Dynamical Modeling of the Biological Regulatory Network of NF-κB Activation in case of HIV-1

Zurah Bibi: 24-FBAS/MSBI/F09

Supervisor: Dr. Shaheen Shahzad

Co-supervisor: Dr. Jamil Ahmad

Department of Environmental Sciences

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

2012

Original for Copy

2 Copy

Dynamical Modeling of the Biological Regulatory Network of NF-κB Activation in case of HIV-1

Submitted By

Zurah Bibi

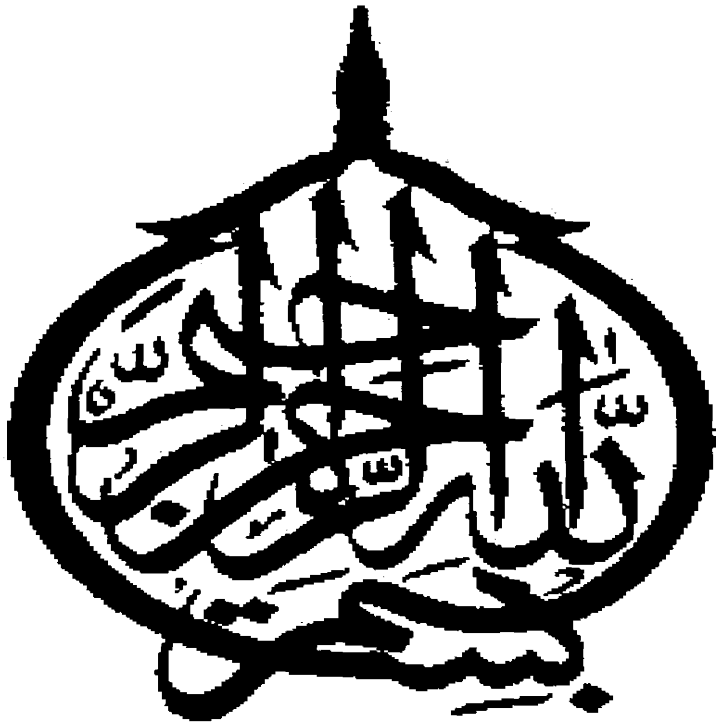
24-FBAS/MSBI/F09

Department of Environmental Sciences

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

2012



In the name of Allah, The most Beneficent, The most Merciful

Department of Environmental Sciences

International Islamic University Islamabad

Dated: 10-02-12

FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Zurah Bibi and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Bioinformatics

COMMITTEE

External Examiner
Dr. Khalid Iqbal
Associate Professor
College of Electrical and Mechanical Engineering
National University of Science and Technology

Internal Examiner
Dr.Sumbul Khalid
Assistant Professor
Department of Environmental Sciences
International Islamic University Islamabad

Supervisor
Dr.Shaheen Shahzad
Assistant Professor
Department of Environmental Sciences
International Islamic University Islamabad

Co-Supervisor
Dr. Jamil Ahmad
Assistant Professor
National University of Science and Technology

Dean, FBAS
Dr. Muhammad Irfan Khan
International Islamic University, Islamabad

A thesis submitted to Department of Environmental Sciences, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Bioinformatics

This thesis is dedicated to my Parents and family

for their endless love, support and encouragement

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

_____

Zurah Bibi

Contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ACKNOWLEDGMENTS

First and foremost, all praise is to Allah S.W.T, the Almighty, on Whom ultimately we depend for sustenance and guidance.

This research work is the part of my MS degree project, which I took under the supervision of *Dr. Shaheen Shahzad* and *Dr. Jamil Ahmad*.

To write the dissertation was not an easy job. The material on the topic was scattered, therefore, to find and arrange the relevant material was a hectic job.

I want to express my unfeigned thanks and praise to Almighty Allah, Who in His great mercy and benevolence has enabled me to undertake and complete this research work. My heartiest praise to the holiest man in the whole galaxies, Almighty's beloved, Hazrat Muhammad (Peace Be upon Him) too, who is the reformer of humanity.

In the completion of this academic endeavor, I would like to thank my supervisor Dr. Shaheen Shahzad for her full guidance and co-operation, whose guidance, careful reading and constructive comments were valuable. Her timely and efficient contribution helped me shape this into its final form and I express my sincerest appreciation for her assistance in any way that I may have asked. I feel pleasure to acknowledge my highly skilled, famous scholar, worthy and learned supervisor, without whose help and assistance, it would have been difficult for me to overcome many problems in the preparation of my thesis.

