Université de Montréal

# Human Herpes Virus-6 Induced Changes in the Expression and Activity of the E2F Family Transcription Factors in Human Cells

Par

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# RÉSUMÉ

Les facteurs de transcription de la famille E2F jouent un rôle important dans la prolifération, l'apoptose et la différentiation cellulaire. Il a été démontré que l'activité de ces facteurs de transcription peut être affectée par plusieurs virus. Ils provoquent entre autre la dérégulation du cycle cellulaire. Par conséquent, il n'est pas étonnant que les virus affectent l'expression et l'activité de E2F dans les cellules infectées. Le VHH-6 (Virus Humain d'Herpès -6) est un microbe pathogène humain omniprésent. Il est un de ces virus qui est connu pour ses propriétés à moduler négativement la progression du cycle cellulaire. Cependant, rien n'est connu au sujet des effets du VHH-6 sur l'expression et les activités fonctionnelles de E2F dans les cellules humaines. Dans le présent projet, nous avons étudié ce problème. À cette fin, nous avons infecté une lignée cellulaire de cellules T de leucémie humaine (HSB-2) *in vitro* avec le VHH-6 (*GS strain*). HSB-2 est la seule lignée de cellules T humaines qui est infecté effectivement par ce virus. En employant les techniques *«Electormobility Shift Assay» (EMSA)* et *«Western Blots»*, nous avons comparé les expressions et les liaisons de E2F avec l'ADN entre les cellules HSB-2 infectées et mock-infectées avec le virus. Les analyses «Western blots» ont montré plusieurs changements qualitatifs et quantitatifs de l'expression de ces facteurs dans les fractions cytoplasmiques et nucléaires des cellules infectées. La plupart de ces changements dans les expressions étaient du à la phosphorylation différentielle des facteurs. Nous avons fait EMSA en utilisant les extraits nucléaires et des oligonucléotides à double brin contenant les séquences consensus de E2F. Dans ces analyses, les protéines des cellules mock-infectées ont donné au moins 3 bandes distinctes dans le gel shift, tandis qu'on a observé deux bandes avec les cellules infectées par le virus. Dans l'EMSA, avec les extraits des cellules infectées, la bande à faible mobilité n'a pas été détectée. L'E2F-oligonucléotide complexe était plus fort dans EMSA avec les cellules infectées qu'avec les cellules mock-infectées. Le « supershift assay » montre que les anticorps contre les facteurs E2F1-6, DP1 et DP2 retardent la migration des complexes préparés à partir des cellules mock-infectées. Dans les cellules infectées ce « supershift » n'a pas été observé avec les anticorps contre les facteurs E2F2, E2F5, E2F6 et DP-1, alors qu'il est réduit avec les anticorps contre les E2F-1, E2F-3 et E2F-4. Nos résultats montrent également que dans les cellules mock-infectées seuls les anticorps contre les protéines p107 et p130 supershift le complexe et non pas le anticorps contre les pRB. Par contre, dans les cellules infectées par le VHH-6 aucun supershift n'était observé avec ces anticorps. Nous concluons par conséquent: Le VHH-6 réduit ou inhibe les interactions des membres d'E2F avec leurs séquences d'ADN spécifique dans les cellules humaines infectées. Les effets viraux sur l'E2F peuvent être importants pour la réplication virale.

**Mots Clés :** cycle cellulaire, DP-1, DP-2, E2F, facteur de transcription, HHV-6.

# SUMMARY

The E2F family of transcription factors plays an important role in cell proliferation, apoptosis and differentiation. The viruses use host cell's transcriptional and translational machinery for their own replication and survival. They deregulate cell cycle by inducing proliferation, quiescence or cell cycle arrest. Therefore, it is not surprising that viruses affect E2F expression and activity in the infected cells. Human Herpes Virus (HHV-6) is a ubiquitously occurring human pathogen. It is one of the viruses, which are known to negatively modulate cell cycle progression in human cells. However, nothing is known about the effects of HHV-6 infection on the expression and functional activities of E2F in human cells. We addressed this issue in this study. For this purpose, we infected a human T cell leukemia cell line HSB-2 *in vitro* with the GS isolate of HHV-6. It is noteworthy that HSB-2 is the only human T cell line that can be productively infected with the GS isolate of the virus. By using Western blots and electromobility shift assays (EMSA), we compared the expression and DNA-binding activities of the E2F family members between the virus-infected and mock-infected HSB-2 cells. The Western blots showed several qualitative and quantitative changes in the expression of these factors in the cytoplasmic and nuclear fractions of the infected cells as compared to the mock-infected ones. Interestingly, most of these changes in the expression pattern were due to differential phosphorylation of the factors, since the treatment of the cell extracts with phosphatase resulted in similar bands between the infected and mock-infected cells. We performed EMSA using nuclear extracts of the cells and double-stranded oligonucleotides containing consensus E2F-specific sequences. In these assays, the mock-infected cells showed at least three distinct gel shift bands, whereas two bands were observed with the virus-infected cells. In the EMSA with the infected cell extracts, the low mobility band was not present. The oligonucleotide-protein complexes were more prominent in EMSA with the infected cells than with the mock-infected cells. The supershift assays showed that antibodies specific for E2F 1-6, DP-1 and DP-2 caused supershifts for the mock-infected cells. The supershifts were, however, not seen for anti-E2F-2, -E2F-5, -E2F-6 and -DP-1 antibodies for the virus-infected cells. Furthermore the supershift by E2F-1, E2F-3 and E2F-4 and DP-2 antibodies were reduced for the infected cells as compared to the mock-infected ones. The p107- and p130-, but not the pRB-specific antibodies, supershifted complexes in the mock infected but not in the virus-infected ells. We conclude that: HHV-6 reduces or abrogates the interactions of E2F members with their cognate DNA sequences in the infected human cells. The viral effects on the E2F may be important for viral replication.

**Key Words:** Cell cycle, DP-1, DP-2, E2F, HHV-6, Transcription factor.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AIDS | Acquired Immune Deficiency Syndrome |
| AP Buffer | Alkaline phosphatase Buffer |
| APAF1 | Apoptotic protease activating factor-1 |
| ATCC | American Type Culture Collection |
| ATM | Ataxia telangiectasia mutated |
| ATR | ATM and rad3 related |
| BCIP | 5 bromo-4 chloro-3 indolyl phosphate |
| bHLH | Basic helix-loop-helix |
| CIAP | Calf intestinal phosphatase |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| CSF | Cerebrospinal fluid |
| DHFR | Dihydrofolate reductase |
| DR | Direct repeats |
| Ds DNA | Double stranded DNA |
| DTT | Dithiothreitol |
| EBV | Epstein-Barr virus |
| EDTA | Ethylenediamine tetra acetic acid |
| EMSA | Electrophoretic mobility shift assay |
| FBS | Fetal bovine serum |
| HBLV | Human B-lymphotropic virus |
| HCMV | Human cytomegalovirus |
| HDAC | Histone de-acetylase |
| HHV-6 | Human Herpes Virus-6 |
| HIV-1 | Human immunodeficiency virus type 1 |
| HPV | Human papillomavirus |
| HSB-2 | Human T cell leukemia cell line-2 |
| HSPG | Heparin sulphate proteoglycan |
| HSV-1 | Herpes simplex virus type 1 |
| IFN | Interferons |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IR | Inverted repeats |
| J JHAN | Human T cell line |
| KCl | Potassium chloride |
| kDa | Kilo Dalton |
| KSHV | Kaposi's sarcoma herpes virus |
| MAb | Monoclonal antibody |
| MDM2 | Mouse double minute 2 |
| Moi | Multiplicity of infection |
| MOLT-3 | Human T cell leukemia cell line |
| mRNA | Messenger ribonucleic acid |
| MS | Multiple sclerosis |

| | |
|---|---|
| NaF | Sodium fluoride |
| NBT | Nitro blue tetrazolium |
| NF-$\kappa$B | Nuclear factor $\kappa$B |
| NK | Natural killer |
| OBP | Origin binding protein |
| ORF | Open reading frame |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PCV | Packed cell volumes |
| Pi | Post-infection |
| PVD | Poly vinyl dichloride |
| RANTES | Regulated upon activation, normal T cell expressed and secreted |
| SIDA | Syndrome immunodéficitaire acquis |
| SupT-1 | Human lymphoma T cell |
| TBE | Tris borate EDTA |
| TCID | Tissue culture infection dose |
| TFDP-1 | Transcription factor DP-1 |
| TGF-$\beta$ | Transforming growth factor-$\beta$ |
| TNF | Tumor necrosis factor |
| UL | Unique long region |
| US | Unique short region |
| UTR | Untranslated region |
| VIH-1 | Virus de l'immunodéficience humaine |
| VZV | Varicella Zoster Virus |
| Wt | Wild type |

**DEDICATED**


*To*


My beloved father who is no more with me to see this accomplishment

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The errors and omissions that remain, I must acknowledge, are my own.

# INTRODUCTION AND REVIEW

# OF LITERATURE

# CHAPTER I

# INTRODUCTION AND REVIEW OF LITERATURE

The present study was conducted to investigate the effects of the Human Herpes Virus-6 (HHV-6) infection on the expression and functional activity of E2F factors in human T cells. Therefore, the pertinent literature on the E2F factors and the virus is reviewed below:

## 1. THE HERPES VIRUSES

The name herpes is derived from the Greek word "herpin" meaning to crawl, to climb or to slip. The herpes viruses have been given this name because they crawl to latent and chronic infections. As of today, more that one hundred herpes viruses have been isolated. They are widely distributed in nature (Ablashi et al, 1991). The history of the discovery of the herpes viruses goes back to the end of the Second World War when the first herpes virus, Varicella Zoster Virus (VZV), was described and found to be the causative agent of chicken pox or shingles. At present it is also called HHV-3. The most recently discovered herpes virus is the Kaposi's sarcoma herpes virus (KSHV) or HHV-8 discovered in 1994 (Chang et al, 1994). On the basis of physiology and morphology, the herpes viruses have been grouped in a single family named Herpesviridae (reviewed by Roizmann et al, 1992). They are all double-stranded DNA viruses with relatively large and complex genomes. They replicate in the cell's nucleus in a wide range of vertebrate hosts, including humans, horses, cattle, mice, pigs, chickens, turtles, lizards, fish, and even in some invertebrates, such as oysters. The viruses tend to have a restricted host range; only a few infect more than one species. At least one distinct herpes virus has been isolated from most of the animal species. Some species may be infected with many herpes viruses. For example,

eight distinct herpes viruses have been isolated from humans (Table 1). The main characteristics of the viruses in this family are: the development of latency in the infected cells, destruction of the infected cells upon viral replication (lytic cycle), and the replication of DNA and the assembly of the capsids in the nuclei of the infected cells. In the latent state, the viral genome becomes a closed circular molecule and only a few viral genes are expressed. The herpes virus genomes encode a variety of enzymes implicated in nucleic acid metabolism, DNA synthesis and protein translation. Based upon the arrangement of the terminal repeat sequences of >100 bp within their genomes, the herpes viruses have been classified into six (A-F) groups as shown in Table 2. Human herpes virus infections are endemic and sexual contact is a significant method of transmission for herpes simplex virus 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HHV-5) and likely for KSHV. HHV-6, however, is not spread by sexual contact (see below).

## 1.1 VIRION STRUCTURE

A typical herpes virus is composed of four structural elements as shown in Figure 1. They include:

- **Core.** The core consists of a single linear molecule of double stranded (ds) DNA in the form of a torus.

- **Capsid.** Surrounding the core is an icosahedral capsid with a 100 nm diameter constructed of 162 capsomeres.

- **Tegument.** Between the capsid and envelope, there is an amorphous, sometimes asymmetrical, feature named the tegument. It consists of viral enzymes, some of which are needed to modulate the cell's biochemical

**Table 1. Human Herpes viruses and the associated diseases**

| Common name | Scientific name | Diseases |
|---|---|---|
| Herpes simplex virus 1 (HSV-1) | Human herpes virus 1 (HHV-1) | Facial, labial and ocular lesions or "cold sores" |
| Herpes simplex virus 2 (HSV-2) | Human herpes virus 2 (HHV-2) | Genital lesions |
| Varicella-zoster virus (VZV) | Human herpes virus 3 (HHV-3) | Chickenpox and shingles |
| Epstein-Barr virus (EBV) | Human herpes virus 4 (HHV-4) | Glandular fever or infectious mononucleosis, Human cancers, e.g., Burkitt's lymphoma, gastric cancer, undifferentiated NPC, Hodgkin's disease. |
| Human cytomegalovirus (HCMV) | Human herpes virus 5 (HHV-5) | Infectious mononucleosis |
| (no common names) | Human herpes virus 6 (HHV-6) | Mild early childhood roseola infantum, MS, CFS, several lymphoproliferative disorders. |
| (no common names) | Human herpes virus 7 (HHV-7) | Rroseola infantum, pityriasis rosea, "Socks and gloves syndrome". |
| Kaposi's sarcoma herpes virus | Human herpes virus 8 (HHV-8) | Karposi's sarcoma, Castleman's multicentric disease. |

CSF, Cerebrospinal fluid; MS, Multiple sclerosis; NPC, Nasopharyngeal carcinoma

**Table 2: Classification of Herpes viruses in different groups**

| Group | Virus | Sub family | Characteristics |
|---|---|---|---|
| A | | β<br><br>α | A large sequence from one terminus is directly repeated in the other terminus |
| B | Saimiriine herpes virus 2<br><br>Bovine herpes virus 5 | γ<br><br>α | The terminal sequence is directly repeated numerous times at both termini |
| C | Epstein-Barr virus<br><br>Pongine herpes virus 1 | γ<br><br>γ | The number of direct terminal repeats is smaller, there may be other unrelated sequences greater than 100 bp that are directly repeated |
| D | Varicella-zoster virus<br><br>Saimiriine herpes virus 1<br><br>Ovine herpes virus 1 | α<br><br>α<br><br>α | One terminus sequences are repeated in an inverted orientation internally |
| E | Herpes simplex virus 1<br><br>Herpes simplex virus 2<br><br>Cytomegalovirus<br><br>Bovine herpes virus 2 | α<br><br>α<br><br>β<br><br>α | The sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two componets |
| F | Tupaia herpes virus | - | The sequences at the two termini are not identical and are not repeated directly |

The classification is based upon the arrangement of repeat sequences within the genomes of the viruses.

**Figure 1. Schematic representation of Human Herpes virus-6**
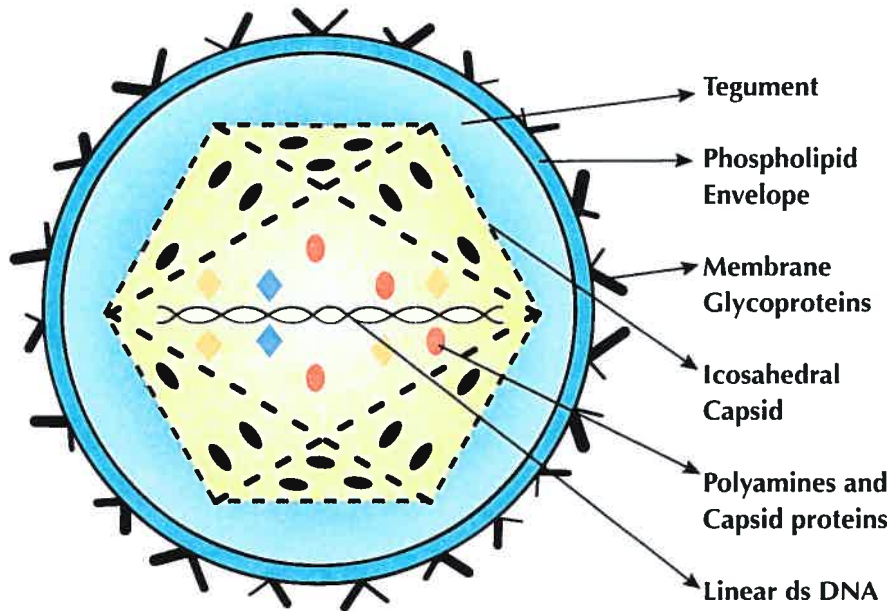


The spikes of membrane glycoproteins are projecting from the surface. Each viral particle contains a copy of the viral genome in the form of a single double-stranded DNA molecule. The viral components are not drawn to the scale.

processes and viral replication. Others are important to counter host cell's immediate responses.

- Envelope. The envelope is the outermost layer of the virion. It is derived from the patches of the altered cellular membranes of the infected cell into which almost a dozen unique viral glycoproteins have been inserted. The viral glycoproteins appear as short spikes embedded in the lipid bilayer of the envelope in electron micrographs. There may be more than 1000 copies of each glycoprotein on a single virion.

## 1.2 GENOME CHARACTERISTICS:

Herpes virus genomes range from 120 to 230 kbp in length with 31 to 75 % G+C content and contain 70 to 120 genes. Because replication takes place inside the nucleus, herpes viruses can use both the host's transcription machinery and DNA repair enzymes to support a large genome with a complex array of genes. Herpes virus genes, like the genes of their eukaryotic hosts, are not arranged in operons and in most cases have individual promoters. However, unlike eukaryotic genes, only a few herpes virus genes are spliced. The genes are characterized as either essential or non-essential for growth in cell culture. Essential genes regulate transcription and are needed for virus assembly. Non-essential or dispensable genes, for the most part, function to manipulate the cellular environment for virus production, to defend the virus from the host immune system and to promote cell-to-cell spread. The large number of dispensable genes is in reality required for a productive in vivo infection. Although the classification of Herpesviridae is based on the differences between the genomic sequences and viral proteins, they all share the same genomic organization. A typical herpes viral genome consists of a unique long region (UL) and a unique short region (US) connected by inverted repeats (IR) as shown in Figure 2. However, HHV-6 and

HHV-7 have only a UL flanked on the right and on the left by direct repeats, which are called DRR and DRL, respectively (Roizman and Pellete, 2001).

## 1.3 HERPESVIRIDAE SUBFAMILIES

As mentioned earlier, eight distinct herpes viruses have been isolated from humans. Based upon their tissue tropism and pathology, they are classified into following three subfamilies:

**1.3.1 Alphaherpesvirinae.** Members of this subfamily have a short reproductive cycle (~18 hr.) with efficient host cell destruction and a variable host range. They tend to become latent in sensory neurons. The subfamily includes HSV-1, -2 and VZV.

**1.3.2 Betaherpesvirinae.** Members are lymphotropic. They have a long reproductive cycle, and a restricted host range. The infected cells become enlarged (cytomegalo). Human Betaherpesvirinae include HCMV, HHV-6, and 7.

**1.3.3 Gammaherpesvirinae.** These herpes viruses are also lymphotropic; however, they are specific for either T or B-lymphocytes. They rarely infect a species other than human beings. Members of the subfamily isolated from humans are EBV and KSHV.

# 2. HUMAN HERPES VIRUS-6

Human herpes virus 6 (HHV-6) was discovered for the first time in 1986 from six patients suffering from lymphoproliferative disorders, two of whom were HIV-seropositive. The virus was initially named as human B-lymphotropic virus (HBLV) as it was isolated from peripheral blood mononuclear cells of patients with B- cell lymphoproliferative disorders (Salahuddin et al, 1986). It

**Figure 2. Genomic organization of Herpes viruses**

Class (Viruses)        Sequence arrangement

A and B          DR                                    DR

(HHV-6, 7)                        UL

C

( EBV)        TR     U2    IR2  U3   IR3     U4          DR     U5  TR

D                                $U_L$                          $U_s$

(VZV)                                                   IR        IR

E            TRL                $U_L$              IRL    IRS $U_S$ TRS

CMV

F

(THV)
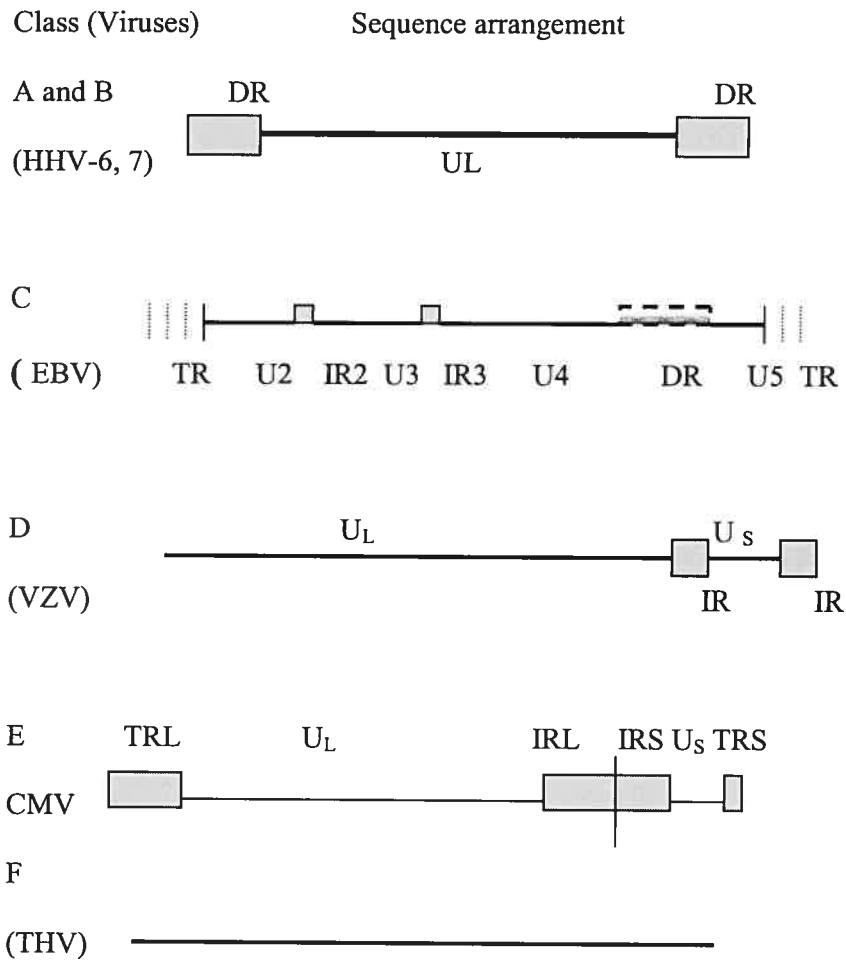
DR: Direct repeat; IR: Inverted repeat; $U_L$: Long unique region; $U_S$: Short unique region; TR: Terminal repeat; IRS: Short inverted repeat; IRL; Long inverted repeat; TRL: Long terminal repeat.

was classified as the sixth member of the herpes virus family and was placed in the gammaherpesvirinae subfamily in common with EBV. After it was realized that HHV-6 preferentially infected T lymphocytes rather than B-lymphocytes, it was reclassified in the betaherpesvirinae subfamily alongwith HCMV. The virus closely resembles HHV-7 and both cause roseola in children, therefore, both were classified in a new genus called Roseola. Some important biological and pathological features of HHV-6 are summarized in Table 3 and 4.

