is a helpful tool for determining VZV ORFs that are dispensable for replication. It has been shown that about 17 ORFs are dispensable for VZV replication in cell culture so far (Marvin *et al*., 2001).

In our system the four overlapping cosmids were used to show that a collection of cloned restriction fragments of VZV DNA can provide all of the functions required for transient replication of plasmid containing VZV origin of DNA replication. It seems clear in Fig. 5 (Lanes 4 & 6) that the four cosmids can support replication of this plasmid as efficient as HSV-1 *ori*-dependant plasmid replication (Fig. 3). Thus the seven (or more) genes of VZV which are homologous to known seven genes of HSV-1 involved in its DNA replication reside in an active state in these cosmids. Hence further analysis with this system could show whether there are any more VZV replication genes that are specific for its origin. Finally, it appears that VZV DNA replication is a complex process involving the interaction of several different components. The work reported in this paper showed that a transient assay system involving co-transfection of plasmids generated by subcloning of the four cosmids can be used to study this multi component system more directly than mutational analysis of VZV genes with cosmid vectors.

Acknowledgements

We thank Dr John Hay for providing the cosmids and Dr Peter Sheldrick for his scientific help and for comments that improved the quality of this report. This work was funded by the Vice-chancellor for Research at Tarbiat Modarres University.

References

1. Arvin A.M. Varicella-zoster virus. *Clini. Microbio. Rev.*, **9**: 361-381 (1996).
2. Birnboin H.C. A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.*, **100**: 243-255 (1983).
3. Challberg M.D. A method for identifying the viral genes required for herpes-virus DNA replication. *Proc. Natl. Acad. Sci. USA.*, **83**: 9094-9098 (1986).
4. Cohen J.I and Seidel K.E. Generation of varicella-zoster viras (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication *in vitro*. *Ibid.*, **90**: 7376-7380 (1993).
5. Davison A.J. and Mc Goach D.J. Evolutionary comparisons of the S segments in the genome of herpes simplex virus type 1 and varicella-zoster virus**.** *J. Gen. Virol.*, **67**: 597-611 (1986).
6. Davison A.J. and Scott J.E. The complete DNA sequence of varicella-zoster virus. *Ibid.*, **67**: 1759-1816 (1986).
7. Graham F.L, Van der Eb A.J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, **53**: 456-461 (1973).
8. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**: 557-580 (1983).
9. Kemble G.W., Annunziato P., Lungu O., Winter R.E., Cha T., Verstein S.J., and Spaete R.R. Open reading frame S/L of varicella-zoster virus encodes a cytoplasmic protein expressed in infected cells. *J. of Virol.*, **74**(23): 11311-11321 (2000).
10. Sommer M.H., Zagha E., Serrano O.K., Ku Ch.Ch., Zerboni L., Baiker A., Santos R., Spengler M., Lynch J., Grose Ch., Ruyechan W., Hay J., and Arvin A.M. Mutational analysis of the repeated open reading frames, ORFs 63 and 70 and ORFs 64 and 69, of varicella-zoster virus**.** *J. of Virol.*, **75**(17): 8224-8239 (2001).
11. Perera L.P., Mosca J.D., Sadeghi-zadeh M., Ruyechan W.T., and Hay J. The varicella-zoster virus immediate early protein, IE 62, can positively regulate its cognate promoter. *Virology*, **191**: 346-354 (1992).
12. Roizman B., Desrosiers R.C., Fleckenstein B., Lopez C., Minson A.C., Staddert M.J. The family Herpesviridae: an update. *Arch. Virol.*, **123**: 425-449 (1992).
13. Sambrook J. and Russell D.W. *Molecalar Cloning: A Laboratoty Manual*. 3rd Ed. CSH Laboratory Press, Cold Spring Harbor, New York (2001).
14. Southern E.M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**: 503-517 (1975).
15. Stow N.D. and Davison A.J. Identification of a varicella-zoster virus origin of DNA replication and its activation by herpes simplex virus type 1 gene products. *J. Gen. Virol.*,: **67**: 1613-1623 (1986).
16. Wu C.A., Nelson N.J., McGeoch D.J., and Challberg M.D. Idenfification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *J. Virol.*, **62**: 435-443 (1988).