I owe a great debt of gratitude and thanks to my co-supervisor, Dr. Jamil Ahmad who was so kind, generous and encouraging that after every meeting with him, I feel new energy and enthusiasm in myself. Long discussions with him gave me new ideas and approach towards my research. Despite his heavy officious responsibilities and tight scheduling in NUST, he provides me an excellent guidance to complete my research work and encouraged me in my interest in this topic.

In particular, I wish to gratitude my father *M. Shafi Tahir* and my mother *Shehnaz Parween* for their love and support throughout my life. Thank you both for giving me

strength to reach for the stars and chase my dreams. I feel proud to express my thanks and deepest sense of appreciation to my brother *Atiq-ur-Rehman* and sister *Hina Shafi*, whose loving and caring attitude encased me to complete this thesis.

I am thankful to all my friends especially Javaria Ashraf, Uzma Ali, Saba Munawar, Mehrin Gul and Yusra Sajid who have never make any excuse for discussion on this research work and provide me with much valued ideas. I also want to thank my classmates Zoya, Arshia, Sheema, Madiha, Fatima, Asma, Sumra and Ambreen for the good company and cherishable moments.

I am very thankful to Dr. Umer Khan Niazi from the Department of modeling and simulation, NUST for his valuable suggestions.

I would also like to express my sincere thanks to my friend Kanwal Iqbal, her back-up provided me a new strength. I am also thankful to all my other friends, well wishers, my other family members, family friends and all my relatives, who have suggested very valuable contribution in the completion of my this work.

I also wish to thank the Research Center of Modeling and Simulation, its leadership and the staff for providing me with an academic base, which has enabled me to take up this study. I am particularly grateful Air Commodore Engineer Tahir Mahmood and Rehan Zafar Paracha for their worthy contribution and assistance.

Special thanks, tribute and appreciation to all those their names do not appear here who have contributed to the successful completion of this study.

Zurah Bibi

List of Abbreviations

Acronym	Abbreviation
Acquired Immunodeficiency Syndrome	AIDS
B cell receptors	BCRs
Biological regulatory network	BRN
Calcium ions	Ca^{2+}
CHEMOKINE RECEPTOR 2	CCR2
CHEMOKINE RECEPTOR 3	CCR3
Cluster of differentiation 4	CD4+
CXC chemokine receptor 4	CXCR4
Cyclo oxgenase 2	COX2
Deoxyribonucleic acid	DNA
Gene regulatory network	GRN
Glycoprotein 120	gp120
Human Immunodeficiency Virus	HIV
Inducible Nitric oxide synthase	iNOS
Inhibitor of kappa B	IκB
Inhibitor of kappa B	IkB
Inhibitor of kappa B kinase	IKK
Inhibitor of kappa B-alpha	IκB-α
Inhibitor of NF κB kinase α	IKKα
Inhibitor of NF κB kinase β	IKKβ
Inter leukin 1	IL1

Interleukin 1 beta	IL1β
Linear Hybrid Automata	LHA
Major histocompatibility complex	MHC
Messenger RNA	mRNA
Negative Regulatory Factor	Nef
Non enzymatic scaffold protein	NEMO
Nuclear Factor kappa beta	NFκB
Peripheral Blood Mononuclear Cell	PBMC
Piecewise Affine Differential Equation	PADE
Receptor interacting protein	RIP
Ribonucleic acid	RNA
T cell receptors	TCRs
T helper cells type 1	Th1
T helper cells type 2	Th2
Transactivator of transcription	Tat
Tumor necrosis factor	TNF
Tumor necrosis factor alpha	TNFα
Viral Protein R	Vpr