## 2.1 HHV-6 VARIANTS:

The first isolate of HHV-6 was termed as GS or AJ. It was isolated from patients from Gambia (Lopez et al., 1988). Several other viral isolates have been obtained from patients from different geographical regions of the world. They include U1102 (from Uganda), Z29 (from Zambia), and HST (from Japan) (reviewed by Krueger and Ablashi, 2003). Based upon their reactivity with anti-HHV-6 monoclonal antibodies, RFLP, and *in vitro* tissue tropism, these isolates have been classified into two variant groups: A and B (Ablashi et al; 1993). The group A variants are represented by GS and U1102, and B variants by Z29 and HST. The A variants replicate in HSB-2 and J JHAN cells and the B variants in MOLT-3 and MT-4. Both variants grow efficiently *in vitro* in IL-2 activated cord blood T cells.

## 2.2 MOLECULAR BIOLOGY:

Complete genome sequences of both A (U1102) and B (Z29, HST) variants of HHV-6 have been determined (Isegawa et al., 1999; Dominguez et al., 1999). The A and B

**Table 3. Important features of Human herpes virus-6**

| Variable | Description |
|---|---|
| Receptor | CD46 is an essential component of the receptor for HHV-6. |
| Variants | HHV-6 A and HHV-6 B variants differ in their *in vitro* cell tropism, reactivity with monoclonal antibodies, and restriction fragment length polymorphisms. The two groups exhibit ~9-12% overall divergence at the nucleotide level. |
| Tropism | HHV-6 is T lymphotropic. HHV-6 infects a broad range of host cells *in vitro* including primary T cells, monocytes, natural killer cells, dendritic cells, astrocytes, and various cell lines of T, B, megakaryocytic, glial, and epithelial origins. |
| Genome size | HHV-6 genome is double-stranded DNA, of ~160,000 bp in length. It has a central unique region of ~144,000 bp, flanked on each end by direct-repeat elements of variable length (~13,000 bp). |
| Herpesviridae family | Subfamily: *Betaherpesvirinae* (along with Cytomegalovirus and HHV-7); genus: *Roseolovirus* (with HHV-7) |
| Replication | In peripheral blood mononuclear cells, viral replication is slow and lytic; syncytia are induced. |
| Transmission | The virus is believed to be transmitted via oral secretions from adults to infants. In utero transmission has also been suggested. |

**Table 4: The pathogenesis and epidemiology of Human herpes virus-6**

| | |
|---|---|
| Paediatric infection | Infects most children at 6–24 months of age (somewhat earlier than HHV-7). |
| Primary infection (infants and children under three years of age) | HHV-6B infection usually manifests as a febrile illness, with or without a rash; presentation with a rash is usually diagnosed as roseola (exanthem subitum). HHV-6A can also cause roseola. |
| Oropharyngeal persistence | DNA can be found in salivary glands and in saliva of a high proportion of adults. |
| Neurological involvement (children and adults) | Primary HHV-6B infection in infants is associated with seizures, particularly in the 12–15-month age range, and with some cases of encephalitis. In adults, HHV-6 DNA has been detected in biopsy samples from some cases of focal encephalitis, and viral antigens have been detected in active CNS lesions from persons with multiple sclerosis. |
| Infection in immunocompromised adults | HHV-6A and HHV-6B can be pathogenic; the viruses can cause pneumonitis, bone marrow suppression and encephalitis. |
| Role in AIDS | Its role is uncertain, both in adults and in children. In vitro, HHV-6 can induce CD4 expression on some CD4⁻ cells; it can also up-regulate HIV-1 gene expression. |
| Other diseases | HHV-6 has been proposed to play a role in CFIDS, but evidence for this is presently equivocal. |
| Prevalence | Over 90% of all adults are infected with both HHV-6 and HHV-7. |
| Reactivation (Children) | Children who have previously been infected with HHV-6 can experience reactivation of HHV-6 in conjunction with primary HHV-7 infection; HHV-7 can also reactivate HHV-6 in vitro. |

AIDS : Acquired immunodeficiency syndrome; CFIDS : Chronic fatigue immunodeficiency syndrome; CNS : Central nervous system; HHV : Human herpes virus; HIV : Human Immunodeficiency virus.

variants are 88% identical at the nucleotide level. Their genomes are about 160-162 kb long. Each genome comprises a central unique (U) region of 143-144 kb length flanked by an 8-9 kb region of direct repeats (DR) on either end. The U region contains a hundred or more open reading frames (ORFs). The terminal and junctional regions of the DR contain human telomerase–like sequences of unknown function. The central region of the genome contains seven blocks of genes that are conserved in all herpes viruses, and a group of genes (U2-U19) to the left of the seven-block region found only in the beta herpes viruses (Figure 2). The genome also contains genes that are only found in HHV-6 and 7. They are located to the left and right of the core genes. HHV-6A (U1102 strain) has 110 ORFs whereas HHV-6B (Z29 and HST strains) contains 119 ORFs. Nine of the B variant (Z29) genes do not have their counterparts in the A variant (U1102) genome and the vice versa is also true. The IE genes occur in two blocks, IE-A (U86-89) and IE-B (U16-19). Their splicing pattern and temporal regulation may differ in two variant groups. The gene product of U89, IE1, of HHV-6B is phosphorylated on ser/thr residues, is sumoylated and localizes to nucleus along with promyelocytic leukemia (PML) proteins (Gravel et al., 2002). The equivalent protein in HHV-6A is 62% identical to it at amino acid level. Some U16/17.transcripts may appear late and act as late genes. U16 activates the LTR of HIV-1 (Flebbe-Rehwaldt et al., 2000; Lusso et al., 1989). The genes involved in DNA replication (E genes) include U27, U41, U43/73/77 (reviewed by Clark, 2000). U94 is one of the only two HHV-6 genes that are not found in HHV-7. It is an IE gene, which encodes a homologue of the human AAV-2 *rep* gene. It plays a role in DNA replication and gene regulation.

It inhibits viral replication and is also expressed during latency (Thompson et al, 1994). It has been reported to inhibit HIV-1 LTR and H-ras-mediated cell transformation (Aroujo et al., 1997; Rotola et al., 1998).

Of the late genes, U39 and U48 encode surface glycoproteins gB and gH, respectively. The HHV-6 gB has 39% sequence homology at amino acid level with HCMV gB, causing immunological cross reactivity between the two viruses. It plays a role in viral attachment and penetration and is conserved in all herpes viruses. In HHV-6A, gB is translated as a precursor molecule which is cleaved to give rise to functional sub units of 64 kd and 58 kD whereas HHV-6B gB forms about 102, 59 and 50 kD proteins (Takeda et al, 1996).

The product of the gene U82, gL, complexes with gH, and plays a role in its transport and processing. The gH-gL complex is involved in the infection and fusion process (Mukai et al, 1997). U72 encodes gM and U100 encodes gp82-100 complex due to differential splicing. U100 of the two HHV-6 variants have only 72% sequence identity, suggesting variant specific roles in infection. gB, gH and gp82-105 contain epitopes for virus neutralization. U11 encodes a phosphoantigen p100, which is the major structural antigen of HHV-6. There is only 80.1% amino acid sequence homology between p100 of A and B variants. The U53 encodes a viral protease, which is auto-cleaved at two sites and is necessary for viral assembly and maturation. A protein kinase encoded by U69 imparts sensitivity to gancyclovir (Ansari et al., 1999 ; reviewed by Clark, 2000.).

Like other herpes viruses, HHV-6 has usurped several host genes, e.g., U83 encodes a chemokine and two genes, U12 and U51, encode chemokine receptors. The DR7 gene encodes a protein, which binds and inactivates p53, transactivates HIV LTR and transforms eukaryotic cells (Kashanchi et al, 1997; reviewed in Dockrell, 2003). It is

noteworthy that several of the HHV-6A ORFs are translated into proteins, which are shorter than their HHV-6B counterparts.

The Roseolavirus-specific genes include U20-24, U24A, U26, and U85 and U100. HHV-6A and HHV-6B variants have 94% amino acid identity in the seven-region conserved block. They differ in the DR and a 24 kb segment to the right of U85 (except U94, which differs only by 2.4%). Because of the differences in the genes between HHV-6A and HHV-6B, there are biological differences between the variants (Krueger and Ablashi, 2003).

## 2.3 HHV-6 BEHAVIOR AND MORPHOLOGY:

HHV-6 virions are 160–200 nm in diameter. Each consists of a central core containing a linear double-stranded DNA, a capsid, and a tegument, which is surrounded by a membrane structure. The capsids are icosahedral and consist of approximately 162 capsomeres. Its tegument is amorphous and the core has smooth appearance. When HSB-2 (A human T cell leukemia cell line; see chapter III) cells are infected *in vitro* by HHV-6 (strain A), the virus binds to 50% of the cells within 15 minutes and to 100% within 30 minutes. The virus binds to coated pits of the cell membrane and is internalized by endocytosis within 6 hours (Torrisi et al., 1999; reviewed in Krueger and Ablashi, 2003). The viral DNA replication is initiated as early as 12 hours post infection. Newly formed uncoated nucleocapsids are visible in cell nuclei by day 3. The virus envelops in the nucleus, de-envelops in the cytosol and re-envelops in the Golgi complex. By day 6 and later, enveloped viruses can be seen in the endoplasmic reticulum. It takes 6 to 10 days for the virus to appear in increasing amounts in the culture medium. It is noteworthy that despite a high degree of infection, it is extremely difficult to obtain cell-free high-titered viral stocks. This suggests that most of the newly formed virions may be non-infectious. HHV-6-

infected HSB-2 cells undergo apoptosis, which progressively increases from 5% on day 1 to 30% on day 6 post-infection (reviewed in Krueger and Ablashi, 2003).

The HHV-6A and HHV-6B variants infect and replicate mainly in CD4+ human T-cells obtained from peripheral or cord blood, and in tissue culture-adapted lines, e. g., HSB-2 (Human T cell leukemia cell line), MOLT-3 (Human T cell leukemia cell line), SupT-1 (Human lymphoma T cell) and J JHAN (Human T cell line) (ClarkDa, 2000). HHV-6 infects and persists in human monocytes/macrophages in a latent state. The virus may also infect B cells, neural cells, and human fibroblasts; however the viral replication is very poor in these cells. Typical cytopathic effects include the appearance of 2-5 times enlarged multinucleated, giant cells, which are refractile and balloon-shaped (Taniguchi et al., 2000). The infected cells tend to aggregate in small-to-medium clusters. The virus has also been reported to induce apoptosis in uninfected bystander T lymphocytes as well as in natural killer cells (Clark, 2000).

## 2.4 HHV-6 REPLICATION

### 2.4.1 Attachment and Entry:

How HHV-6 attaches to host cells and what is the mode of attachment are still not very clear. It is thought that, similar to HSV-1 gB, the HHV-6 gB binds to haparan sulphate proteoglycans (HSPG) on the cell surface before attaching to the viral receptor, CD46. However, it is noteworthy that heparin, which inhibits interaction between HSPG and the HSV gB protein and consequently inhibits infection of the human cells by HSV-1, has no effect on HHV-6 infection (Pellet and Dominurez, 2001; Santoro et al., 1999). Although CD46 has been identified as a receptor for HHV-6, its expression alone is not sufficient for viral fusion and infection, suggesting that the virus requires other coreceptor (s) for infection. The chemokine receptors CXCR4 and CCR5 (which act as essential co-receptors for T-tropic and

monocytotropic HIV-1 strains, respectively) were also studied and were found non-essential for HHV-6 infection (Yasukawa et al., 1999; reviewed in Dockrell, 2003). Furthermore, it was found that although HHV-6 preferentially infects CD4+T cells, CD4 does not serve as a viral receptor. The penetration of HHV-6 in the infected cells is mediated by endocytosis and is pH sensitive. It can be inhibited by a monoclonal antibody to the viral glycoprotein complex gp100 (Cirone et al., 1992; Foa-Tomasi et al., 1991).

## 2.4.2 Transcription:

HHV-6 genes belong to either latent or lytic category. The latent genes are expressed in the latent phase of the infection and usually comprise a very restricted set of genes. The lytic genes are expressed during a productive viral infection. Depending upon their temporal sequence of expression, they are classified into immediate early (IE), early (E) or late (L) genes (reviewed in Clark, 2000). The IE genes are transcribed first to encode proteins needed for regulation of gene expression. IE genes are synthesized within minutes to hours post-infection and do not require *de novo* protein synthesis. Only virion-associated proteins may be sufficient for their expression. The E genes encode proteins for DNA replication and the L genes encode structural proteins needed for viral assembly. The transcription of E genes requires IE gene activity and transcription of L genes is dependent on viral DNA replication or the expression of E genes. U83 and U89/90 encode IE (A locus) genes in HHV-6 (Rapp et al, 2000, French et al, 1999). U16 through U19 have been designated as IE (B locus) genes. U42 encodes a homolog of the HSV IE gene alpha27 and homolog of HCMV U69. However, it is not transcribed in the absence of *de novo* protein synthesis.

Some HHV-6 transcripts are spliced several times. Most important of them are IE locus and U100. The use of some splice sites is kinetically regulated whereas others may use non-canonical donor and acceptor sequences. In HHV-6 the DNA polymerase promoter has no TATA box and solely depends on the presence of a palindromic ATF/CREB transcription factor-binding site in the virus infected cells (Agulnick et al, 1994).

## 2.4.3 Genome Replication:

HHV-6 genes for lytic phase viral replication include DNA polymerase, DNA binding protein (DBP), the DNA polymerase processivity factor, a helicase/primase complex and origin binding protein (OBP) (Agulnick et al., 1993; reviewed in Pellet and Dominurez, 2001). HHV-6 has oriLyt region similar to other herpes viruses. The origin is located in the region between 5'end of U41 and 3'end of U42. U41 encodes the major DNA binding protein. There is a region in the centre of the oriLyt that contains two sites, OBP-1 and OBP-2, separated by an AT rich region. The OBP binds to these sites. The protein is encoded by U73. It was shown in transient replication assays that both OBP-1 and OBP-2 are required for efficient plasmid DNA replication (Dewhurst et al, 1993; Dewhurst et al, 1994; reviewed in Pellet and Dominurez, 2001).

## 2.4.4 Genome Packaging:

Circularized viral genomes, which are present in about 5% of the viral ncleocapsids, provide templates for rolling circle replication. The replication of viral DNA by rolling circle mechanism results in the production of long concatemers of nascent DNA (Martin et al., 1991). The juxtaposition of DRR and DRL in a concatemer provides complete cleavage and packaging signal. This results in the packaging of a viral DNA unit into a single nucleocapsid.

## 2.4.5 Viral Assembly and Release:

In HHV-6, nucleocapsids are formed in the nucleus of the infected cell. Then, there is a successive envelopment, de-envelopment and re-envelopment when nascent virion moves from one cell compartment to other (Figure 3). After 3 days of infection we can see the nascent capsids containing DNA in cell nuclei (Black et al, 1997). They are enveloped as they pass through the inner nuclear membrane. The enveloped capsids lose their envelopes in the cytoplasm. At this stage abundant nonenveloped and tegumented nucleocapsids can be seen in the cytoplasm (Torrisi et al, 1999). In some cases naked capsids may acquire tegument in the cytoplasm. The viral glycoproteins gB, gH-gL and gp82-gp105 are concentrate in the annulate lamella. The nucleocapsids acquire envelopes from the glycoprotein-studded annulate membranes. (Cardinali et al., 1998; Torrisi et al., 1999). The annulate lamellae are cytoplasmic structures related to endoplasmic reticulum and are the sites for envelopment and glycoprotein maturation. HHV-6–infected cells do not express viral glycoproteins on their plasma (and nuclear) membranes. Therefore, viral capsids do not envelope at the plasma membranes. The mature virions pass through the Golgi complex and are released by exocytosis or cell lysis, and not by budding from the cell membrane.

## 2.5 Fate of the HHV-6-Infected Host Cells:

The infection causes several deleterious effects on the host cells. It shuts off DNA synthesis, marginates chromatin and stimulates synthesis of macromolecules in the infected cells. The cells enlarge and can be recognized by the physical characteristics like ballooning, refractility and the presence of multinucleated and giant cells. The virus-infected cells are arrested in the G2/M phase of the cell cycle (De Bolle et al., 2004). The infected cells die by necrosis. The virus induces apoptosis in uninfected bystander cells. However, in primary NK and T cells, the infection may cause

apoptosis. The infection inhibits proliferative responses of human peripheral blood mononuclear cells (PBMC) to antigens and mitogens. It inhibits IL-2 but enhances IFN-γ production from activated T cells (Flamand et al, 1995). The infection induces the expression of CD4 on CD4-negative cells, e.g., NK cells and CD8+ T cells. This makes these cells susceptible to HIV infection (Lusso et al., 1991). The infection also down regulates expression of CD3 and CXCR-4 molecules on T cells (Secchiero et al, 1997; Secchiero et al, 1998; Yasukawa et al, 1999) but enhances expression of many T cell adhesion molecules, e.g., HLA-DR, CD49d, CD44, CD11a and CD2. The infection suppresses formation of erythroid and granulocyte-macrophage colonies by interacting with CD34 positive progenitor cells (Isomura et al., 2003; Carrigon and Knox, 1995).

## 2.6 BIOLOGICAL PROPERTIES OF HHV-6:

### 2.6.1 Cell Tropism:

HHV-6 has the ability to infect a variety of cells and cell lines but it is predominantly regarded as a T-cell tropic virus. The virus infects and replicates preferentially in activated CD4+ T cells. However, it can also infect CD8 T lymphocytes, γδ TCR+ T lymphocytes, dendritic cells, natural killer (NK) cells and monocytes of the peripheral blood. This virus can also infect established cell lines of the megakaryocytic, glioblastomal, neural, epithelial and fibroblastic origin but replication is poor (Clark, 2000; Asada et al., 1999; Luppi et al., 1999). In vivo, the virus has a wider tropism; HHV-6 genomes or antigens can be detected in lymph nodes, PBMC, tubular epithelial cells, endothelial cells and histiocytes in kidney, salivary glands and CNS. Chimps and certain species of monkeys can be infected with HHV-6, which causes rashes in them (Dockrell, 2003).

**Figure 3. The replication cycle of HHV-6**
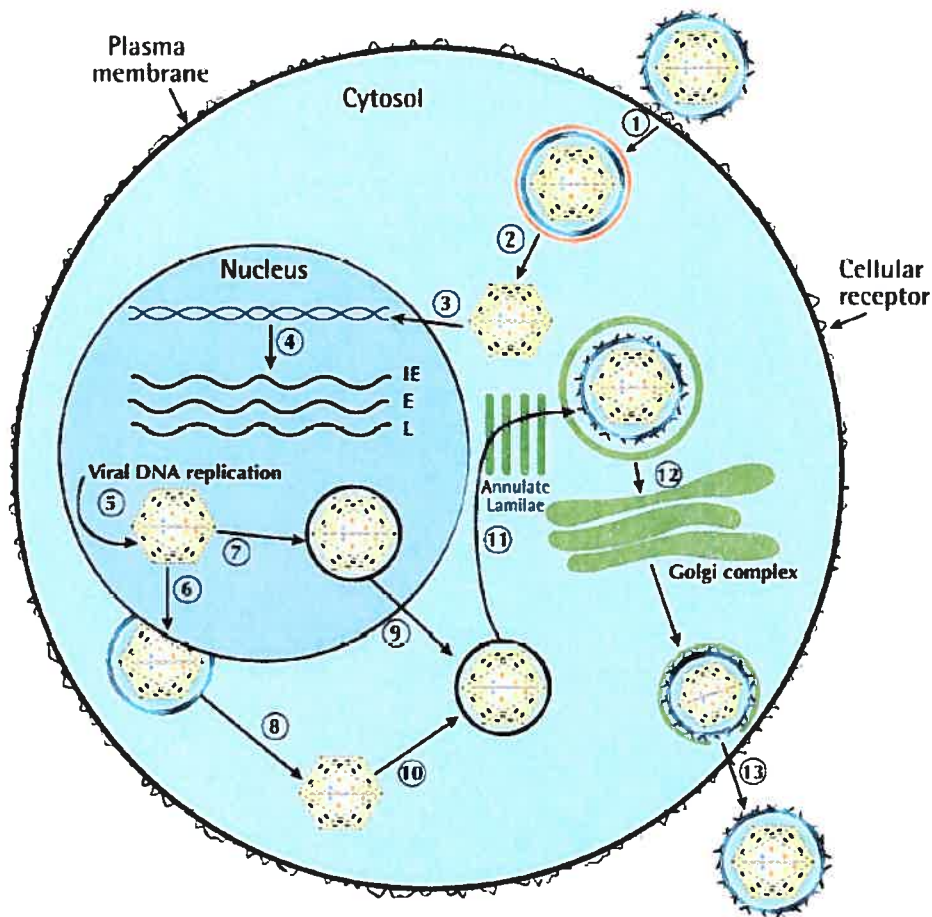


A single viral particle is shown here initiating the infection process. However, it is more likely that several viral particles are involved in this process. The process includes following steps: Attachment of the virus and its entry into the cell by endocytosis (1); de-envelopment of the virus particle (2); transport of the capsid to the nucleus (3); transcription of immediate early (IE), early (E) and late (L) genes followed by DNA replication (4); packaging of viral DNA into icosahederal capsid (5); envelopment of capsid and egress to cytosol (6); packaging and tegusome formation (7); de-envelopment of the virus to form capsid (8); transport of the virus particle into the cytoplasm (9); acquisition of tegusome (10); re-envelopment and acquisition of membrane glycoproteins while passing through ER (11); transport and glycosylation through Golgi complex (12); egress of the virus from the infected cell (13).The endosomal membrane is shown in red. ER, Endoplasmic reticulum.