List of Figures

List of Tables

Abstract

The BRN of NF-κB activation in HIV-1 replication and subsequent immune suppression leads to a stable steady state depicting vicious cycle of HIV causing its rapid replication due to uncontrolled trans-activation. The model generated two qualitative cycles that characterize the normal immune response against HIV-1, representing a healthy fight between the virus and the immune cells, showing the state of clinical latency. Modeling of the gene regulatory network behavior is a difficult task due to the presence of large number of unknown biological parameters, i.e., numerical values of dynamical features related to biochemical reactions. From a computational perspective, these modeling approaches employ the structure of the network (e.g., interlocked feedback loops) rather than relying on the numerical values of biological compound concentrations during chemical interactions. The logical formalism of René Thomas is a powerful approach for the discrete (qualitative) modeling and these techniques facilitate to automatically investigate the qualitative properties of the genetic regulatory networks. The biological regulatory network model shows stable steady states and cycles, that gives insight into the abnormal and normal behaviors respectively. High levels of HIV-1 genes expression, verified by the model deadlock state (1,1,2) were seen due to the increased levels of intracellular NF-κB and it thus offers a favorable environment for HIV-1 replication. The continuous production of inflammatory cytokines also stimulate NF-κB domain Rel A to replicate HIV-1. New experimental techniques like micro-arrays examine the gene expression over different intervals of time. It drags attention towards validating the model through model checking to temporal aspects of a biological phenomenon that takes place in all biological scales, i.e., the 'time delay'. HYTECH tool synthesizes parametric constraints characterizing cyclic conditions in form of invariance kernels or non-cyclic time delays. Equality and inequality constraints were produced, the former one is sensitive, i.e., cannot tolerate slight fluctuations in the threshold values. These constraints confirmed that the production time delay of HIV must be greater than other two elements. A new technique called protein knock out was applied using GENOTECH tool which helps to check stability of the system. The production of the two domains (Rel A and Rel B) of NF-κB is dependent upon Receptor interacting protein

(RIP), playing a key role in the regulation of these domains. Knocking out Rel A takes the system into a state of equilibrium, i.e., slowing the progression of viral infection. While absence of Rel B lead towards a lethal condition where HIV devastating effects take control of the immunity. Biological regulatory network verification techniques which combine the qualitative and quantitative properties were used as a guarantee that no condition was mistakenly ruled out.

CHAPTER NO. 1

INTRODUCTION

1. INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is one amongst the major pandemic diseases, caused by the Human Immunodeficiency Virus (HIV). HIV has spreaded out rapidly, reached to every region of the world and is the fourth leading cause of deaths globally. Currently, AIDS presents the world's most severe public health challenge and it is responsible for mortality mainly among people ranging from 15-59 years of age (Peters *et al.*, 2008). Asia's eventual contribution to this pandemic disease is determined by the two giants, India and China, having Africa as the leading source (De Cock and Weiss, 2000). More than 60 million people have been infected with the HIV virus causing death of more than 25 million people, since determination of HIV as the cause of AIDS. It has a global prevalence of about 0.8% estimated at the end of 2007, having 33 million people (approx.) living with HIV globally (Cohen *et al.*, 2008). According to a survey report conducted by UNAIDS in year 2000, each day an estimated 15,000 people become infected. On the other hand, although treatment and prevention methods have been improved but still the number of affected individuals is rising, encompassing about 2.5 million new infections and 2.1 million deaths in 2007 declared by WHO, UNAIDS and UNICEF report in year 2008.

In order to devise new therapies, it is important to understand its mechanism of activity. It attacks CD4+ T lymphocytes primarily and macrophages (responsible for cytokine induced immunity), which functions to regulate and amplify elements of the immune system. CD4+ T lymphocytes would greatly decrease when no therapy is applied during AIDS and results in a weakened immune system. Thus, the body's ability to fight

infections including cancers would be damaged, resulting in death (Alimonti *et al.*, 2003). HIV-1 is one major subtype of HIV, used in the current investigation.

Huge efforts have been made to understand the origin of AIDS, its mechanism of demolishing immunity but there are still many landmarks to achieve in order to design some cure for it. Current treatments only mean to prolong life or delay the onset of AIDS (Whiteside, 2008). So a laborious effort has to be done to dig out the appropriate prevention. Although HIV/AIDS is a worldwide issue, its dynamics stand differently in different parts of the world along with the consequences (Whiteside, 2008). How HIV spreads its infection after getting into an individual remains to be uncertain.

1.1 Key elements of Biological Regulatory Network (BRN) of HIV-1

To investigate the role of HIV in human body and its mode of immune activation, several signaling pathways have been carefully reviewed to construct a biological regulatory network (BRN) regarding HIV intervention of immune system. Initially three key elements were extracted including HIV-1, NF-κB and TNF-α, being the focus of the study, while the BRN was further extended by adding new entities.