## 2.6.2 Immune Response:

During a primary infection in children, HHV-6-specific antibodies appear in serum in 3-7 days post-infection (pi). IgM titre is high in the second week and can remain detectable for 2 months pi. IgG antibodies peak by two weeks. The infected persons remain seropositive for their entire lives (Dockrell et al., 1999). The infected host elicits both humoral and cellular immunity against the virus. Antiviral neutralizing antibodies recognize both linear and conformational epitopes in gB, gH and gp82-105. Cell mediated immunity against HHV-6 is considered an important element in the virus control by the host. Analysis of IFN-γ production by T-lymphocyte clones in response to β-herpes viruses confirms that reacting clones respond to HHV-6 antigens. Individuals having defects in NK cell function are susceptible to repeated herpes virus infections. HHV-6 infection of human PBMC induces IL-15 production, which activates NK cells and enhances NK activity of the PBMC (Flamand et al., 1996). Other virus-induced cytokines include IL-18, IL-1β, TNF-α but not IL-6. HHV-6 suppresses mitogen-induced proliferation of human T cells and inhibits IL-2 secretion from them. It stimulates IFN-γ production from CD4 T lymphocytes (Gosselin et al, 1999).

HHV-6 infection causes CD4+ T cell depletion. Both HHV-6 variants induce apoptosis in the CD4+ T-cell lines in vitro. As mentioned earlier, apoptosis in these cells is not because of viral replication as most of the apoptotic cells are uninfected cells. However, the induction of apoptosis in CD4+ cord blood lymphocytes is caused directly by the infected cells (Ichimi et al., 1999). HHV-6A can also deplete CD4 T-lymphocyte by inducing CD46 mediated cell fusion (Mori et al., 2002). The viral receptor CD46 is down-regulated in HHV-6 infected cells. Since CD46 acts as a complement regulatory protein, its down-regulation in the infected cells results in

complement activation. Moreover HHV-6 decreases production of reactive oxygen in monocytes (Burd and Carrigan, 1993; reviewed in Dockrell, 2003).

### 2.6.3 Clinical Pathology:

Primary infections in children usually cause roseola infantum, which is characterized by high fever and the development of a skin rash upon defervescence (Yamanishi et al., 1988; and reviewed by Abdel-Haq and Asmar, 2004). It is noteworthy that HHV-7 also causes roseola in children. According to Hall et al. (1994), HHV-6 was the cause of febrile illness in 21% of the 6-12 month old children, who visited emergency units. The infection is usually self-limiting. However, complications, e.g., febrile seizures, encephalitis, etc may also occur. The infection in adults may cause undifferentiated febrile illness or infectious mononucleosis-like disease. The infection usually becomes latent and the infected persons usually become life-long virus carriers. Primary infections differ from reactivated ones in clinical symptoms. HHV-6 B variants are often associated with roseola; however, A variants are a common cause of infections in children in Zambia. Moreover the symptoms caused by type HHV-6A may vary than those of HHV-6B. A variety of clinical complications and diseases are associated with HHV-6 infection (see Table 4).

## 2.7 HHV-6 IN THE IMMUNO COMPROMISED HOST:

### 2.7.1 HIV-1 Infection:

HHV-6 is considered a co-factor in the development of AIDS in HIV-1 infected individuals. The HHV-6 U3 protein trans-activates the HIV-1 LTR *in vitro* (Mori et al, 1998). It belongs to the HCMV US22 proteins and is expressed in the cytoplasm and endoplasmic reticulum of HHV-6-infected cells at one-day pi. HHV-6 infections are frequently activated in. AIDS patients. Specific symptoms of the reactivation may include pneumonitis and encephalitis. The infection contributes to the development of

immunosuppression by infecting and depleting CD4+ T cells. In this regard, HHV-6A but not B variants induce fusion of infected CD4+ T cells with uninfected cells via interaction between CD46 and gpB-H. As mentioned earlier, HHV-6 can also increase host cells' susceptibility to HIV-1 infection by inducing and up-regulating CD4 expression on them (Dockrell et al, 2003). The HHV-6 trans-activates HIV-1 LTR by its several gene products, e.g., U16/17 and U3 (Flebbe-Rehwaldt, 2000). It also upregulates IL-1β and TNF-α and other cytokines, that increase HIV-1 replication (Dockrel et al., 2003).

In the tissues coinfected with HHV-1 and HHV-6, HIV-1 proviral DNA levels are higher (Emery et al., 1999). In children with vertically acquired HIV-1 infection, the primary HHV-6 infection has been associated with more rapid progression of the disease (Caserta et al., 2001). HIV-1 causes suppression of CCR5-tropic (M-tropic) virus by up-regulating RANTES, which is known to inhibit infection by CCR5-tropic viruses and enhance replication of CXCR4-tropic (T-tropic) virus (Griverl et al., 2001). Therefore, HHV-6 co-infection promotes evolution of HIV-1 virus towards more pathogenic T-tropic syncytium-inducing viruses. HHV-6 reactivation has also been implicated in the neuropathogenesis of AIDS in children.

It has been shown by using the immunomicroarray chip assay that HHV-6 modulates the inflammatory response to HIV infection. Both A and B variants of HHV-6 induce a type-1 response in the host T cells (Mayne et al., 2001). As a result of this infection, IL-12, IL-15, IL-1β, TNF-α and several members of the TNF-α receptor family are activated. The infection also up-regulates the transcription of the IL-8 gene and adhesion molecule in liver cell lines (reviewed in Clark, 2000).

**2.7.2 Chronic Fatigue Syndrome:**

Chronic fatigue syndrome (CFS) is characterized by generalized fatigue accompanied by fevers, sore throat, myalgia, lymphadenopathy, sleeplessness, depression and neurocognitive difficulties. Patients suffer from a number of immunologic abnormalities including immunosuppression. It has been observed that there is reactivation of HHV-6 viruses in the individuals having CFS. Thus, HHV-6 reactivation may be involved in the pathogenesis of this syndrome (reviewed in Abdel-Haq and Asmar, 2004).

### 2.7.3 Bone Marrow Transplantation:

The incidence of HHV-6 infection after bone marrow transplantation varies from 28-75% depending upon the diagnostic method. The majority of the HHV-6 infections post-transplantation are due to reactivation of HHV-6B and the peak incidence of the infection is 2-4 weeks post transplantation (Dockrell and Paya, 2001). There is fever and rash associated HHV-6 viremia after transplantation. Bone marrow suppression, pneumonitis, encephalitis and graft versus host disease have been seen in some individuals. The virus infects stem cells and exerts suppressive effects on engraftment of these cells in transplant recipients. The reactivations may also increase pathogenicity of an existing viral infection or autoimmune condition without becoming a pathogen itself. The use of OKT3 and anti-thymosine globulin in the transplantees to prevent graft rejection is related to the reactivation (reviewed in Caserta et al., 2001). HHV-6 reactivation may also lead to HCMV and EBV reactivations.

### 2.7.4 Solid Organ Transplantation:

Up to 66% of renal transplant patients have reactivation of HHV-6 infection. As mentioned above in the case of bone marrow transplantees, the reactivation occurs most commonly following treatment with OKT3 or antithymocyte globulin and may

be related to a significant degree of immunosuppression associated with these two products. HHV-6 reactivation has also been described in liver transplant patients (Harma et al., 2003). Co-infections with HCV and HHV-6 may lead to enhanced fibrosis in liver.

## 2.8 HHV-6 AND THE CNS

### 2.8.1 HHV-6 and Encephalitis:

HHV-6 has been implicated as a cause of encephalitis in transplantation recipients (Singh et al., 2000). Its reactivation may cause meningitis and encephalitis in the **immunocompetent** individuals and the clinical outcomes in these HHV-6 associated encephalitis range from complete recovery to moderate impairment and death (Caserta et al, 2001). Clinical features of encephalitis include headache, confusion, seizures, abnormal movements and disturbance in higher mental function. Both HHV-6 variants can be detected in the specimens and HHV-6 DNA can be detected in the CSF. The presence of HHV-6 DNA in CSF correlates with the presence of central nervous system symptoms.

### 2.8.2 Effect of HHV-6 Infection on CNS White Matter:

HHV-6 has a potential role in the demyelination of white matter of CNS. Multiple sclerosis (MS) is the most common demyelinating disease of the human CNS. It has been strongly associated with HHV-6 infection. The viral antigens can be detected in the nuclei of oligodendrocytes from MS patients but not from control subjects. Increased levels of anti-HHV-6 IgM and soluble CD46 in MS patients have been detected. However, some workers were unable to support a role of this virus with MS (Luppi et al., 1994; reviewed by Clark, 2000). The virus may also play a role in progressive multifocal leukoencephalopathy, which is a demyelinating disease of the CNS in individuals with impaired cellular immunity and is caused by JC virus.

## 2.9 FEBRILE SEIZURES:

HHV-6 infection causes febrile seizures in infants and young children having more pronounced effect on children of 12-15 months of age. Affected children may have convulsions and other febrile diseases. Among the children whose first febrile seizures were caused by HHV-6, the incidence of recurrent febrile seizures was significant. Frequency of severe forms of convulsions and postictal paralysis is significantly higher among children with primary HHV-6 infection (Jee et al., 1998; reviewed by Abdel Haq and Asmar, 2004).

## 2.10 HHV-6 AND CARDIOVASCULAR SYSTEM:

HHV-6 infection has been detected in the endothelium of aorta, umbilical vein and capillaries of the bone marrow (Takatsuka et al., 2003). The infection is also related to thrombic microangiopathy (Toyabe et al., 2002). Several reports have also associated primary HHV-6 infection with idiopathic thrombocytopenic purpura (Hashimoto et al., 2002; reviewed in Koichi Yamanishi, 2001).

## 2.11. HHV-6 AND OTHER VIRUS INFECTION

HHV-6 can activate other viral infections, for instance those induced by EBV, HCV, measles, papillomavirus and parvovirus and may contribute to the pathologic effects of these viruses. Dual active infections frequently appear especially with other herpes viruses.

## 2.12. EPIDEMIOLOGY

HHV-6 is ubiquitous in human populations and up to 90% of the adult population is seropositive all over the world (reviewed by Krueger and Ablashi, 2003). It usually infects children in the later half of the first year of age, peaking at 3-9 months (Clark, 2000; Campadelli-Fiume et al, 1999). Maternal antibodies decline by the age of 6 months and infants become increasingly susceptible to HHV-6 infection at this age. In

Europe and the USA, the HHV-6 seroprevalence is 72-95% both in adults and children. In Africa, Asia and Latin America, the seroprevalence is 60-95%. Majority of the clinical infections in immunocompetent host are due to HHV-6B variants. The infections with A variants are frequently present in patients with immunosuppression and neurological manifestations (Hall et al., 1998). Infections usually occur via contaminated oral secretions (Krueger and Ablashi, 2003; Clark, 2000). In the salivary gland tissue, the more frequent strain is 6B. These variants are also more frequently found in the peripheral blood mononuclear cells. Co-infections with both A and B variants were detected in 22-34% of lung specimens (Clark, 2000; Cone et al., 1996).

## 2.13 ANTIVIRAL TREATMENT

Many compounds effective against HHV-6 have been reported to date but no controlled clinical studies are available (reviewed by Clark, 2000). Gancyclovir and acyclovir have shown some inhibitory activity on HHV-6 viral infections. Gancyclovir blocks HHV-6 infection in bone marrow transplant patients with HHV-6 encephalitis. Valacyclovir is used for prophylaxis against HHV-6 reactivation. Foscarnet and phosphonoacetic acid inhibit viral DNA polymerase. They have no effect on latent infections. Type 1 IFN has also been shown to reduce disease activity in patients with multiple sclerosis (Hong et al, 2002).

# 3. E2F TRANSCRIPTION FACTORS

E2F factors comprise a family of related transcription factors that play a key role in the regulation of cell cycle progression in many different species including mammals, flies, nematodes, amphibians and plants. The first E2F was originally identified as a factor with transcriptional activity that binds to the sequence 'TTTCGCGC' in the

E2 promoter of the adenovirus (Kovesdi et al, 1986). Hence the term E2F was coined; 'E2' comes from the E2 promoter and 'F' stands for factor. Later on, similar sequences, which are involved in the regulation of gene expression, were identified in the promoters of several cellular genes, e.g., dihydrofolate reductase (DHFR), c-myc and cyclin E, (Blake et al, 1989). Most of these sequences are in the regulatory regions of the genes that modulate growth, cell cycle progression and DNA synthesis, e.g., cyclin-dependent kinases (CDK), cyclins, proliferating cell nuclear antigen (PCNA), DNA polymerase, etc. The activity of E2F transcription factors is under the control of pRB, and cyclins-CDKs as shown in Figure 4. The proliferative E2Fs (E2F-1, E2F-2 and E2F-3) are also regulated by acetylation.

E2Fs also regulate their own activity positively due to the presence of the E2F cognate sites in the E2F gene promoters. Recent studies have revealed that E2Fs target various gene functions not only during the G1/S transition and DNA replication but also during mitosis and DNA damage and repair checkpoints (Ren et al, 2002; reviewed in Cam and Dynlacht, 2003).

### 3.1 The E2F Family:

E2F family is made up of DNA binding heterodimeric proteins containing one E2F subunit and one DP subunit. E2Fs bind DNA as dimers (Huber et al, 1993). They may form homodimers among themselves or heterodimers with DP subunits. The homodimers of E2F can bind to their cognate DNA sequence but this binding is very weak as compared with the E2F/DP heterodimers.

### 3.2 STRUCTURE OF E2F AND DP PROTEINS:

The structure of a typical E2F family member is shown in Figure 5. It contains distinct domains for binding to DNA, cyclin A, and "pocket" proteins (see

section 3.2.5), a transactivating domain, a dimerization domain as well as an evolutionarily conserved region called "marked box." The individual family members may vary from the typical structure and may lack one or more of these domains. The E2F members range from 345 to 904 amino acids in length. All the family members have a DNA binding domain (comprising a basic helix-loop-helix region), a dimerization domain and a "marked box" except the recently discovered E2F-7, which is the biggest member of this family and has a very unusual structure (see Figure 7)he members except E2F-6 and E2F-7, there is a C-terminal transactivation domain. E2F-1-3 also have an N-terminal cyclin- A binding domain. The E2F partners, DP-1 and DP-2, have weak homology with E2F in the DNA binding and dimerization domains. All E2F members can heterodimerize either with DP-1 or DP-2 allowing, at least 14 possible DNA-binding complexes. The functional domains of the E2F family members are detailed below.

### 3.2.1 DNA Binding Domain:

E2F belongs to the basic helix-loop-helix (bHLH)-containing family of proteins. The bHLH region of the protein is necessary for DNA binding, and has a different structure than that of many other bHLH proteins, e.g., Myc/Max.

Because of the structural differences, E2F members bind to a specific DNA sequence TTTCGCGC, which is different from the DNA sequences of other bHLH proteins. The DNA binding domains of all the E2F family members are located close to the N termini and are 70% identical. The DNA binding domains of DP1 and DP2 are also at the N terminus and are 90% identical to each other.

# Figure 4. E2F and cell cycle progression
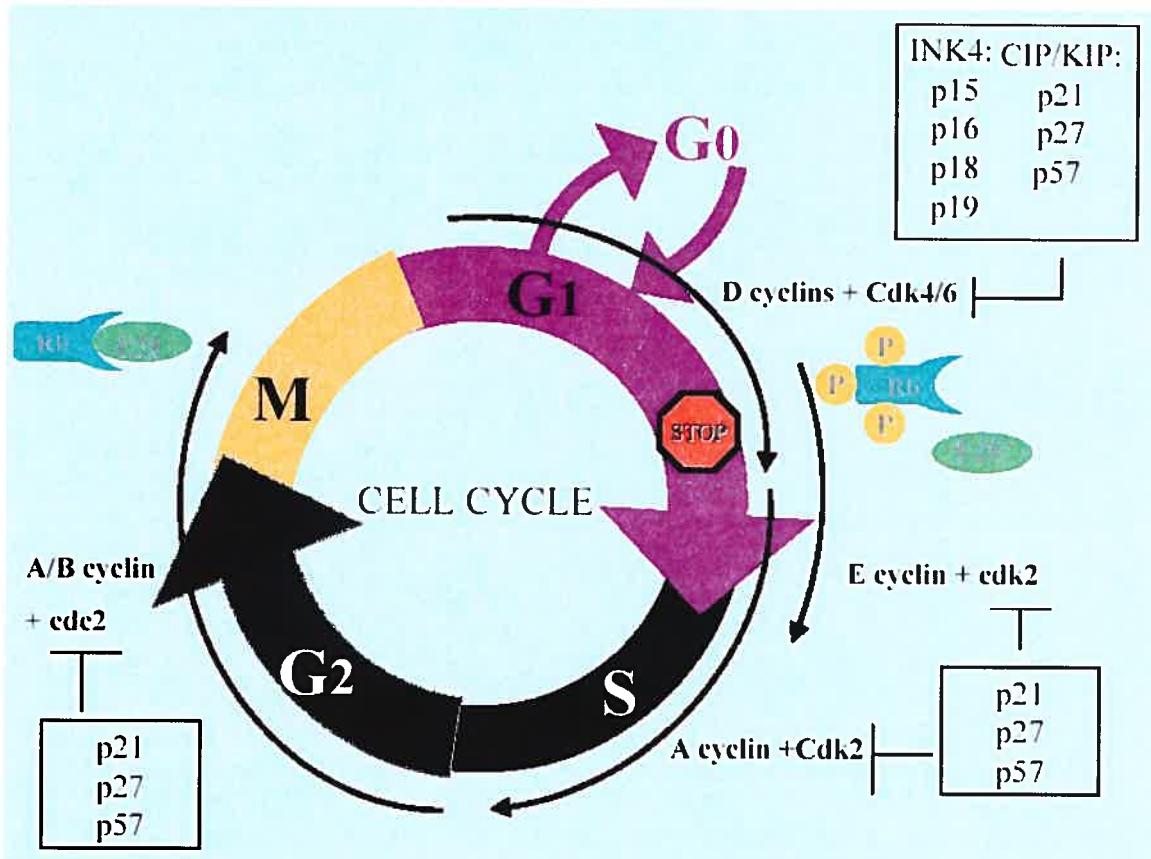


E2F are inactivated in G2 and M phases by pRB. Starting early in G1, pRB is phosphorylated by CDK2 and 4. Phosphorylated pRB releases E2F, which activate transcription of genes involved in DNA replication. INK4: inhibitors of CDK4; CIP: CDK inhibitor proteins, KIP: Kinase inhibitor proteins.

But the E2F and DP DNA binding domains are only about 40% identical (Salansky et al, 1996).

### 3.2.2 Dimerization Domain:

E2F binds DNA as a heterodimer with DP proteins. Both E2F and DP have homodimerization and heterodimerization domains. In E2F-1, the homodimerization domain spans 150-191 amino acids and heterodimerization domain spans amino acids 188-241. As stated earlier, the heterodimers of E2F and DP are more active than their homodimeric forms in DNA binding and transactivating functions. They also exhibit increased affinity for "pocket" proteins. DP proteins themselves cannot bind to the "pocket" proteins. Due to their ability to increase the binding of E2F to the "pocket" proteins, DP might function to repress E2F-1 transactivation functions at certain times in cell cycle progression. The leucin–zipper region in E2F, which is adjacent to the DNA binding domain, is responsible for heterodimerization. The region of DP proteins required for heterodimerization is also adjacent to their DNA binding domains (Figure 5 and Figure 6).

### 3.2.3 Marked Box:

All E2F family members except E2F-7 have an evolutionarily conserved region adjacent to the dimerization domain called as "marked box". This region has been found in insects, Caenorhabditis elegans, yeasts and vertebrates. It is thought to have a role in dimerization and DNA bending. The heterodimer partners of E2F, protein DP-1 and DP-2 do not have this box

### 3.2.4 Transactivation Domain:

E2F family members other than E2F-6 and E2F-7 have C-terminal highly

**Figure 5. The structure of a prototypic E2F family member**
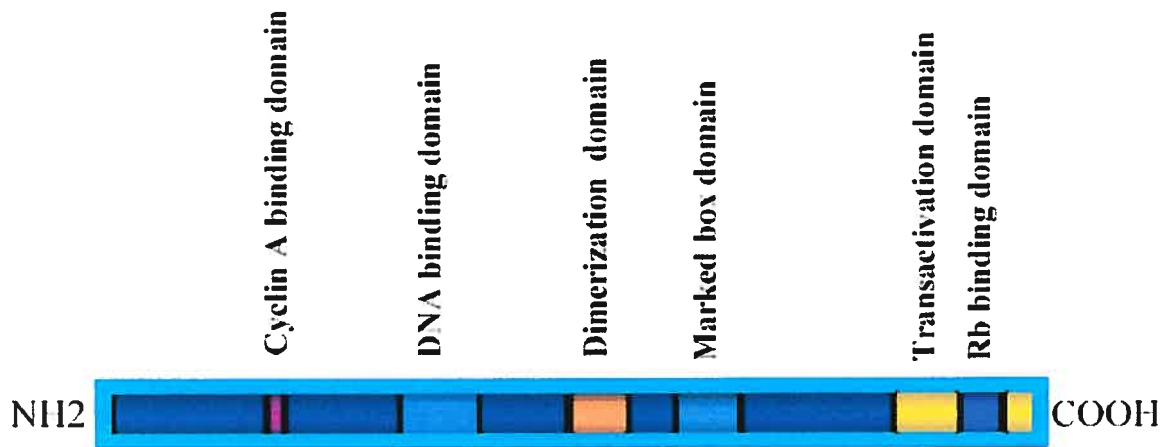


NH2 and COOH represent N and C termini of the protein, respectively. The functions of various domains are detailed in the text.

conserved transactivation domain, deletion of which abolishes the transcriptional activity of these proteins. The transactivation domain comprises the "pocket" protein binding domain as well as the elements upstream of and downstream of it (Figures 6 and 7).

### 3.2.5 The "Pocket" Protein Binding Domain:

E2F/DP heterodimers are stably bound to one of the members of "pocket" proteins, which include pRB, p107 and p130. They vary in their preferences for binding to different "pocket" proteins. E2F-1, E2F-2 and E2F-3 bind exclusively with pRB; E2F-4 binds with high affinity to p107 and p130 but also associates with pRB, whereas E2F-5 associates only with p130 (Figure 6). The "pocket" protein-binding domain is a highly conserved domain at the C-terminus of E2F, and is sandwiched between two segments of the transactivation domain. Both E2F-6 and E2F-7 lack this domain and hence, they cannot bind "pocket" proteins (Dyson et al, 1998).

### 3.2.6 Cyclin Binding Domain:

As the name implies, this domain binds cyclin A. E2F-1-3 each have a domain near the N-terminus close to the DNA binding domain that binds to cyclin A. Cyclin A/CDK2 complex binds E2F and phosphorylates DP-1 at the end of S phase. The phosphorylation of DP-1 lowers the DNA binding activity of the E2F/DP heterodimer and therefore reduces activation of E2F responsive genes (Krek et al, 1995; Xu et al., 1994). The cyclin A binding motif is conserved in E2F-1, E2F-2 and E2F-3 (Figures 7 and 8). A canonical nuclear localization signal (NLS) lies within the cyclin A binding domain of proliferative E2Fs.

# Figure 6. A schematic comparison of the structures of different E2F family members and their interaction with different pocket proteins
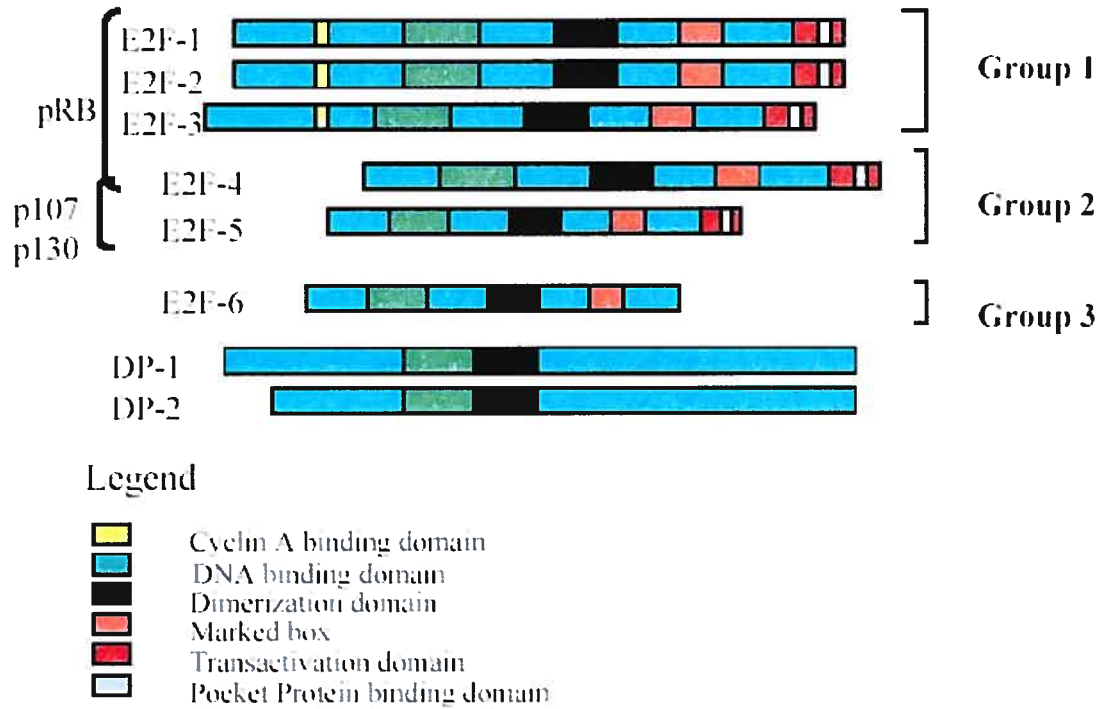


It may be noted that E2F-1, E2F-2 and E2F-3 interact only with pRB whereas E2F-5 may interact with either p107 or p130. E2F-4 may interact with all members of the family. E2F-6 has no pocket protein-binding domain. DP-1 and DP-2 have close structural homology with their E2F partners.

## 3.3 THE E2F FAMILY MEMBERS:

There are seven known E2F and two DP members in the E2F family (Figures 7, 8 and 9). Individual family members are described below.

### 3.3.1 E2F-1

This is the founding member of the family and was first to be identified and characterized. Its gene is composed of seven exons and spans 11kb. The position of E2F gene is 20q11.2. Like other members of E2F family it has N-terminal DNA binding domain and a C-terminal acidic transactivation domain (Neuman et al, 1996). It is a critical determinant of the G1/S phase transition during the mammalian cell cycle. It activates the transcription of a group of genes that encode proteins necessary for DNA replication. The accumulation of G1 cyclins is regulated by E2F1 (Ohtani et al, 1995). E2F-1 can induce apoptosis by a variety of mechanisms, which may or may not be p53-dependent (see below in the apoptosis section for details). It is this apoptosis-inducing ability of E2F-1 that qualifies it as a tumor suppressor molecule. This is supported by the phenotype of the E2F-1 KO mice, which suffer from many tumors (La Thangue, 2003).

The repression of E2F activity is important in the differentiation of many cell types including neutrophils, keratinocytes and adipocytes. There is evidence that E2F-1 can also play a direct role in the regulation of early adipocyte differentiation (Fajas et al, 2002). It is noteworthy that the master regulator of adipogenesis, peroxiosome proliferator-activator receptor (PPAR-$\gamma$), is an E2F target gene.

### 3.3.2 E2F-2

The cDNA of E2F-2 was cloned using a probe containing the DNA binding domain of E2F-1. This cDNA has 46% amino acid sequence similarity to E2F-1 and has DNA and retinoblastoma (Rb) recognition sites (Ivey-Hoyle et al, 1993). The gene map locus is 1p36. E2F-2, along with E2F-1 and E2F-3, belongs to a subclass of E2F factors thought to act as transcriptional activators important for progression through the G1/S transition. However, unlike E2F-1, E2F-2 is a weak inducer of apoptosis. The E2E-2 KO mice suffer from many immunological defects but not from tumors (reviewed in Stevens and La Thangue, 2003). The transcription factor MYC induces transcription of the E2F-2. However, the MYC induced S phase and apoptosis require distinct E2F activities. MYC induced S phase was impaired in the absence of E2F-2 but not E2F-1. In contrast, the ability of MYC to induce apoptosis was markedly reduced in cells deleted for E2F-1 but not for E2F-2 (Leone et al, 2001).

### 3.3.3 E2F-3

Screening a NALM-6 cell cDNA library with an E2F-1 probe identified this member of E2F family that has both DNA and retinoblastoma protein recognition sites (Lees et al, 1993). The gene map locus for E2F-3 is 6p22.3. There are two variants in E2F-3: E2F-3A and E2F-3B. E2F-3B is expressed via an alternative translation start site and lacks 101 N-terminal amino acids relative to the full-length protein, including a moderately conserved sequence present only in the growth promoting E2F family members (E2F-1, -2, and -3). In contrast to E2F-3A, which is expressed only at the G1/S boundary, E2F-3B is expressed throughout the cell cycle, with peak levels in G0 when it is associated with pRB (He et al., 2000). E2F-3 is essential for embryonic viability. The E2F-3-/- mutants die prematurely with congestive heart failure. This kind of defect

was not seen in E2F-1 or E2F-2 null mice (He at al., 2000). Lizhao et al. (2001) by using a floxed E2F-3 allele demonstrated that the floxed E2F-3 allele did not have any adverse effect on the development of animals but the Cre mediated ablation of E2F-3 from MEFs showed reduced rate of proliferation (Lizhao et al. 2001).

Like E2F-2, E2F-3 has no tumor suppressor activity. Both alone as well as in combination with loss of E2F-1, E2F-3 mutants do not show an increase in the incidence of tumor formation (Cloud et al, 2002).

### 3.3.4 E2F-4

Ginsberg and collegues identified E2F-4 in 1994 (Ginsberg et al., 1994). Its cDNA encodes a 2.2 kb RNA and has a protein of 411-416 amino acids. The locus of the E2F-4 gene is 19q22.1. E2F-4 is expressed in almost all tissues and this expression is constitutive. It forms heterodimer with DP-1. Unlike other members of the E2F family, E2F-4 interacts with p107 *in vivo* and this interaction diminishes E2F-4's transactivating capacity. This interaction also diminishes E2F4's transforming activity (Ginsberg et al, 1994). The ability of Myc to induce S phase was impaired in the absence of either E2F2 or E2F3 but not of E2F-1 or E2F-4. Both E2F-4 and E2F-5 are involved in MYC repression in cells. These factors pre-exist in cells as a complex containing SMAD3, E2F-4, E2F-5, DP-1, and the co repressor p107 in the cytoplasm. In response to TGF-$\beta$, which activates SMAD3, this complex moves into the nucleus and associates with SMAD4, recognizing a composite SMAD-E2F site on MYC gene promoter (Chen et al, 2002).

In the mouse model, loss of E2F-4 has no detectable effects on either cell cycle arrest or progression. However, E2F-4 is essential for normal development

(Humbert et al, 2000). The simultaneous inactivation of E2F-4 and E2F-5 in mice results in neonatal lethality, suggesting that E2F-4 and E2F-5 perform overlapping functions during development. E2F-4 and E2F-5 are dispensable for cell cycle progression but are necessary for pocket protein mediated G1 arrest of cycling cells (Gaubatz et al, 2000).

### 3.3.5 E2F-5

E2F-5 was cloned from a human fibroblast using p130 as bait (Sardet et al, 1995). Like E2F-4, E2F-5 also does not bind to pRB. E2F-5 gene encodes a 345 amino acid protein that is 69% identical to E2F-4. It binds to p130 and p107 pocket proteins. The gene locus for E2F-5 is 8q21.13. As mentioned in the above section, it forms a complex with SMAD3, along with p107 and other E2F members in the cytoplasm. TGF-$\beta$ causes them to move to the nucleus, where they join SMAD4 and represses MYC expression.

### 3.3.6 E2F-6

Trimarchi's group identified E2F-6 in 1998 (Trimarchi et al, 1998). E2F-6 is expressed as two mRNAs species, 2.5 and 3.5 kb, in humans. The molecular weight of E2F-6 protein is 35 kDa. The DNA binding and dimerization domains of E2F-6 are highly related to those of other E2F family members but it lacks transcriptional activation and pocket protein binding domains. It also lacks the cyclin A binding domain. E2F-6 can dimerize with DP1 or DP2, and bind to DNA as a heterodimer in a sequence specific manner. E2F-6 is unable to activate transcription and does not interact with "pocket" proteins; rather it acts to repress the transcription of E2F responsive genes by countering the activity of other E2F complexes.

E2F-6 causes gene silencing in a manner that is not dependent on the retinoblastoma family of "pocket" proteins. E2F-6 is found in multimeric protein complexes that contain MGA and MAX, and thereby the complexes can bind not only to the E2F binding site but also to MYC and SV40 T protein binding sites. This complex also contains chromatin modifiers such as histone methyltransferases, which modify lysine-9 of histone H-3 and creates a binding site of high affinity for heterochromatin repressor proteins, e.g., HP1-$\gamma$ and polycomb group proteins (PcG). The E2F-6 complex occupies target promoters in G0 cells rather than in G1 cells. The chromatin modifiers contribute to silencing of E2F and MYC- responsive genes in quiescent cells (Ogawa et al, 2002). E2F-6 null mice display homeotic transformations of the axial skeleton (Storre et al, 2002). Overexpressed E2F-6 can inhibit entry into S phase of cells that are stimulated to exit G0 phase (Gaubatz et al, 1998) and can also delay the exit from S phase (Cartwright et al, 1998). E2F-6 also regulates genes that are involved in the pathogenesis of neoplasia. In some cases it can repress target promoters in a manner that does not require histone H3 methylation at lysine 9 (Oberly et al, 2003).

### 3.3.7 E2F-7

This is the recently identified member of the E2F family (de Bruin et al., 2003). This newly discovered E2F is structurally and functionally different from other family members. The most important feature of E2F7 is that it contains two DNA binding domains and lacks the heterodimerization domain. This suggests that it binds to the E2F consensus DNA sequence independently of DP proteins. In other features it resembles E2F-6, e.g., it lacks the C-terminal domains necessary for transactivation and binding to the retinoblastoma family proteins.

Therefore, E2F7 is unable to activate E2F responsive genes; Instead E2F-7 blocks the endogenous E2F-dependent transcriptional activity and the transactivating activity of an overexpressed E2F-1. The mechanism of its transcriptional repression is not clear, but might involve competition between E2F7 and other E2Fs for the same DNA target sites. E2F-7 may function as a tumor suppressor gene as its overexpression slows down cellular growth and mutation in this gene might cause cancer formation (de Bruin et al, 2003 and Di Stefano et al, 2003). The presence of two DNA binding domains enables it to bind cognate DNA sequences independently of heterodimerization with DP proteins (Logan et al, 2004).

### 3.3.8 DP-1

DP-1 is one of the heterodimerization partners of E2Fs (Figure 6). The cDNA for the DP-1 transcription factor (TFDP-1) was cloned by Girling et al., (1993). The gene map locus is 13q34. The mature protein has 410 amino acids. The antibodies against DP1 supershift a majority of the protein species that bind to the E2F site, suggesting that most of the cellular E2F complexes contain DP1. As mentioned above, DP-1 is a component of the large repression complex for MYC (Chen et al, 2002). This complex contains, in the nucleus, SMAD3, E2F4, E2F5, DP1, p107 and SMAD4 (Leone et al, 2001).

### 3.3.9 DP-2

Transcription factor DP-2, another E2F dimerization partner, was cloned from a human kidney cDNA library (Zhang and Chellappan, 1995). Its gene TFDP-2 encodes a 386 amino acid protein that is 68% identical to TFDP-1. The gene map locus for DP-2 is 3q23. Antibodies specific for DP2 supershift the

remainder of the E2F activity that is not reactive with the DP1 antibody suggesting that DP-1 and DP-2 may be the only DP proteins that heterodimerize with E2F (reviewed in Slansky and Farnham, 1996).

## 3.4 CLASSIFICATION OF THE E2F FAMILY:

The E2F family comprises of seven members in mammals. On the basis of their structural organizations and functions, they have been placed in three different groups (Figure 6).

1. **GROUP I:** Group I consists of E2F-1, E2F-2 and E2F-3 members. They have a domain N-terminal to the DNA binding domain that binds to cyclin A (Black et al., 1999). They have a transactivation and a "pocket" protein-binding domain at the C-terminus. The latter domain is responsible for binding to the retinoblastoma family of tumor suppressors proteins. The Group I E2Fs are required for cell cycle progression at the G1/S checkpoint.

2. **GROUP II:** It comprises E2F-4 and E2F-5. They do not have the cyclin A binding domain but contain transactivation and pocket protein-binding domains on the C-terminus. E2F-4 and E2F-5 are expressed at steady state levels during cell cycle and their biological activities are dependent on relocalization in different cellular compartments during the cell cycle. Unlike the group I E2F, these E2F are poor transcriptional activators. They can't induce quiescent cells to enter the cell cycle. The members of this group are important for the repression of E2F responsive genes by binding pocket proteins (pRB, p107 and p130) and to recruit histone deacetylases to the promoters of E2F responsive genes (Brehm and Kouzarides, 1999). E2F-4 and E2F-5 have nuclear export signals within their DNA-binding domains. They could, however, be imported to cell nuclei by association with DP-2.

3. **GROUP III:** E2F-6 is the main member of this subclass. It has heterodimerization and DNA binding domains, which are homologous with other E2F member domains. The N-terminal domain of E2F-6 bears no homology to those of other E2F family members. It also lacks the transcriptional activation and pocket protein binding sequences. Therefore it causes gene silencing in a manner independent of the retinoblastoma protein family members. Recently Ogawa et al., (2002) described the molecular mechanism of E2F-6 functioning in gene silencing in G0 cells. They found that E2F-6 exists as a component of a multimeric protein complex with MGA and Max. This complex can bind E2F as well as Myc binding sites (Ogawa et al., 2002).

The newly discovered member of the E2F family, E2F-7 may also be placed in this group on the basis of structural and functional homologies. It lacks the sequences necessary for dimerization, transactivation and "pocket" protein binding. Instead it has two DNA binding domains (Figure 7), which bind to the E2F consensus sites. It occupies these sites and acts as a transcriptional repressor and, therefore, inhibits cellular proliferation (De Bruin et al., 2003).

Last month while I was doing the final corrections in my thesis, one more member of this family E2F-8, was discovered (Maiti et al, 2005). It posseses two distinct DNA binding domains. These domains have no homology to any DNA binding domain present in the E2F family members. The gene for E2F-8 is located on chromosome 7 and shares many characteristic with E2F-7 gene concerning homodimerization and E2F dependent gene repression activities.

## 3.5 REGULATION OF E2F

The functional activities of E2F are regulated by "pocket" proteins and cyclin A as these proteins can bind to their specific sites present in these factors. Another level of regulation of E2F is imposed by their acetylation.

### 3.5.1 The "Pocket" Protein-Mediated Regulation of E2F:

The family of pocket proteins comprises pRB, p107 and p130. The best-known member of this family is pRB, the protein product of the human retinoblastoma tumor susceptibility gene (reviewed in Dyson, 1998). This tumor suppressor gene is absent or mutated in one third of human tumors. It was originally identified as a tumor suppressor gene which, when mutated, causes retinoblastoma (a malignant tumor of the retina). The three members of the pRB family differ in their ability to interact with different E2F members. Whereas pRB can interact with E2F-1, E2F-2, E2F-3 and E2F-4, p107 and p130 can interact only with E2F-4 and E2F-5. The basis for this specificity is unclear. Their differential binding specificities for E2F are likely to be important with regard to the biology of these proteins. They all bind E2F through a sequence of amino acid called "pocket", which is comprised of non-contiguous regions near their C- terminus. This "pocket" region can be differentiated into two components: the larger component in pRB (aa 395-876) and the smaller component (aa 379-792). The larger component is important for interaction with E2F, whereas the smaller one binds to the peptide LXCXE. This peptide motif is present in viral oncoproteins, histone deacetylases, and cyclin D1, and provides the molecular basis for their interaction with the "pocket" proteins. The expression pattern of the various pocket proteins is different in the different phases of the cell cycle. p130 is highly expressed in quiescent and differentiated cells. Its level drops rapidly when cells enter the cell cycle. In contrast, p107 levels are very low in terminally differentiated cells and its levels arise when quiescent cells are stimulated to proliferate. The levels of

**Figure 7. A schematic depiction of the E2F-7 structure**



Note the presence of two DNA-binding domains (DBD1 and 2). NH2 and COOH
represent N and C termini of the protein, respectively.

pRB are moderate in most cell types and are present in both quiescent and cycling cells. As the expression of "pocket" proteins fluctuates during the different phases of the cell cycle, the levels of various E2F family members also fluctuate. The levels of E2F-1, E2F-2 and E2F-3 increase during G1 to S progression. These proteins are strong activators of transcription and when overexpressed, are capable of driving cells into S phase. In contrast, E2F-4 and E2F-5 are weak transcriptional activators. E2F-4 and E2F-5 are the major forms of E2F found in quiescent cells, where E2F mediated gene expression is repressed. In summary, p107 and p130 interact with E2Fs that are primarily considered to be co repressors, whereas pRB regulates E2Fs that are strong activators.

The "pocket" proteins are phosphorylated by cdk. This phosphorylation is the molecular event associated with the progression of the cell cycle through the "restriction point" in G1. Beyond this point, cells become committed to entering in S phase event if they are deprived of growth factors. In G0 and early G1 phases, the pRB is hypophosphrylated and this form actively binds and inactivates E2F family members. When bound with pRB, the transactivation domain of the E2F is concealed within the "pocket" of the pRB proteins, as a result E2F is unable to activate transcription. In mid to late G1 phase, the cyclin D-cdk4/6 and cyclin E-cdk2 phosphorylate pRB proteins. Because of this phosphorylation, the conformation of pRB changes in such a way that it is no longer able to hold E2F. The released E2F accumulate and activate transcription from the E2F responsive genes. Thus, the phosphorylation of pRB regulates E2F-dependent gene transcription during cell cycle progression (reviewed in Dyson, 1998).

There are at least two different mechanisms of transcriptional repression by pRB. In the first place it can bind with E2F and suppress its ability to transcribe responsive genes. Secondly the repressor complex of pRB and E2F that is formed at gene promoters actively blocks the transcription by recruiting histone deacetylases (HDAC), the histone methyl transerases, e.g., SUV39H1, and other chromatin remodeling enzymes. The HDAC deacetylates histone octamers, which facilitate nucleosomes condensation into chromatin. This inhibits gene expression by blocking the access of transcription factors to the promoter. Moreover as the acetylation of E2F increases the binding of the E2F/DP complex to DNA (see below), the recruitment of HDAC to E2F via pRB may deacetylate and inhibit E2F's binding to DNA.

**3.5.2 Cyclin A-Mediated Regulation of E2F: The** cyclin A activity is very critical in S/G2 phase to reduce the activity of E2F responsive genes. At the end of S phase, the cyclinA/CDK2 complex phosphorylates DP-1. This phosphorylation reduces the binding of the heterodimer to specific DNA sequences. Consequently, the E2F responsive gene transcription is reduced. Moreover cyclinA/cdc2 complex hyperphosphorylates DP-1 in G2 phase causing the disruption of E2F/DP complex and sequestration of E2F by pRB in G2/M (Krek et al., 1994).

**3.5.3 Acetylation-Mediated Regulation of E2F:**

Several workers have demonstrated that E2F-1-3 are acetylated by p300/CBP and associated histone acetyl transferases (HATs) at three conserved lysine residues located at the N-termini of their DNA-binding domains (Martinez-Balbas et al., 2000; Marzio et al., 2000). The acetylation increases the DNA binding ability of the E2F. It, however, can be reversed by HDACs.

## 3.6 E2F AND APOPTOSIS:

E2F is not only involved in cell growth and the cell cycle modulation but it also plays a prominent role in apoptosis. Results from a variety of experiments suggest that E2F can induce apoptosis in various cell types in p53 dependent and p53 independent manners. The overexpression of E2F-1 in transgenic megakaryocytes causes increased apoptosis (Guy et al, 96). Furthermore, E2F-1 knockout mice show reduced apoptosis and emergence of various tumors (Black et al, 1999). Of all the E2Fs, E2F-1 is the most potent inducer of apoptosis. However, ectopic expression of E2F-2, E2F-3 and to some extent E2F-4 could also induce apoptosis in different cell lines (Ginsberg, 2002).

The ectopic expression of E2F-1 induces p53 via transactivation of p19ARF (ARF; Sherr, 1998), which in turn interacts with MDM2, the negative regulator of p53. This stabilizes and activates p53. There is an additional p53-dependent pathway of E2F-1- induced apoptosis, which is independent of ARF. This pathway involves p73, a homolog of p53. E2F-1 activates transcription of p73 that leads to activation of p53 responsive genes and, therefore, p53-dependent apoptosis. Not surprisingly, the disruption of p73 functions by gene targeting or by dominant negative mutants prevents E2F-1 induced apoptosis (Irwin et al, 2000). E2F-1 directly activates the expression of the apoptosis-activating factor-1 (Apaf-1), which combines with cytochrome c and activates procaspase 9, causing apoptosis via downstream effector caspases (Irwin et al., 2000; Furukawa et al., 2002). Augmented E2F activity also causes the release of cytochrome c from mitochondria to the cytoplasm that seems to be p53 dependent (Hickman et al., 2002). DNA microarray studies have revealed that augmented ectopic expression of E2F-1 upregulates the proapoptotic members

of the Bcl-2 family including Bad, Bik and Bid (Ma et al., 2002). E2F-1 also disrupts activation of NF-$\kappa$B, which is a well characterized transcription factor that regulates cell survival by activating various anti apoptotic genes.

## 3.7 E2F AND DNA DAMAGE:

Recent studies have shown the involvement of the E2F, particularly E2F-1, in DNA damage checkpoints (Cam and Dynlacht, 2003). Ionization radiation up regulates the expression of E2F-1 and induces S phase and cell death. DNA damage causes the induction of E2F, which are then phosphorylated by ATM/ATR. The ATM/ATR are two related protein kinases, which are recruited and activated in response to DNA damage resulting from genotoxic insults. They coordinate the DNA damage response by phosphorylating several proteins involved in DNA repair, cell cycle arrest and apoptosis. The ATM/ATR-mediated phosphorylation stabilizes E2F-1 (Lin et al 2001). E2F-1 is needed for DNA damage-induced apoptosis and the DNA damage responses make use of signals from the pRB/E2F cell cycle pathway (reviewed in Stevens and La Thangue, 2003). In response to DNA damage, checkpoint kinase 2 (Chk2) also regulates E2F-1 activity by phosphorylating E2F-1, which stabilizes the factor, increases its transcriptional activity and localizes it in discrete nuclear structures. Some studies have shown that dominant negative Chk2 mutants block induction of E2F-1, suggesting an important role of E2F-1 in checkpoint control and explain why E2F-1 acts as a tumor suppressor protein. In another study, it was shown that E2F-1, E2F-2 and E2F-3 could cooperate and interact with p53 to promote its apoptotic function but cyclin A prevents E2F-1 from interacting with p53 to induce apoptosis. It is noteworthy that in response to DNA damage cyclin A level decreases and the E2F-1-p53 interaction increases

in cells. Under these conditions, E2F-1 may promote p53-mediated apoptotic functions. Thus, E2F may play a novel role in the event of DNA damage (Hsieh et al 2002).

## 3.8 E2F AND TUMORIGENESIS

Several recent studies have shown that an overexpression of E2Fs not only forces the cell out of the quiescence but also may cause its transformation (Cam and Dynlacht, 2003). The tumor-causing activities of E2F are restrained by their interaction with pRB. E2F-1-3 can transform cells into tumors in the absence of pRB or in the presence of mutated pRB. On the other hand, E2F-1-/- mice develop a broad spectrum of tumors, suggesting a dual role of E2F-1 as an oncogene as well as a tumor suppressor gene (Zhu et al 2001). It is not yet known what role, if any, the repressor E2Fs (E2F-4 and E2F-5) play in cell transformation and tumorigenesis.

## 3.9 E2F AND VIRUSES

Several viruses, particularly DNA viruses, affect E2F activity directly or indirectly. These viruses use the host cell's transcriptional and translational machinery for their own replication and survival. The viruses deregulate cell cycle by inducing proliferation, quiescence or cell cycle arrest and viral latency. They also have the ability to transform and immortalize cells by overriding certain checkpoints, which monitor cell division and DNA damage. The viruses frequently target pRB and p53, and inactivate or degrade them. They may also stimulate cyclin D and increase cdk activity. A few viruses that are known to affect the E2F pathway in the infected host cells are mentioned below:

### 3.9.1 Human Papillomavirus (HPV):

Human papillomavirus (HPV) is the major cause for warts and are believed to contribute to the development of many cancers mainly cervical cancer. Human papillomavirus E7 protein share a short amino acid sequence that constitutes a domain required for the transforming activity of this protein. These sequences are also required to bind to the retinoblastoma gene product (pRB) (Chellappan et al., 1992). HPV protein E7 binds strongly with Rb, releasing permanently E2F transcription factor and causing the cell to be tumorous (Figure 8).

### 3.9.2 Adenoviruses:

The adenovirus E1A gene product has similar amino acid sequence as of E7 of HPV that binds to the pRB protein. E1A dissociates the E2F-pRB complex releasing active E2F permanently for the cell cycle so that the viral replication can proceed efficiently and in the time course in which a lytic infection does not occur, oncogenic process may initiate (Chellappan et al., 1992).

### 3.9.3 Simian Virus 40 (SV40):

Large T antigen of SV40 binds pRB and prevents its interaction with E2F, which become dissociated from pRB and are free to transcribe genes necessary for the continuation of cell cycle (Figure 8). In this way, the antigen overrides the pRB regulated cell cycle checkpoints. SV40 small T antigen acts upstream of pRB and promotes cell cycle progression. It activates cyclin D1 expression, which in turn increases the activity of cdk4 and cdk6, phosphorylation of pRB, and causes release of E2F from pRB. The SV40 large T antigen has also an indirect effect on E2F regulation. It disrupts p53 DNA complex. By targeting

p53, this viral protein inhibits p53-mediated transcription of p21, which is an upstream negative regulator of cdks (reviewed in Ludlow and Skuse, 1995).

### 3.9.4 Epstein Barr Virus (EBV)

EBV is a causative agent for many lymphoproliferative diseases in humans and *in vitro* induces immortalization of human B cell and transforms primary

cells into anchorage-independent growth. Its major oncoprotein, latent membrane protein -1 (LMP-1), blocks the E2F-4 and E2F-5 functions by exporting them to the cytoplasm (Ohtani et al., 2003). Since E2F-4 and E2F-5 are downstream mediators for a p16 INK-mediated cell cycle arrest, LMP-1 plays an important role in cell proliferation and tumor formation by blocking the functions of these transcription factors. It also downregulates the expression of the tumor suppressor gene p16 INK (Ohtani et al., 2003). Two immediate early proteins of EBV, BZLF1 and BRLF1, act as viral transactivators, which mediate switch from latent to lytic viral infection. Both these proteins have been found to enhance the expression of E2F-1, suggesting their requirement for EBV lytic replication (Swenson et al., 1999; Mauser et al., 2002).

### 3.9.5 Human Immunodeficiency Virus type 1 (HIV-1):

E2F-1 represses transcription of the HIV-1 long terminal repeat (LTR) and diminishes the extent of the Tat-induced activation of the viral promoter (Kundu et al., 1997). HIV-1 Tat plays a pivotal role in the regulation of viral replication and transcription. It also regulates the expression of many cellular genes. Tat modulates many cellular processes involved in proliferation,

**Figure 8. Activation of E2F by different viral proteins**
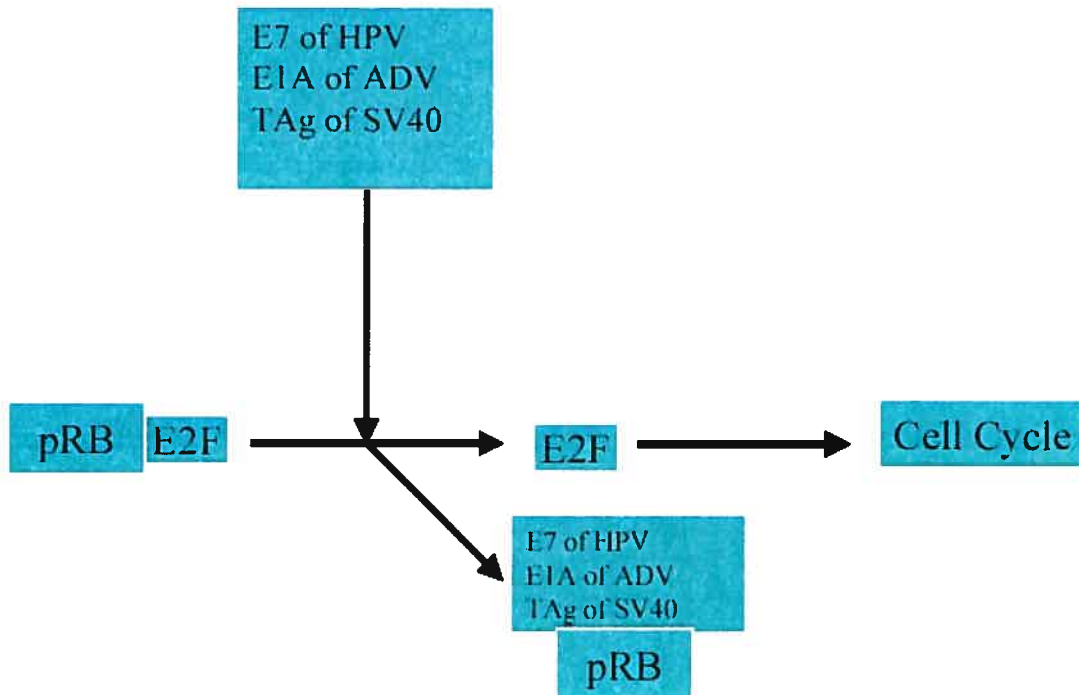


The viral proteins shown here sequester pRB family members and prevent inactivation of E2F in G2/M phase.

apoptosis and differentiation, and therefore may act as a co-factor in AIDS-related tumors. E2F-4 is known to act as a Tat-binding protein. The Tat-E2F-4 complexes bind E2F cis regions more strongly than E2F-4 alone and increase E2F-dependant activation of HIV-1 LTR and other promoters, e.g., of cyclin A gene. This suggests that Tat acts as an adapter protein, which recruits E2F-4 and other cellular factors to regulatory regions of various cellular genes to modulate their biological activities (Ambrosino et al., 2002).

### 3.9.6 Herpes Simplex Virus 1 (HSV-1):

HSV-1 induces intracellular redistribution and modification of E2F4 and increases E2F-pRB interaction by modifying pRB (Olgiate et al, 1999). As a result of HSV-1 infection, all E2F members are post-translationally modified and translocated to nucleus. The binding ability of these proteins to their cognate DNA sites is also decreased in HSV-infected cells (Advani et al, 2000).

### 3.9.7 Hepatitis C Virus:

Hepatitis C virus (HCV) has been reported to cause hepatocellular carcinoma, liver cirrhosis and acute and chronic hepatitis. In a recent study it was found that HCV causes a significant reduction in E2F mediated transcription in a murine normal liver (CL2) cell line (Ohkawa et al, 2004).

### 3.9.8 Human Herpes Virus-6 (HHV-6):

HHV-6 is known to deregulate cell cycle progression in human cells. This suggests that the virus may have the potential to modulate the expression and functional activities of the E2F. However, our literature search on PubMed could not reveal any study on this subject.

# OBJECTIVES

# CHAPTER II

## Objectives :

This research project was focused on the effect of HHV-6 infection on E2F/DP transcription factors. These transcription factors play key roles in cell cycle progression, synthesis of nucleotides, DNA replication and apoptosis. Moreover, their deregulation has been implicated in tumorigenesis. A variety of viruses have been reported to modulate these factors by binding pRB, the negative regulator of E2F, and activate E2F responsive genes. HHV-6 (Human herpesvirus-6) is a T-cell tropic virus and is the etiologic agent of roseola. It establishes latency in the host, and can infect a variety of cells including T cells, B cells and macrophages. HHV-6 infection induces profound changes in the infected cells. It inhibits proliferation of mitogen activated T cells and induces cell cycle arrest in G2 phase of the infected cells. It is noteworthy that E2F are inactivated in this phase of the cell cycle. This virus, therefore, may potentially deregulate E2F expression and activity in the infected cells. Since to the best of our knowledge no study has addressed this issue, the present study was designed with the hypothesis that HHV-6 infection induces changes in the expression and functional activity of E2F/DP transcription factors. The objectives were to examine the effects of HHV-6 infection on the expression and the functional activities of the E2F factors in human cells.

# MATERIALS AND

# METHODS

# CHAPTER III

## Materials and Methods

### 3.1 Cells:

The human T cell leukemia cell line (HSB-2) was used in this study. To date, this is the only known cell line, which can be efficiently infected *in vitro* by Human Herpes virus-6 A variants. It was initially obtained from American Type Culture Collection (ATCC, Bethesda, MD). It was maintained in RPMI 1640 (Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 2.5mM L-glutamine, 10U/ml penicillin (Nova Pharma, Toronto, Canada), 100$\mu$l/ml gentamicin (Schering, Montreal, Canada), and 2$\mu$g/ml fungizone (Squibb, Montreal, Canada). They were incubated at 37°C in a humidified incubator and buffered with 5% $CO_2$. The cells were split 1:3 every 3-4 days.

### 3.2 Virus Preparation:

GS strain (a prototype isolate) of human herpes virus-6 (HHV-6) was used in this study. The virus was prepared as described with some modifications(Flamand et at, 1995). In brief, aliquots of 5 million cells were each infected with 5 ml of the virus preparation having a titre of $5 \times 10^5$ tissue culture infectious doses TCID50/ml. The TCID50 represents viral titre in 50% tissue culture infectious doses and was determined as described (Flamand et al., 1995). The cells were incubated at 37°C for 1.5 hours with frequent gentle shaking, washed with sterile PBS and then resuspended in 10 ml of the culture medium with 5% FBS. The infected cells were incubated at 37°C in a humidified incubator buffered with 5% $CO_2$ until the cells started showing cytopathic effects (large balloon-shaped cells). At this point, the cultures were harvested for the virus. The cells were centrifuged at 1000xg for 10 minutes at 4°C

and the supernatants were collected and pooled. The cell pellets were suspended each in 1ml culture medium, pooled together and subjected to 3 cycles of freeze and thaw on dry ice. The cell lysate was centrifuged again at 1000xg and added to previous supernatant. The supernatant was passed through $0.45\mu$ filter. The virus titre of the supernatant was measured in TCID50 as described above, aliquoted and stored at $-80°$ C. This served as a viral stock. Similarly obtained supernatant from growing uninfected HSB-2 cells was used as mock virus.

### 3.3 Cell Infection:

Five million cells were pelleted and resuspended in 5 ml of the viral preparation (at 0.5 multiplicity of infection; moi), incubated at 37°C for one hour with intermittent shaking, washed and resuspended in the culture medium containing 5% FBS. The infected cells were incubated at 37°C in an incubator having 85% relative humidity and 5% $CO_2$ atmosphere. The infected cells usually showed typical cytopathic effects of the infection on day 9 -11 post-infection (pi). At this time point, usually 90% of the cells were infected with HHV-6 as determined by indirect immunofluorescence assay with a virus-specific monoclonal antibody (mAb) 2D6 (Pfeiffer et al, 1995). The infection was also verified by Western blots using the virus-specific monoclonal antibody (data not shown).

### 3.4 Nuclear and Cytoplasmic Protein Fractionation:

In order to analyse the expression and DNA-binding activity of E2F factors in the nuclear and cytoplasmic compartments, the extraction of proteins from these two compartments was performed by the method as described (Advani et al, 2000). Briefly, the virus infected and mock-infected cells were counted and 5 million cells

were harvested by pelleting at 500xg for 10 minutes at 4°C. The cells were washed with chilled, sterile phosphate buffer saline (PBS) and again pelleted by centrifugation for 10 minutes at 4°C. The pellet was resuspended gently by pipetting up and down 10 times in 3 packed cell volumes (PCV) of hypotonic lysis buffer containing 10mM HEPES (pH 7.5), 3mM $MgCl_2$, 1mM EDTA, 10mM KCl, 0.05% NP-40, 10mM NaF, 10mM β-glycerophosphate, 1mM dithiothreitol (DTT), 0.01mM sodium orthovanadate, 0.5mM phenylmethylsulfonyl fluoride and was kept on ice for half an hour. The partially lysed cells were centrifuged at 500xg for 5 min at 4°C. The supernatant, which contained the cytoplasmic fraction of proteins, was transferred to another chilled eppendorf tube. The pellet, containing nuclei, was washed gently in the hypotonic lysis buffer and pelleted again as mentioned above. The nuclei were lysed by resuspending the pellet in a high salt buffer containing 50mM HEPES (pH 7.9), 250mM KCl, 0.1% NP40, 0.1 mM EDTA, 10mM NaF, 10mM β-glycerophosphate, 1mM DTT, 0.1mM sodium orthovanadate, 0.5mM phenylmethylsulfonyl fluoride, 5% glycerol at 4°C for 30 min. The nuclear lysate was centrifuged at 18000x g for 10 minutes at 4°C.

Fractionation of the cytoplasmic and nuclear extracts of the mock-infected cells was performed in the same way as for the virus infected ones. Protein concentrations of the fractions were determined by using a commercial protein detection kits (BCA protein assay kit; Pierce, Rockford, IL) and were equalized between mock and HHV-6 infected cells. Both the cytoplasmic and nuclear extracts were stored at −84°C until used for analysis.


## 3.5 Preparation of E2F Specific Oligonucleotide Probes

The DNA-binding activity of the E2F factors was determined by using a $^{32}$P-labelled double-stranded (ds) 27 base pair oligonucleotide probe in gel-shift and supershift assays. The probe contained the canonical E2F-binding sequence motif and was purchased from a commercial source included in the Gelshift Cell Cycle Regulator Kit (Active Motif, Carlsbad, CA; Cat no. 37331). The sequence of wild type E2F consensus element is 5' – G G T T TG T G T T **T A G G C G C G A A A** A C T G A A- 3'. The boldfaced nucleotides in the sequence represent the E2F binding motif. A mutant ds oligonucleotide was also included in the kit and was used as a control for the binding specificity of the E2F factors with the wild type oligonucleotide probe in gel shift assays. The mutant oligonucleotide has the same sequence as the wild type one except that two key bases in the E2F binding site are mutated in it. Its sequence is 5' – G G T T T G T G T T T A G G **T A** C G A A A A C T G A A- 3'. The two boldfaced nucleotides in the sequence represent mutated ones with respect to the wild type oligonucleotide. E2F do not bind the mutant oligonucleotide probe.

The wild type oligonucleotide probe was end labelled with $^{32}$P as follows: 50 ng of the unlabeled probe, 2 μl of 10x kinase buffer, 40 μCi of [γ-$^{32}$P] ATP [(specific activity 10 mCi/ml) Amersham Biosciences, Piscataway, NJ] and 10U of T4 polynucleotide kinase (MBI Fermentas Inc., Ontario, Canada; Cat # EK0031) were mixed in the final volume of 20μl with deionised water. The mixture was incubated for 30 minutes in the water bath at 37°C. At the end of the reaction, 5 μl of a solution containing 1% (w/v) SDS and 100Mm EDTA was added to stop the kinase reaction. Labelled oligonucleotide probe was separated from free [γ-$^{32}$P] ATP by column chromatography using Sephadex G-25 spin columns (Microspin G-25; Amersham Biosciences, Piscataway, NJ Cat. # 27-5325-01) and stored at -20° C.

### 3.6 E2F Gel Mobility Assays and Supershift Assays

To find out the effect of HHV-6 infection on the binding efficiency of E2F with its cognate DNA sequence, electromobility shift assay (EMSA) was performed. For this purpose, a commercially available kit was used (Gel Shift Cell Cycle Regulator kit; Active Motif, Carlsbad, CA, Cat. # 37331). The assay was performed according to manufacturers' instructions. For each assay, 10 $\mu$g of the protein, 1ng of $^{32}$P-labelled E2F-specific probe were mixed with the reaction mixture in 25 $\mu$l final volume. The reaction mixture contained sheared salmon sperm DNA to reduce non-specific binding of proteins to the probe. The specificity of the E2F binding to the probe was verified by competition with 100-fold molar excess of unlabeled E2F-specific oligonucleotide. The binding reactions were carried out for 30 minutes at room temperature. After the incubation, the entire content of each reaction tube was loaded directly onto a 5% non-denaturing polyacrylamide gel. The gel was prepared by mixing 6.7 ml 30% polyacrylamide (29 gram polyacrylamide and 1 gram bis-acrylamide in 100 ml of $H_2O$), 29.0 ml water, 4 ml 10x Tris, Borate, EDTA buffer (TBE), 280 $\mu$l of 10% (w/v) ammonium persulfate solution and 40 $\mu$l TEMED in 40 ml gel. In order to equilibrate the gel, it was run empty (without samples) for one hour in pre cooled 1x TBE buffer. After loading the samples (the reaction mixtures), the gel was subjected to electrophoresis in 1x TBE buffer at 100 V. The gel migration was monitored by the migration of the loading buffer into a separate well. When the dye of the loading buffer reached within the bottom third of the gel (in about an hour), the electrophoresis was stopped. The gel was dried on 3 mm Whatman paper (VWR Scientific Products, West Chester, PA) and exposed to Kodak Biomax film at –20°C until desired signal intensity was achieved (1-3 days).

In order to identify the individual transcription factor bound to oligonucleotide probe, gel supershift assays were performed. For this purpose, the factor-specific or control antibodies (1.0 $\mu$g) were added to the reaction mixture approximately 20 minutes prior to the addition of the $^{32}$P labelled E2F-specific oligonucleotide probe as described (Hilton et al, 1995). Then the probe was added and the reaction mixture was incubated for an additional 30 minutes at room temperature and loaded onto the gel as described above.

**3.7 Western Blots:**

The expression of different E2F and their partner DP proteins in the HHV-6 infected and mock-infected cells were compared in the whole cell lysates as well as in nuclear and cytoplasmic fractions, by Western blots as described {Xu, Ahmad, et al. 2000 556 /id}). Briefly, whole cell lysates, nuclear and cytoplasmic fractions (20-25 $\mu$g) were mixed with SDS gel loading buffer containing 100mM Tris–Cl (pH 6.8), 4%(w/v) SDS, 0.2%(w/v) bromophenol blue, 20%(w/v) glycerol and 200mM DTT, boiled for 10 minutes and loaded on 12% SDS-PAGE in mini PROTEAN-II polyacrylamide gel electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA; Cat # 165-2957). The resolved proteins were transferred onto polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Nepean, Ontario, Canada; cat # IPVH 00010) using a semi-dry transfer system (Transblot; Bio-Rad Laboratories, Hercules, CA). Equal loading of proteins in different lanes of the gel was visually examined on membrane blots by Ponceau S (Sigma Aldrich, Milwaukee, WI; cat # 141194) staining as described (Bannur et al, 1999). After removing the stain with distilled water, the unbound sites on the membrane were blocked by one hour incubation at room temperature in PBS (pH 7.3) containing 5% non-fat milk and 0.005% Tween 20

(called blocking buffer). Thereafter, the blots were incubated with protein-specific primary antibodies. The bands were revealed with alkaline phosphatase (AP)-conjugated secondary antibodies and chromogenic substrates BCIP and NBT (both from Promega, San Luis Obispo, CA) as described (Xu et al., 2000). In some cases, protein bands were revealed on blots by enhanced chemiluminiscence by using a commercial kit (Vectastain, ABC-AMP, Vector Laboratories, Burlingame, CA; cat # AK-6601).

In order to determine which bands on the SDS-PAGE were phosphorylated, the nuclear extracts from the infected and mock-infected cells were treated with calf intestinal alkaline phosphatase (CIAP). For this purpose, 25ug of the extracts were incubated with 5 U of the phosphatase in the dephosphorylation buffer containing 10mM Tris-Cl, 1mM $ZnCl_2$ and 1mM $MgCl_2$ at 35°C for 30 minutes. The treated and untreated extracts were then loaded on SDS-PAGE as described above.

In order to compare the amounts of proteins loaded into different wells of the gel, the membranes were stripped off the bands by incubating them in a buffer containing 0.2 M Glycine-HCl (pH 2.5) , 0.05% Tween-20 and 100 mM 2-Mercaptoethanol at 65°C for an hour. The membranes were later developed for $\beta$ -actin using a specific mAb (Sigma) and AP-conjugated secondary antibodies as described above.

## 3.8 Antibodies and Reagents

Alkaline phosphate (AP)-conjugated anti-rabbit IgG (Fc) was from Pierce (Pierce, Rockford, IL; Cat. # 31235). Antibodies for pRB (cat # SC 50X), p130 (cat # SC 317X), p107 (cat # SC 318X), p300 (cat # SC 584), HDAC1 (cat # SC 7872), E2F-1 (cat # SC 193), E2F-2 (cat # SC 632), E2F-3 (cat # SC 878), E2F-4 (cat # SC 860),

E2F-5 (cat # SC 999), E2F-6 (cat # SC 8175), DP-1 (cat # SC 610) and DP-2 (cat # SC 829) were all purchased from Santa Cruz (San Jose, CA).

# RESULTS

# CHAPTER IV

## Results

### 4.1 HHV-6 INDUCED CHANGES IN THE EXPRESSION OF E2F TRANSCRIPTION FACTORS

In order to study the expression of various E2F factors, we performed Western blots on nuclear and cytoplasmic extracts of HHV-6 and mock infected HSB-2 cells using factor-specific antibodies as described in the Materials & Methods. Below, are the results obtained for each of the factors.

#### 4.1.1 E2F-1:

Despite more loading of proteins for mock-infected lanes (as judged by the intensity of actin bands), the E2F1 is expressed relatively more in the nuclear fraction of the infected cells as compared to the mock-infected ones, especially for slowly migrating forms of the factor (Figure 9). Of note is the presence of a relatively higher molecular weight E2F-1 species (near 105 kDa) in the cytoplasm of the mock-infected cells. This band is replaced by novel band near the 75 kDa mark in the cytoplasm of the infected cells.

#### 4.1.2 E2F-2:

The expression of the most prominent band of E2F-2 is clearly decreased in the cytoplasm of the infected cells. Its comparable band runs as a doublet in the nucleus of the mock-infected cells. In the infected cells, this doublet runs as a single band representing its upper isoform (Figure 10). Furthermore, note the presence of 3 bands in the nuclear fractions of the infected cells as compared to 5 bands in the same fraction of the mock-infected cells.

#### 4.1.3 E2F-3:

In the cytoplasm of infected cells the overall expression of E2F-3 is decreased. The virus infected cells showed three major bands in the cytoplasm as compared to four bands in the mock-infected cells. The slow-migrating band

seems to have disappeared from the infected cells' cytoplasm. The E2F-3 present in the nucleus migrated as a doublet in the mock-infected cells, but only the upper of the doublet was present in the nuclear fraction of the infected cells (Figure 11).

### 4.1.4 E2F-4:

It appears that the overall expression of this factor is increased in the infected cell extracts. Prominent qualitative changes were seen in the expression of E2F-4 in the cytoplasm between the infected and mock-infected cells. The HHV-6 infected cells expressed six bands in their cytoplasmic fraction, whereas only five bands were present in the same fraction of the mock-infected cells. Two novel bands of E2F-4 migrating close to 50 kDa were exclusively found in the cytoplasm of the infected cells. Overall the E2F-4 expression was increased in the cytoplasmic fraction in the virus-infected cells. Of note is the presence of a band near 75 kDa in the cytoplasmic fraction of the mock-infected cells. The equivalent band in the cytoplasmic fraction of the infected cells migrated more slowly. Significant qualitative changes were also observed in the expression of this factor in the nuclear fractions between the infected and mock-infected cells (Figure 12). It is noteworthy that the two novel bands detected in the cytoplasmic fractions of the virus-infected cells are also present in the nuclear fractions of the infected cells.

### 4.1.5 E2F-5:
E2F-5 expression remains unchanged in both cytoplasmic and nuclear compartments of the mock and HHV-6 infected cells (Figure 13).

### 4.1.6 E2F-6:
The expression of E2F-6 is increased in both fractions of the infected cells. There appears a relatively higher molecular weight species of this factor in the

nucleus of infected cell (See lane 5, Figure 14). Three bands of the factor are relatively more abundant in the cytoplasm of the HHV-6 infected cells as compared to the mock-infected cells (Figure 14).

### 4.1.7 DP-1:

Four bands were observed on Western blots for this protein in the cytoplasmic extracts of the mock-infected cells, whereas only three bands were observed in the cytoplasmic extracts of the virus-infected cells.

A band migrating near the 25 kDa mark in the cytoplasmic fraction of the mock-infected cells disappeared in the infected cells. Notably, the most prominent band was increased in intensity in the cytoplasm of the infected infected cells. No significant changes were observed in the nuclear fractions between infected and mock-infected cells except for the appearance of a novel slow migrating band (near the 75 kDa mark) in the infected cells (Figure 15).

### 4.1.8 DP-2:

The expression of this E2F partner, particularly of its major band running close to the 50 kDa mark, was significantly decreased in the nuclear fractions in the virus-infected cells as compared to the mock-infected ones. Furthermore, a prominent dublet band running near the 50 kDa mark in the cytoplasmic fraction of the mock-infected cells was seen as a single band in the virus-infected cells. The single band represented lower partner of the doublet of the mock-infected cells (Figure 16). The overall expression, however, was remained the same in the infected and mock-infected cells (Figure 16).

### 4.2. EFFECT OF ALKALINE PHOSPHATASE ON THE EXPRESSION PATTERNS OF E2F ON WESTERN BLOTS

**Figure 9. The expression of E2F-1 in the nuclear and cytoplasmic fractions of HHV-6-infected and mock-infected cells.**
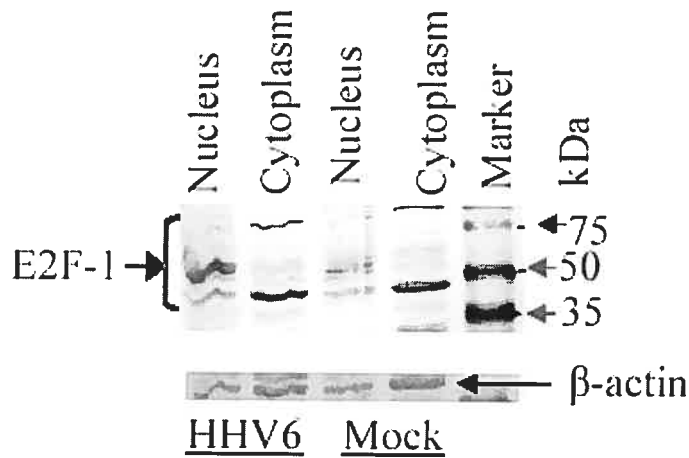


Twenty $\mu$g of proteins from each of the nuclear and cytoplasmic extracts of the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. After blotting onto the PVD membrane, the blots were developed using E2F-1 specific antibodies and AP-conjugated anti rabbit antibodies. After stripping, the membranes were developed for $\beta$-actin. The E2F1-specific bands and molecular weight markers are indicated by arrows. Due to incomplete stripping some previous bands reappeared on the blot after staining for $\beta$-actin.

**Figure 10. E2F-2 immunoblot of cytoplasmic and nuclear fractions of mock and HHV-6 infected HSB-2 cells**
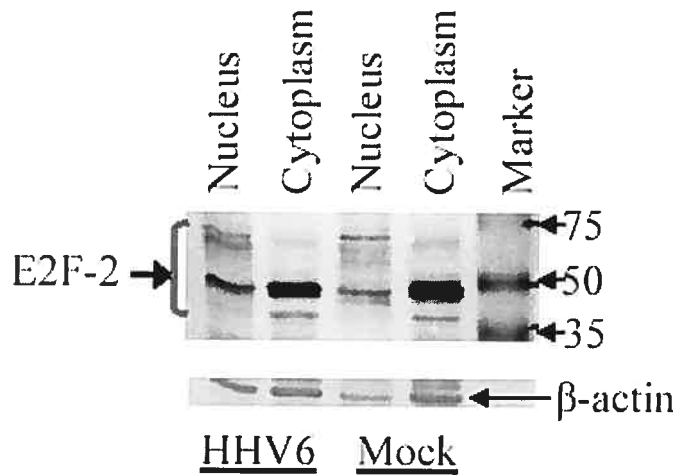


Proteins from the nuclear and cytoplasmic extracts (25 µg) of the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. After blotting onto the PVD membrane, they were developed using E2F-2 specific antibodies and AP-conjugated secondary rabbit antibodies. After stripping, the membrane was developed for $\beta$-actin for loading control. The the bands of interest and molecular weight markers are indicated by arrows. Some of the previous bands can be seen on the membrane developed for $\beta$-actin due to incomplete stripping.

**Figure 11. The expression of E2F-3 in the nuclear and cytoplasmic fractions of HHV-6-infected and mock-infected cells**



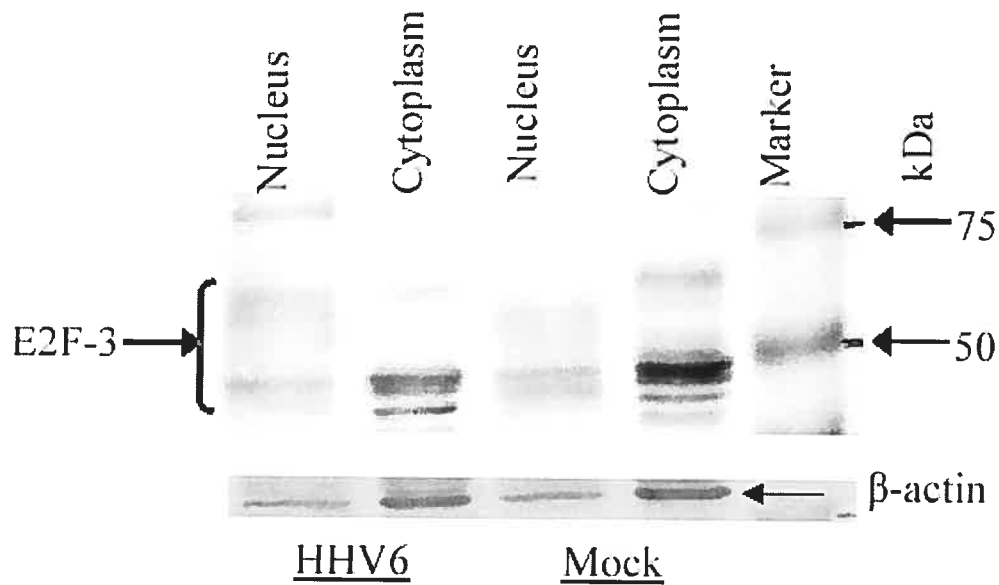Nuclear and cytoplasmic extracts (20 μg each) of the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. The proteins were then transferred onto the PVD membrane and developed using E2F-3 specific antibodies and AP-conjugated secondary rabbit antibodies. After, the membrane was stripped and developed for β-actin. The molecular weight markers and the bands of interests are indicated by arrows. Some of the previous bands can be seen alongwith the β-actin due to incomplete stripping of the membrane.

**Figure 12. E2F-4 immunoblot of cytoplasmic and nuclear fractions of mock and HHV-6 infected HSB-2 cells**
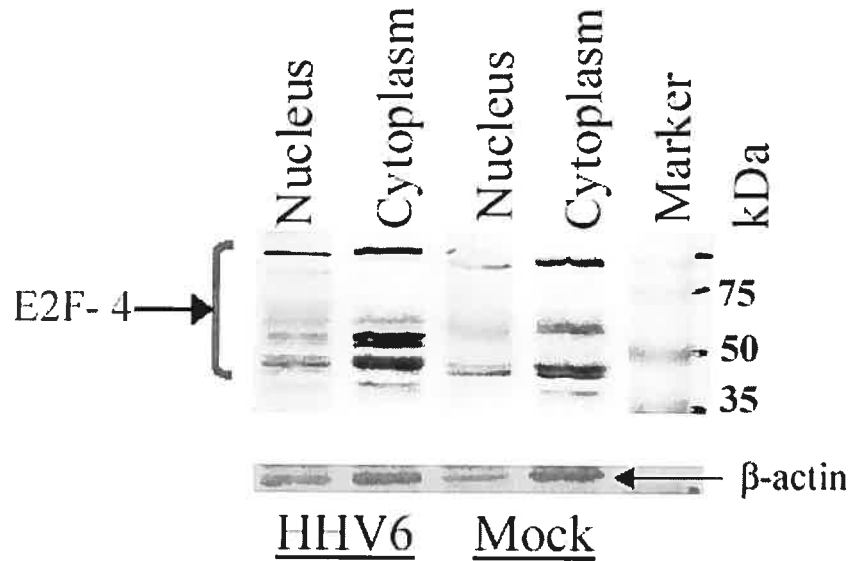


Twenty five µg of proteins from the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. The proteins from the gel were transferred onto the PVD membrane and were developed using factor specific antibodies and AP-conjugated secondary antibodies. After, the membrane was stripped and developed for β-actin. The arrows indicate the E2F-4-specific and β-actin bands.

**Figure 13.    E2F-5 expression in cytoplasmic and nuclear fractions of mock and HHV-6 infected HSB-2 cells**



For each extract eighteen $\mu$g of proteins of the virus-infected and mock-infected cells were run on 10% SDS-PAGE. After, they were bloted onto the PVD membrane, and developed by using factor specific antibodies. then, the membrane was stripped and developed for $\beta$-actin as loading control. The arrows indicate the bands of interests and molecular weight markers. Above the $\beta$-actin band some of the bands from the previous development can be seen due to incomplete stripping.

**Figure 14.  E2F-6 immunoblot of cytoplasmic and nuclear fractions of mock and HHV-6 infected HSB-2 cells**
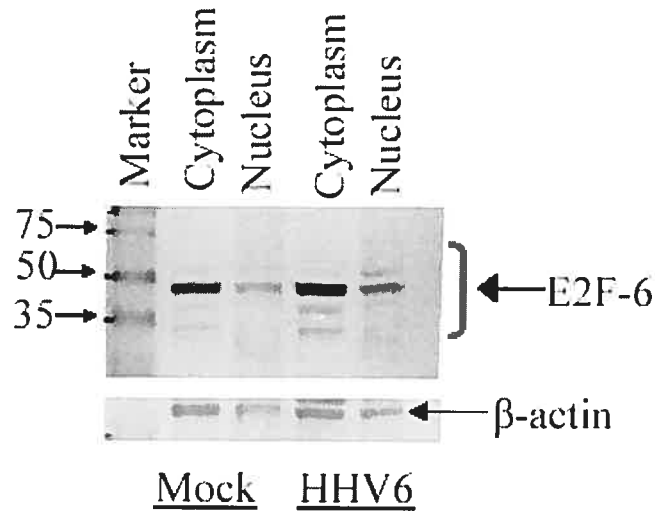


Twenty µg of proteins from each of the fractions from virus-infected and mock-infected cells were run on 10% of SDS-PAGE. After blotting onto the PVD membrane, the blots were developed using E2F-6 specific antibodies and AP-conjugated secondary antibodies. Then the membrane was stripped and developed for β-actin as loading control. The bands of interests and molecular weight markers are indicated by arrows. Some of the previous bands are visible alongwith β-actin bands as the stripping of all the bands from the previous development was not possible.

**Figure 15.** **The expression of DP-1 in the nuclear and cytoplasmic**
**fractions of HHV-6-infected and mock-infected cells**
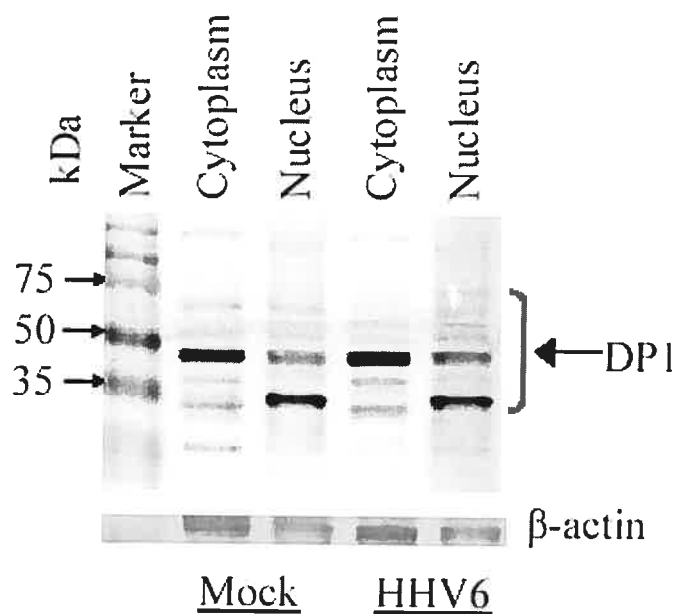


The extracts (25 $\mu$g each) from the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. After blotting onto the PVD membrane, the blots were developed for DP-1 using specific polyclonal antibodies. After stripping, the membranes were developed for $\beta$-actin. The DP-1 specific bands and molecular weight markers are indicated by arrows.
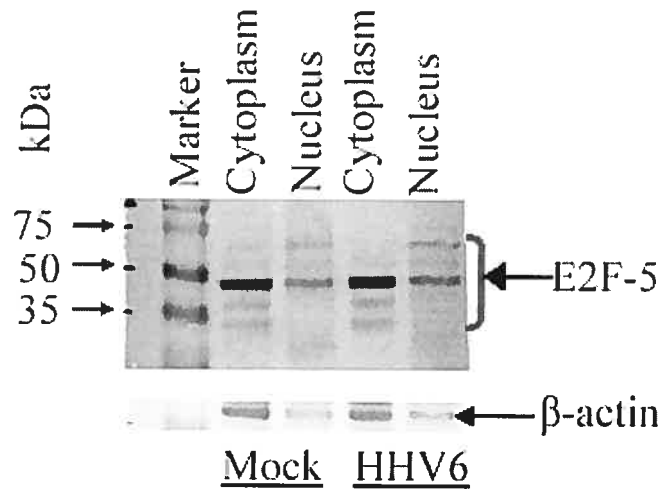
**Figure 16. DP-2 immunoblot of cytoplasmic and nuclear fractions of Mock- and HHV-6-infected HSB-2 cells**
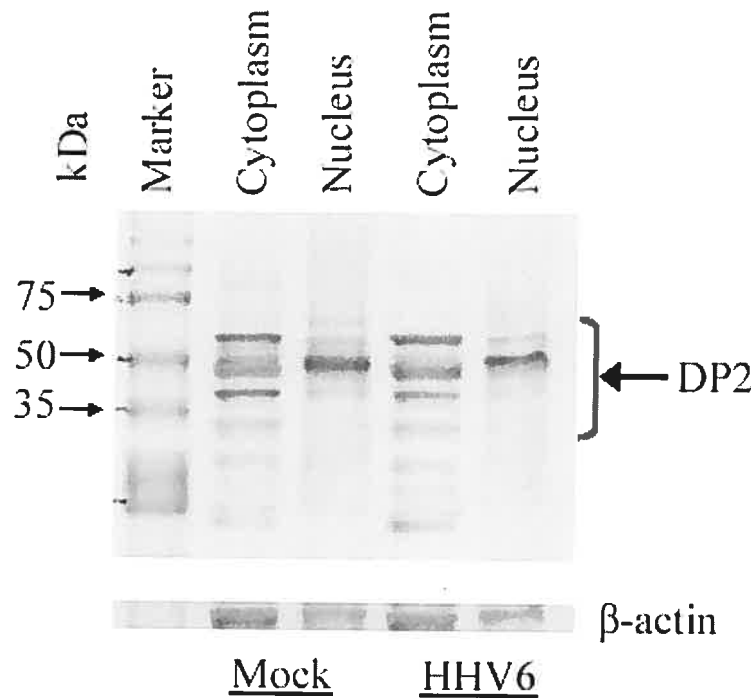


Eighteen $\mu$g proteins from each of the nuclei and cytoplasm of the virus-infected and mock-infected cells were run on 10% of SDS-PAGE. After they were blotted onto the PVD membrane and developed using DP-2 specific polyclonal antibodies and AP-conjugated secondary antibodies. After stripping, the membranes were developed for $\beta$-actin. The DP-2-specific bands and molecular weight markers are indicated by arrows.

As mentioned in the INTRODUCTION section, the E2F transcription factors are mainly regulated by phosphorylation. Therefore, in order to find out the phosphorylated status of these factors, we treated nuclear extracts from the infected and mock infected cells with Calf Intestinal Alkaline phosphatase and determined the expression pattern of these factors on Western blots as described in Materials and Methods. The multiple bands observed on the Western blots in the case of non treated extracts for E2F-1, E2F-4 and DP-1 were reduced to one or more bands migrating near the 50 kDa mark after the phosphatase treatment (see Figures 17, 19 and 20). However in the case of E2F2, the only faint bands were seen close to the 50 kDa mark. Most of the bands were reduced to one or two prominent bands seen below the 35 kDa mark (see Figure 18). These results suggest that most of the bands seen in the nuclear extracts of both virus and mock infected cells may be due to differential phosphorylation.

## 4.3 EFFECT OF THE VIRUS INFECTION ON THE DNA BINDING ACTIVITIES OF E2F:

In order to determine the effects of HHV-6 infection on the DNA binding activities of the E2F and their partner proteins, we performed gel shift assays using double stranded oligonucleotides containing E2F binding consensus sequence as described in Materials & Methods. The nuclear extracts of mock-infected cells showed at least three distinct gel shift bands. In the infected cell nuclear extracts, the low mobility band was not present. Moreover binding to the labelled oligonucleotide was highly increased in the infected cells as reflected by a higher density of the bound complexes (Figure 21). The binding activity is specific as it is being competed by non-labelled wild type

**Figure 17.** **The effect of phosphatase treatment on the expression pattern of E2F1 in the HHV-6-infected and mock-infected cells**
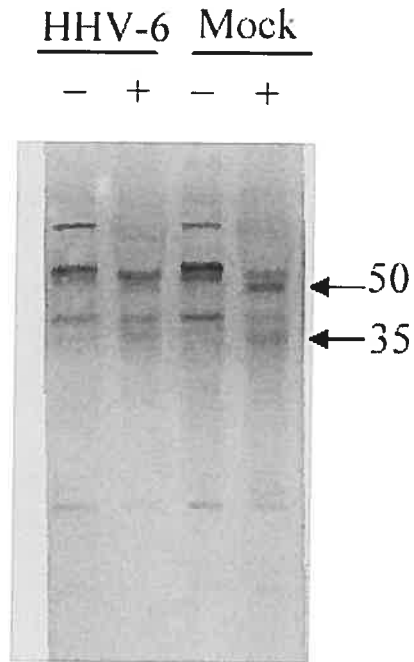


Twenty five μg proteins from the nuclear fraction of the cells were treated with calf intestinal alkaline phosphatase, and run on 10% SDS-PAGE. After transfer of the proteins onto the PVD membrane, the blots were developed for E2F-1. The + and − above each lane indicates treatment with the phosphatase or not respectively. The arrows on the right show molecular weight.

**Figure. 18  The effect of phosphatase treatment on the expression pattern of**

**E2F-2 in the HHV-6-infected and mock-infected cells**
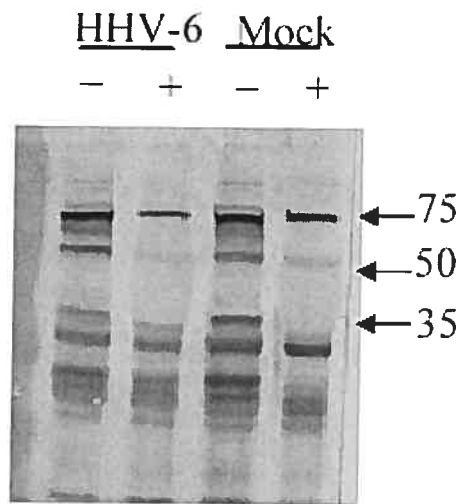


Nuclear proteins (25μg each) from the virus-infected and mock-infected cells were treated with calf intestinal alkaline phosphatase, and run on 10% SDS-PAGE. After transfer of the proteins onto the membrane, the blots were developed with antibodies against E2F-2. The + and − above each lane indicates treatment with the phosphatase or not respectively. The arrows on the right show molecular weight.

**Figure 19.  The effect of phosphatase treatment on the expression pattern of**

**E2F-4 in the HHV-6-infected and mock-infected cells**
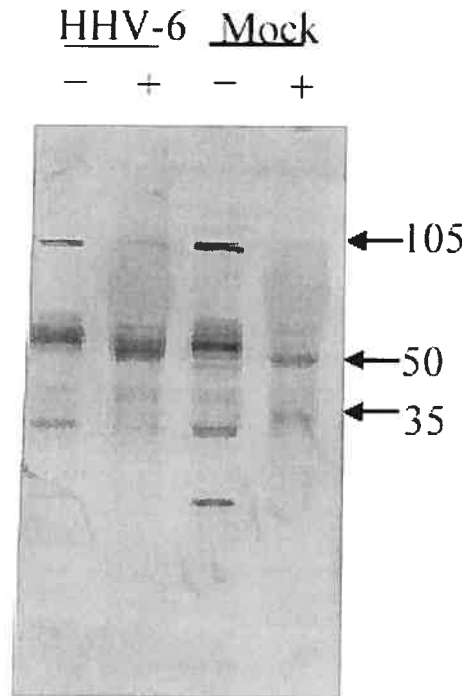


Equal amounts of nuclear proteins (25 μg) from each of the virus-infected and mock-infected cells were treated with calf intestinal alkaline phosphatase, and run on 10% SDS-PAGE. After transfer of the proteins onto the PVD membrane, the blots were developed for E2F-4. The + and – above each lane indicates treatment with the phosphatase or not respectively. The arrows on the right show molecular weight.

**Figure 20.** **The effect of phosphatase treatment on the expression pattern of**

**DP-1 in the HHV-6-infected and mock-infected cells**
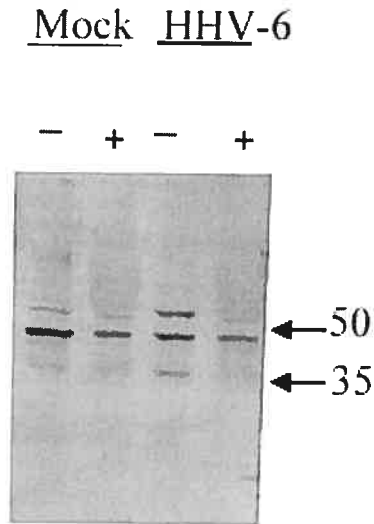


Twenty five μg nuclear proteins cells were treated with calf intestinal alkaline phosphatase, and run on 10% SDS-PAGE. The proteins were transferred to membrane and were developed for DP-1. The + and – above each lane indicates treatment with the phosphatase or not respectively. The arrows on the right show molecular weight.

**Figure 21.** Electrophoretic mobility shift assay for the nuclear extracts of HHV-6- and mock-infected HSB-2 cells
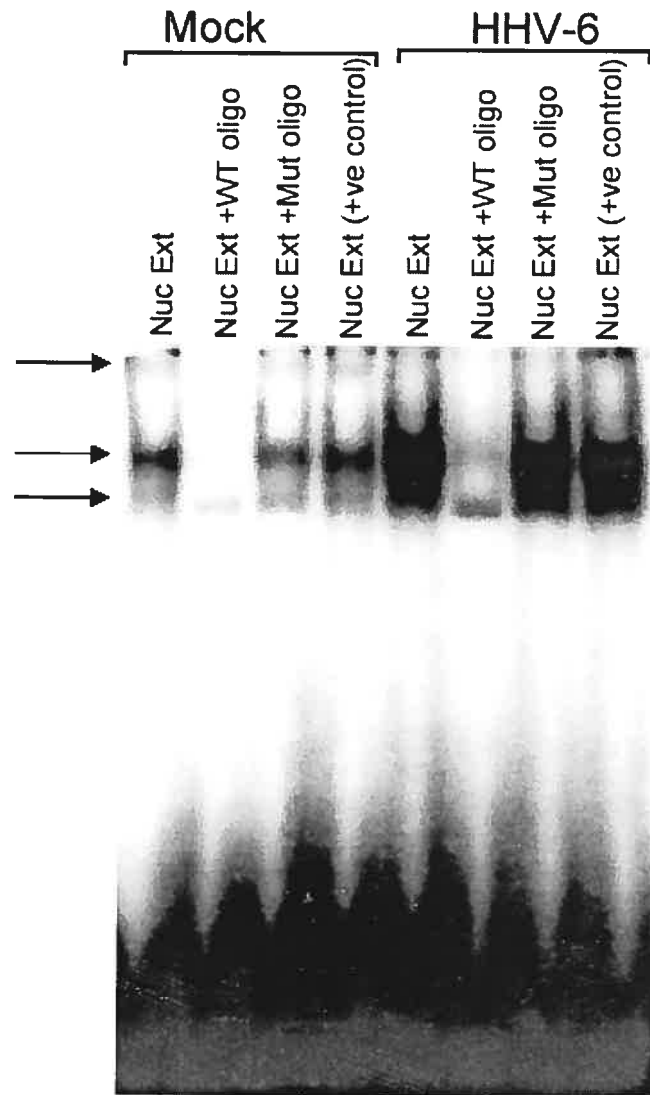


The nuclear extracts from the HHV-6- and mock-infected cells(10 $\mu$g each) were incubated with the $^{32}$P end-labelled ds oligonucleotide containing E2F-specific consensus sequences and run on 5% non-denaturing acrylamide gel. The gels were auto-radiographed using Kodak Biomax film. The arrows indicate the DNA-protein complexes. Wt, Wild type; Mut, Mutant; Nuc Ext, Nuclear extract.

oligonucleotide but not by non-labelled mutant oligonucleotide (Figure 21). The cytoplasmic fractions also showed the same banding pattern but lesser intensity as compared to the nuclear fractions (data not shown). Furthermore, a fast migrating complex appears in the infected cell extracts on longer exposure. This complex is not present in the mock-infected cells (Figure 22-23).

## 4.4 IDENTIFICATION OF THE E2F MEMBERS BOUND TO THE COGNATE DNA SEQUENCES:

To find out which E2F factors or their interacting proteins are present in the gel shift complexes in the infected and mock-infected cells, we performed supershift assays using antibodies specific for these proteins as described in the Materials & Methods. The antibodies specific for E2F 1-6, DP-1 and DP-2 supershifted the low migrating complex in the mock-infected cells (Figures 22 and 23). These supershifts were not seen with antibodies for E2F-2, E2F-5, E2F-6 and DP-1 for the HHV-6-infected cells. Furthermore, the supershifs by E2F-1, E2F-3 and E2F-4-specific antibodies are reduced in the infected cells. The E2F-5-specific antibodies supershift a relatively feeble band in the mock-infected cells. This supershifted band almost disappears in the infected cells. The E2F-6-specific antibodies supershift a relatively stronger complex, which is completely vanished in the infected cell nuclear extracts. The same pattern is seen for DP-1-specific antibodies in the infected cell nuclear extracts. For DP-2-specific antibodies, no change is seen in the binding pattern between mock- and HHV-6 infected cells.

**Figure 22. E2F DNA Electrophoretic Mobility Super Shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibodies against E2F-1,-2,-3 and –4**



The nuclear extracts from the HHV-6 and mock-infected cells were incubated with the $^{32}$P end-labelled ds oligonucleotide containing E2F-specific consensus sequences and antibodies against E2F-1, E2F-2, E2F-3 and E2F-4 and run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed using Kodak Biomax film. The arrows indicate the DNA-protein and E2F antibody complexes. Asteriks (*) to the left of the bands indicate the supershifted ones.

**Figure 23.** **E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibodies against E2F-5,-6,DP-1 and DP-2**
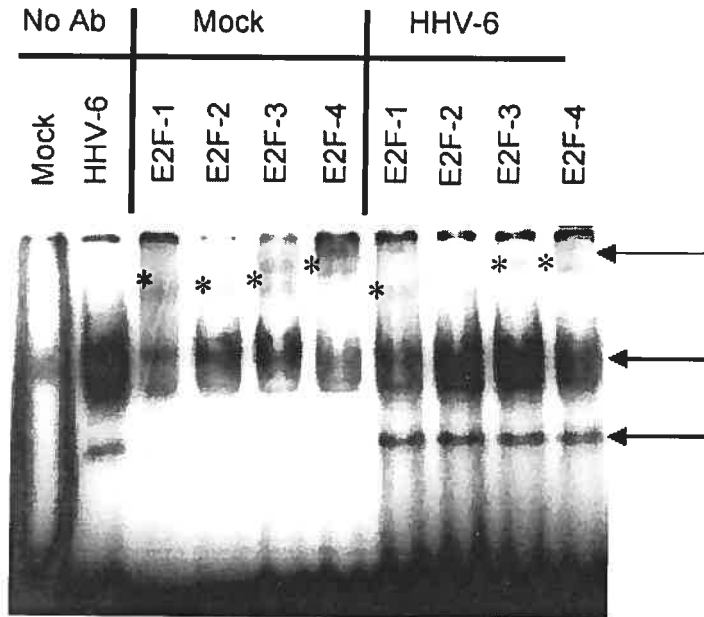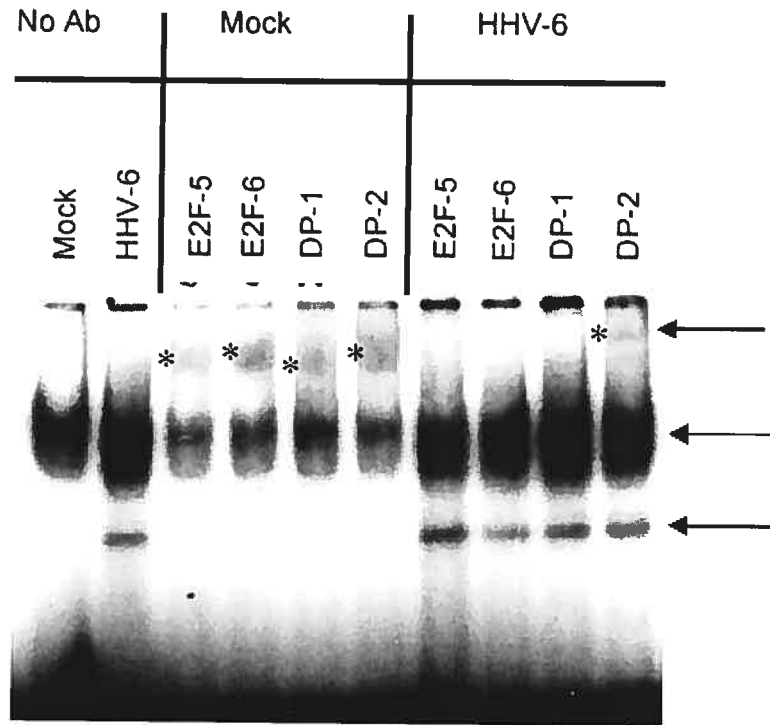


The nuclear extracts from the virus and mock-infected cells were reacted with the $^{32}$P labelled ds oligonucleotide containing E2F-specific consensus sequences and antibodies against E2F-5, E2F-6, DP-1 and DP-2 and run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed. The arrows indicate the DNA-protein and antibody complexes. Super shifted bands has been shown by asteriks (*) on the left of the band.

**Figure 24. E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibody against pRB**



The extracts from the were incubated with the $^{32}$P end-labelled ds oligonucleotide containing E2F-specific consensus sequences and antibody against pRB run on 5% non-denaturing acrylamide gel. The gels were auto-radiographed using Kodak Biomax film. The arrows indicate the DNA-protein and antibody complexes. Rabbit IgG (Rab IgG) and E2F-3 are used as negative and positive controls respectively. Asteriks (*) on the left of the bands show the supershifts.

**Figure 25. E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibody against p107**



The extracts from the nucleus of virus and mock-infected cells were made to react with the $^{32}$P end-labelled ds oligonucleotide containing E2F-specific consensus sequences and antibody against p107. It was run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed. The arrows indicate the DNA-protein and antibody complexes. Rabbit IgG (Rab IgG) and E2F-3 are used as negative and positive controls respectively. Super shifted complex has been shown by asteriks (*) on the left of the band.

**Figure 26.** E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibody against p130
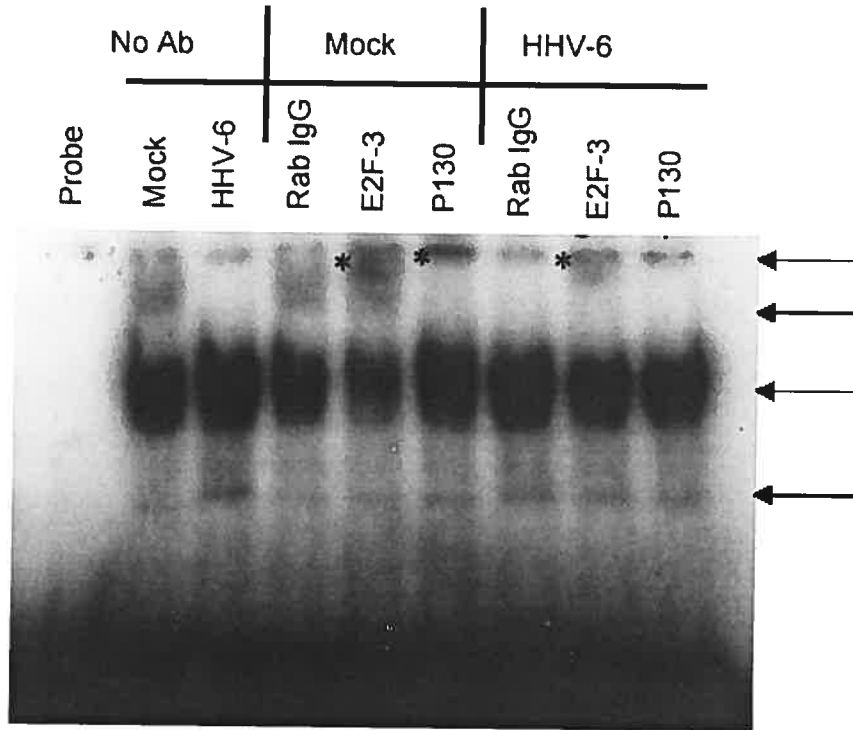


Mock and HHV-6 infected cells' nuclear extracts were incubated with the $^{32}$P end-labelled oligonucleotide containing E2F-specific consensus sequences and antibody against p130, run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed using Kodak film. The arrows indicate the DNA-protein and antibody complexes. Rabbit IgG (Rab IgG) and E2F-3 were used as negative and positive controls respectively. Asteriks (*) on left side of each band show the super shifted one.

**Figure 27. E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibody against HDAC1**
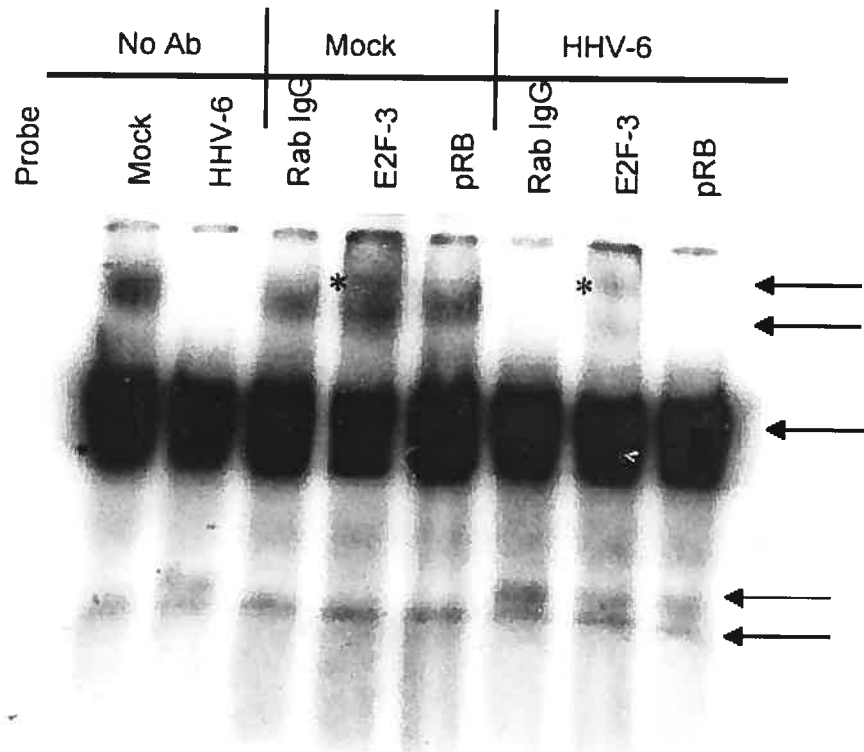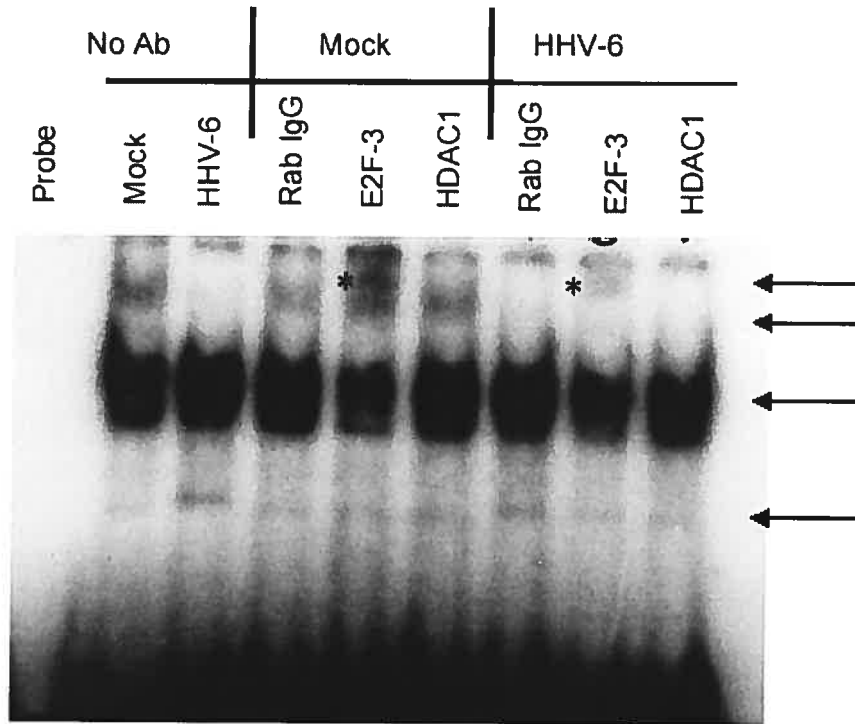


The extracts from the HHV-6 and mock-infected cells were reacted with the $^{32}$P end-labelled oligonucleotide containing E2F-specific consensus sequences and antibody against HDAC1 run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed. The arrows indicate the DNA-protein and antibody complexes. Rabbit IgG (Rab IgG) and E2F-3 are used as negative and positive controls respectively. Asteriks (*) on the left of each band show the super shifted complex.

**Figure 28.  E2F DNA Electrophoretic mobility super shift assay for the nuclear extract of mock infected and HHV-6 infected HSB-2 cells using antibody against P300**
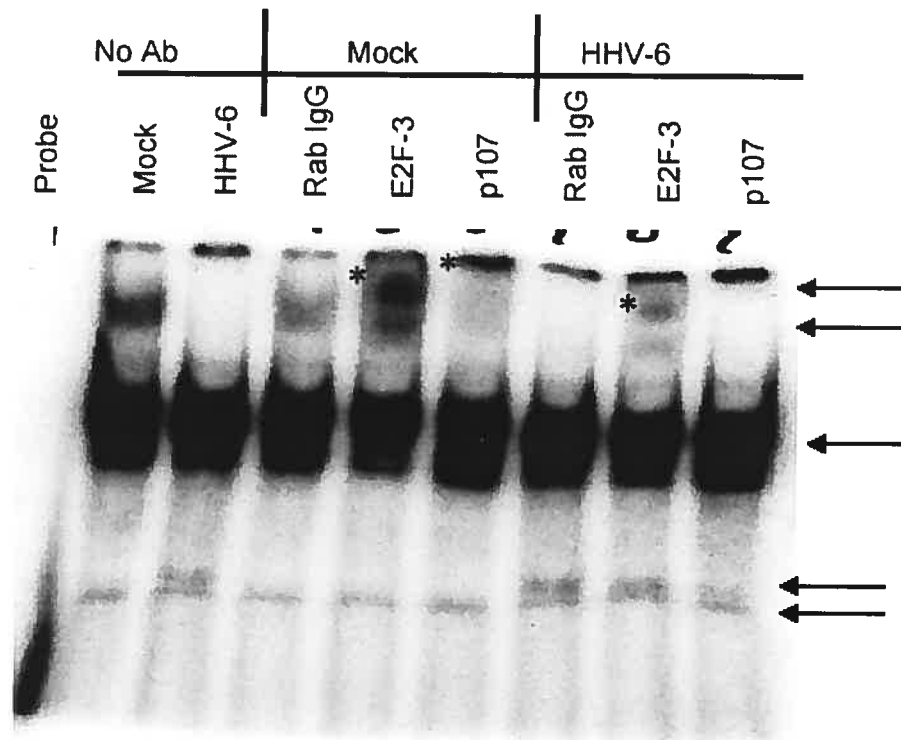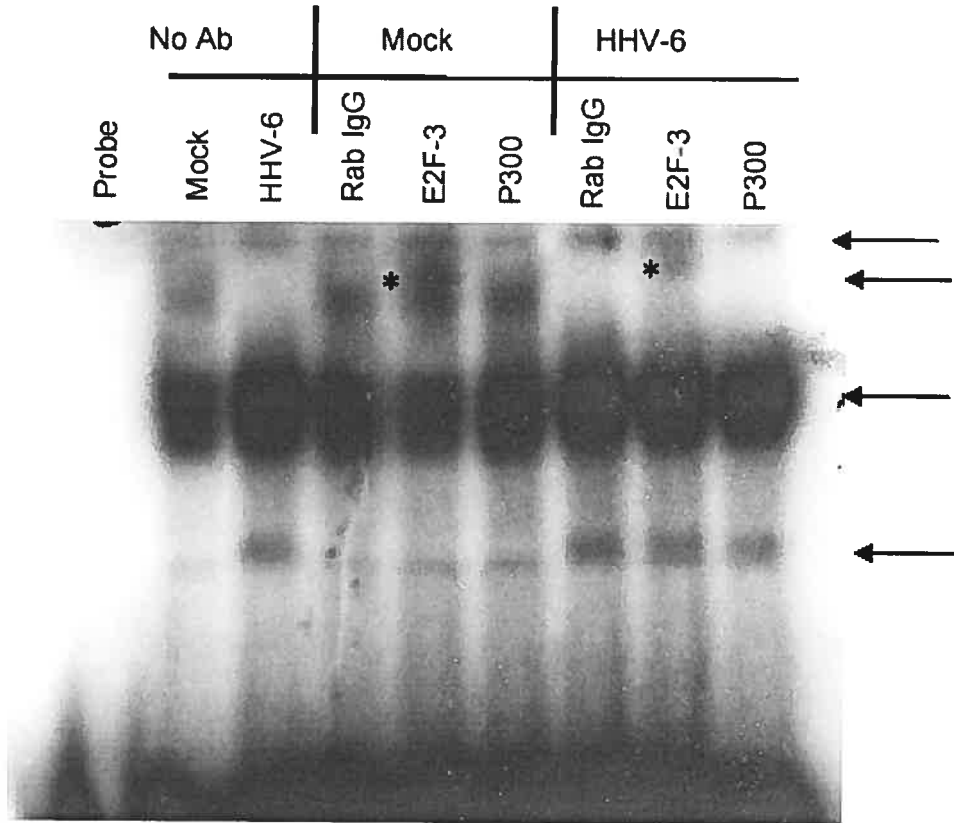


The nuclear extracts from the cells were incubated with the $^{32}$P labelled ds oligonucleotide containing E2F-specific consensus sequences and antibody against p300 and run on 5% non-denaturing acrylamide gel. The gels were dried and auto-radiographed. The arrows indicate the DNA-protein and antibody complexes. Rabbit IgG (Rab IgG) and E2F-3 are used as negative and positive controls respectively. Super shift has been shown by the asteriks (*) on the left of the bands.

The antibodies specific to p107 and p130, but not to pRB, supershifted the oligonucleotide-bound slow migrating complexes in the mock-infected cells. Interestingly, the complexes were completely supershifted with anti-p130 and p107 antibodies. This supershift is not seen in infected cells (Figures 24, 25 and 26). It is noteworthy that, antibodies against p300 and histone deacetylase (HDAC-1) did not react with the complexes from both the infected and mock-infected cells (Figures 27 and 28).

We also performed gel shift and supershift assays for the cytoplasmic fractions of mock- and HHV-6 infected cells. The results were almost similar to those obtained with the nuclear extracts (data are not shown).

# DISCUSSION AND CONCLUSIONS

# CHAPTER V

## Discussion and Conclusions

### Discussion:

The E2F comprises a family of transcription factors that functions as heterodimers with DP-1 or DP-2. Their role in the progression of cell cycle and DNA replication has been well characterized. They also play a role in the differentiation, growth and apoptosis of cells (reviewed in Stevens and La Thangue, 2003; reviewed in Ginsberg, 2002). As stated above, these factors have been divided into three functional groups. The members of a group perform distinct as well as overlapping functions. Because of their importance in cell cycle progression and DNA synthesis, it is not surprising that viruses have evolved strategies to utilize E2F for manipulating cell cycle progression in the virus-infected cells and to use them for their own replication. E2F are functionally regulated largely by their interaction with pRB. Three families of DNA viruses, papillomaviruses, adenoviruses and papovaviruses exploit these interactions. They target pRB, disrupt pRB-E2F interactions and activate E2F. The adenovirus E1a, the papillomavirus E7 and the simian virus-40 T antigen have been demonstrated to bind pRB (Decaprio et al, 1988; Dyson et al, 1989; Whyte et al, 1988). The virus-activated E2F factors play an important role in the cell transforming functions of these viral antigens.

In the case of Herpesviruses, the family to which HHV-6 belongs, several viruses have been known to induce changes in the expression and functional activities of E2F factors. For example, LMP-1, the major oncoprotein of the Epstein-Barr virus inhibits p16 INK mediated cell cycle arrest by downregulating the expression of E2F-4 and

E2F-5. LMP-1 plays an important role in cell proliferation and tumor formation by blocking the functions of these transcription factors (Ohtani et al., 2003).

Another member of this family, the human cytomegalovirus (HCMV), encodes a viral kinase (IE 72) that phosphorylates F2F1-3, p130 and p107 (Pajovic et al 1997). This increases the DNA binding ability of the E2F and decreases association of E2F-4 with p107 and p130. Similarly, E2F-1, E2F-4 and E2F-5 are post-translationaly modified and show a reduction in their DNA binding activities in the HSV-1 infected cells (Advani et al, 2000; Olgiate et al, 1999; Hitton et al, 1995). The overall aim of the viruses is to modify the internal microenvironment of the infected cells in order to favor viral replication.

HHV-6 is a member of the Herpesvirus family. It infects mainly CD4+ human T cells. The virus induces profound changes in the infected cells: it causes accumulation of the infected cells in G2/M phase, induces apoptosis, inhibits proliferation of mitogen-activated T cells and stimulates host cell macromolecule synthesis (De Bolle et al., 2004; Ichimi et al., 1999). Since E2F are known to play a role in all these cellular processes, it is quite conceivable that HHV-6 infection may cause changes in the expression and functional activities of these transcription factors. The present study, therefore, was undertaken to address these issues.

In order to investigate changes in the expression of various E2F factors, we performed Western blots using specific antibodies on cell lysates from mock- and HHV-6 infected cells. Since these factors may be localized in the nuclear and/or cytoplasmic compartments of the cell, we isolated nuclear and cytoplasmic fractions and determined the expression of the factors in these fractions separately. Our results show that HHV-6 infection induces significant quantitative as well as qualitative changes in the expression of these factors in the infected cells. We observed that:

The expression of E2F-1, which is involved in the cell proliferation as well as apoptosis, was slightly increased in both compartments in the infected cells. Of note was the disappearance of a slow migrating band (near 105 kDa mark) in the cytoplasm of the infected cells (Figure 9). This was replaced by a novel band of about 75 kDa in the cytoplasm in these cells. We speculate that these two bands may represent differentially phosphrylated forms of E2F-1. No other changes were observed in the expression of E2F-1 in the nuclear fractions between infected and mock-infected cells. The DNA binding activity of this factor was decreased in the nuclear extracts of the infected cells (also see below). The changes observed in the expression of E2F-1 in the nuclear and cytoplasmic fractions of the infected cells cannot explain reduced activity of this fctor in gel shift assays. Clearly further studies are needed to learn why the DNA binding activity of E2F-1 is reduced in the nuclear extracts of the infected cells.

We observed qualitative changes in the expression of E2F-2 in the nuclear fractions of the virus-infected cells (3 bands) as compared to the mock-infected cells (5 bands). It appears that posttranslational modifications of the factor are responsible for the changes observed on the Western blots. It may be noted that phosphatase treatment of the nuclear fractions eliminated most of these bands on Western blots suggesting that differential phosphorylation of this factor in the nuclear extracts of the infected and mock-infcted cells are the major post-translational modification responsible for the qualitative changes observed on the Western blots here. Interestingly, anti-E2F-2 antibodies supershifted DNA-protein complexes in the supershift assays when nuclear extracts from the mock-infected cells were used but not when the extracts from the HHV-6-infected cells were used. These data suggest that within the nuclei of the infected cells E2F-2 are unable to bind their cognate DNA sequences. It is likely that

sequences. It is likely that these factors are modified in the infected cells in such a way that they are unable to bind their cognate DNA sequences. Alternatively, they may be competed out by other family members, which may bind these DNA sequences more efficiently. However, given the fact the supershifts with the antibodies for other E2F family members are weaker in the infected cell nuclear extracts then for similar extracts from the mock-infected cells, the latter possibility is very unlikely.

The changes observed in the expression of E2F-3 in the nuclear fractions of the infected and mock-infected cells were minimal and mainly of the quantitative nature. Interestingly, this factor bund to the DNA-protein complexes both in the infected and mock-infected cells in the gel shift assays, albeit less so in the case of the virus-infected cells, as the E2F-3-specific antibodies-mediated supershift was less prominent in the nuclear extracts of the infected cells as compare to the mock-infected cell extracts (Figure 11).

It is noteworthy that E2F are frequently modified post-translationally by phosphorylation. Furthermore E2F-1, -2 and -3, but not other members of the family, are also modified by acetylation at lysine residues (Martinez-Balbas, 2000). Clearly, further studies are needed to determine whether these E2Fs are acetylated in the virus-infected cells. Collectively, our DNA binding tests using gel shift and supershift assays indicate that E2F-1, -2 and -3 bind cognate DNA sequences very efficiently in mock-infected cells but not in the case of HHV-6-infected cells. These data also suggest that E2F1-3 are modified in the nuclei of the infected cells in a way that they either do not bind or bind less efficiently with their cognate DNA sequences. How the virus achieves this, in addition to the differential phosphorylation of these factors, is not known.

We observed very interesting changes in the E2F-4 in the virus-infected cells as compared to the mock-infected ones. The overall expression of the factor seemed to be increased in the infected cells. The most interesting change was the appearance of two novel bands in the cytoplasmic as well as in the nuclear fractions of the virus-infected cells. Upon treatment of the nuclear fractions from the virus-infected cells with CIAP, all the differentially migrating bands were reduced to a prominent band near 50 kDa. The nuclear fraction of the mock-infected cells led to several diffuse bands around the 50 kDa mark. These data suggest that the two novel bands present in the nuclear and cytoplasmic fractions of the virus-infected cells resulted from the differential phosphorylation of E2F-4.

E2F-4, unlike E2F1-3, prevents cell proliferation and the transcription of E2F-responsive S-phase genes by recruiting p130 and p107 and hence histone deacetylases to the E2F binding sites into the cell nuclei. However our gel shift assays show that this factor is contained in the E2F-oligonucleotide complexes in the mock and virus-infected cells. Interestingly, anti-E2F-4 antibodies supershift relatively smaller amounts of the complex in the infected cell nuclear extracts as compared to these extracts from the mock-infected cells. Therefore, it is unlikely that HHV-6 uses this factor to counter the activities of other members of the family. Further work is needed to understand the real significance of the HHV-6-induced post-translational changes in E2F-4.

We did not note any significant change in the expression of E2F-5 between mock-infected and virus-infected cells both in the nuclear as well as in the cytoplasmic fractions of the cells. Despite this anti-E2F-5 antibodies could not supershift protein-olignucleotide complexes in the nuclear extracts of the virus-infected cells. These antibodies did supershift these complexes in the nuclear extracts of the mock-infected

cells. It is possible the subtle changes induced in this factor in the virus infected cells cannot be detected by Western blots.

The overall expression of the anti-proliferative E2F-6 was increased in both cytoplasmic and nuclear fractions of the virus-infected cells as compared to the mock-infected ones. We observed a novel slow migrating band (above the 50 kDa mark; see Figure 14) in the nuclei of the infected cells. As described above for E2F-5, anti-E2F-6 antibodies could not supershift any DNA-protein complex in the nuclear extracts of the virus-infected cells. Therefore, the significance of HHV-6-induced changes in this factor is not yet known.

Of the E2F partners, the expression pattern of DP-1 showed some changes both in the nuclear and cytoplasmic fractions between HHV-6-infected and mock-infected cells: the disappearance of a fast migrating band (near the 25 kDa mark) in the cytoplasmic fraction of the virus-infected cells that was present in the the mock-infected cells, the appearance of a novel slow migrating band (near the 75 kDa mark) I the nuclear fraction of the infected cells. However, the latter band is rather faint. Interesingly, the CIAP treatment of the nuclear fractions from the infected and mock-infected cells reduced most of the bands to a major band close to the 50 kDa mark (Figures 17-20). This suggests that the bands of DP-1 observed on the Western blots are mainly due to differential phosphorylation events. Interestingly, anti-DP-1 antibodies were able to supershift protein-DNA complexes in gel shift assays from nuclear extracts of the mock-infected cells but not from the virus-infected cells. It may be interesting to compare the ability of DP-1 from the infected and mock-infected cells to heterodimerize with E2F members.

We observed some changes in the expression of DP-2 between the virus-infected and mock-infected cells (Figure 16). The major band running close to the 50 kDa mark

was significantly reduced in the nuclear fractions of the virus-infected cells. The factor-specific antibodies were able to supershift DNA-protein complexes from the nuclear fractions of both the virus-infected and mock-infected cells. The supershifted complexes were, however, less prominent, which could be due to reduced expression of this factor in the nuclei of the infected cells.

The DP proteins, like their E2F partners, are phosphorylated by the cyclinA/CDK1 complex at the end of S phase (Krek et al., 1994). The overexpression of phosphorylated DP partners may induce cell cycle arrest in the G2/M phase. The accumulation of a hyperphosphorylated DP-1 (near the 75 kDa mark) in the nuclear fractions of the virus-infected cells is in accord with the observations that HHV-6 induces cell cycle arrest in the G2/M phase in the infected cells (De Bolle et al., 2004). Consistent with these observations, we did not observe any supershift with anti-DP-1 antibodies in the virus-infected cells. However, further studies are needed to confirm this conclusion.

We observed significant changes in the results of the gel shift assays with nuclear extracts between mock-infected and virus-infected cells. Firstly, three DNA-protein complexes were observed for the mock-infected cells as compared to the two for the virus-infected cells: the slow migrating complex being absent from the gel-shift assays for the virus-infected cells. Upon prolonged exposure, a fast migrating complex appeared only in the infected cell assays (Figures 22-23). This complex did not supershift with any of the antibodies used. The exact identity of the complex remains unknown.

While supershifts were observed with specific antibodies for all E2F members and their partners in the nuclear extracts from the mock-infected cells, no supershifts were observed with antibodies specific for E2F-2, E2F-5, E2F-6, and DP-1. These results

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suggest that these factors may be prevented from their inclusion into E2F-oligonucleotide complexes in the virus-infected cells. Alternately, they may have become inaccessible to these antibodies in these cells. It is noteworthy that all these factors, except E2F-5, did show some qualitative changes in their expression patterns on Western blots between mock-infected and virus-infected cells. There is no direct evidence to suggest that the changes observed in the expression of these factors on the Western blots may have been responsible for the lack of their supershifts with specific antibodies. It is surprising why E2F-5 was excluded from these complexes in the virus-infected cells despite showing similar expression pattern in the infected and mock-infected cells. On the other hand very significant changes were observed in the expression of E2F-4 in the virus-infected cells, i.e., two novel bands both in the cytoplasmic and nuclear fractions of these cells that were absent in the Western blots with the mock-infected cells (Figure 12). However, despite these changes, E2F-4 were present in the oligonucleotide-protein complexes both in the virus-infected and mock-infected cells, as the supershifts were observed with E2F-4-specific antibodies EMSA. However the supershifts were not as prominent for the virus-infected cells as in the case of the mock-infected cells. It is also noteworthy that the supershifted complexes were also relatively less in abundance with anti-E2F-1, E2F-3, and DP-2 in the case of the virus-infected cells. This suggests that the infection tends to decrease E2F-oligonucleotide complex formation.

With regard to the pocket proteins, which bind to and negatively regulate the transcriptional activities of E2F factors, supershifts were observed with anti-p130 and anti-p107 antibodies in mock-infected cells. No supershifts were observed with anti-pRB. This suggests that in asynchoronously growing HSB-2 cells, E2F activities are mainly regulated by p107 and p130 and not by pRB. This is consistent with the

reported mutation of pRB in these cells (Furukawa et at, 1991). Thus, we confirm that pRB is incapable of interacting with E2F in these cells. Interestingly, we observed that anti-p107 and anti-p130 antibodies did not supershift E2F-oligonucleotide complexes in the virus-infected cells. These results suggest that HHV-6 infection inhibits interaction of E2F with these pocket proteins. Alternately, it may be possible that the infection inhibits expression of these pocket proteins in the infected cells. Further studies are required to address this issue. The significance of these results in the virus replication is not clear.

Histone acetylation and deacetylation are catalyzed by histone acetyltransferases (HAT) and histone deacetylases (HDAC), respectively. HDAC convert the $\varepsilon$-amino group of lysines into neutral $\varepsilon$-acetamido group causing changes in chromatin structure that favor gene transcription. Since E2F may regulate transcription by recruiting histone deacetylaes (HDAC) or via histone acetylases (p300) to the E2F binding sites within promoter regions of the E2F-responsive genes. We performed supershift assays using antibodies specific for a histone deacetylase (HDAC-1) and an acetylase p300. These antibodies did not supershift any E2F-oligonucleotide complexes in mock infected as well as in the virus-infected cells, suggesting that these proteins may not be involved in the regulation of E2F transcriptional activities in HSB-2 cells, whether they are infected with HHV-6 or not.

Moreover there may the possibility of the involvement of E2Fs in the regulation and expression of HHV-6 genes that has to be investigated in detail. This project in future can be extended to see the functional consequences of HHV-6 infection for E2F responsive cellular genes. Furthermore, one could identify viral encoded proteins in the induction pathway and study whether CDKs/cyclins activities are modulated or not as a result of HHV-6 infection.

## Conclusions:

The infection with HHV-6 induces significant quantitative and qualitative changes in E2F transcription factors in HSB-2 cell line. These changes can be observed on the Western blots performed with cytoplasmic and nuclear fractions of cell lysates. Most of the changes observed were due to differential phosphorylation of the factors, since a prior treatment of the lysates with a phosphatase eliminated most of the differentially migrating bands on Western blots. The infection also modifies formation of protein complexes with E2F-specific ds DNA oligonucleotides: two complexes were observed with nclear extracts from the virus-infected cells as compared to three in the case of mock-infected cells. Furthermore, upon prolonged exposure of the films, a fast migrating complex was observed in the gel shift assays with nuclear extracts from the virus-infected cells. Prior incubation of the cell extracts with antibodies to all members of the E2F family supershifted the complexes in the case of mock-infected cells suggesting the presence of these factors in these complexes. No supershifts were observed with antibodies for E2F-2, E2F-5, E2F-6, and DP-1 in the cell extracts from the virus-infected cells. Furthermore the supershifts observed with anti-E2F-1, E2F-3, E2F-4, and DP-2 antibodies in the case of these cells were relatively less intense as compared to those seen with these antibodies in the case of mock-infected cells. We conclude from these data that HHV-6 somehow downregulates and/or abrogates DNA binding activities of the E2F members. The inactivation of the E2F members in HHV-6-infected cells may be important for viral replication. Moreover our results show that p130 and p107 are actively recruited into DNA-protein complexes in gel shift assays performed using nuclear extracts from the mock-infected cells, since anti-p130, and anti-p107 caused visible supershifts in the EMSA. These antibodies, however, were not able to cause any supershifts in the

EMSA when nuclear extracts from the virus-infected cells were used. We conclude from these results that HHV-6 infection also inhibits E2F interactions with these pocket proteins.

The virus-induced changes in the functional activities of the E2F members may be important for efficient viral replication in the infected cells.

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# CHAPTER VI

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