1.1.1 Human Immunodeficiency Virus (HIV)

HIV is a retrovirus that belongs to the lentivirus family. Retroviruses have the capability to exploit host DNA and their RNA for replication and encompass long incubation periods or latency and finally establishes their complete invasion (i.e., with signs and symptoms). For its replication HIV uses CD4+ cells and after that kills these immune cells, thus eliminating body's immunity. Using this mechanism HIV can cause

rigorous harm to the immune system and ultimately devastates it completely. HIV-1 infection progresses in a diverse pattern of disease succession (Janewa *et al.*, 2004; Yokota, 2005). Longitudinal studies represent along and variable periods of latency (asymptomatic) from the time of infection and appearance of AIDS (Mellors, 1998; Tassie *et al.*, 2002; Ageron *et al.*, 2004). Asymptomatic phase can linger for 15 or more years in some cases while some develop AIDS in just 2 years (Cecilia *et al.*, 1999; Meissnera *et al.*, 2004).

Its mechanism of progression is not clearly understood, different factors govern its development mainly viral replication and immune proliferative abilities (De Boer *et al.*, 1994; Altes *et al.*, 2002; Galvani 2005; Hogue *et al.*, 2008; Iwami *et al.*, 2008). In the early 1990's the progression of AIDS and viral diversity was well established, particularly by Martin A. Nowak's work (Nowak *et al.*, 1991; Nowak, 1992; Nowak and May, 1993; Nowak and May, 2000; Nowak, 2006). The existence of viral diversity depends upon the viral load explosion when it exceeds the threshold number (Iwami *et al.*, 2009).

CD4+ T cell depletion is mainly caused by programmed cell death (apoptosis), which can be caused by HIV, through multiple pathways (Judie *et al.*, 2003). Immune deregulation occurs when CD4+ levels drop from a normal of 1000 cells mm 23 of whole blood to less than 200 mm 23 causing opportunistic infections. Research has proven that Th responses play a key role in the control of HIV infection (Rosenberg *et al.*, 1997; Rosenberg and Walker, 1998; Phenix and Badley, 2002).

Apoptosis plays a significant role in many normal physiological mechanisms, e.g., maintaining homeostasis of lymphocyte populations, tissue differentiation and also

removes tumorigenic, mutated or virus-infected cells (Golstein, 1998; Everett and McFadden, 1999).

HIV-1 infection results in persistent activation of the immune system. Macrophages and other cell types that are permissive to HIV-1 infection may be infected by this virus, leading to a signaling modulation (Coleman and Wu, 2009). HIV-1 proteins such as Negative Regulatory Factor (Nef), Transactivator of transcription (Tat) and Viral Protein R (Vpr) have been found in serum of HIV-1 infected patients released by infected/apoptotic cells. These HIV-1 proteins enter the macrophages and transform both cellular machinery and viral transcription. It is vital to translate the signaling pathways involved in HIV infection for a better understanding of AIDS pathogenesis as this could lead to novel therapeutic approaches (Herbein *et al.*, 2010).

For efficient transcription of viral genes and for viral replication of the HIV-1, Tat protein is indispensable as well as the expression of several cellular genes (that interfere with the intracellular signaling) is regulated by it (Noonan *et al.*, 2000; Gautier *et al.*, 2009). The fully grown Tat protein size ranges from 86 to 101 amino acids. It is organized in functional domains required to carry out transactivation activity. There is a motif in C-trerminus that mediates cell adhesion and Tat binding to integrin receptors (Chang *et al.*, 1995). At least three cell surface molecules including heparin sulfate, beta-integrin and chemokine receptors are shown to be specific for Tat binding as well as peptides spanning its cysteine-rich region compete with cognate ligands to bind CXCR4, CCR2, and CCR3 chemokine receptors in primary human monocytes and PBMCs. Tat can also trigger Ca2+ mobilization in macrophages, causing several complications of neuronal cells (Albini *et al.*, 1998; Xiao *et al.*, 2000; Li and Verma, 2002).

1.1 Nuclear Factor kappa beta (NF-κB)

It is a transcription factor that performs significant roles in immune activation (Ghosh *et al.*, 1998; Li and Verma, 2002; Bonizzi and Karin, 2004). NF-κB/IκB family of transcriptional regulators, promotes the expression of more than 100 genes, most of these plays a part in immune defense against pathogens (Ghosh *et al.*, 1998). The encoded proteins comprise a variety of cytokines, chemokines, receptors involved in immune recognition, proteins required for antigen presentation and adhesion molecules that take part in transmigration across walls of blood vessels. As NF-κB entails a primary role in immune activation, it is also known as the central mediator of immune system (Hiscott *et al.*, 2001). Different studies on NF-κB state that it also has a role during oncogenesis and in the regulation of programmed cell death (Barkett and Gilmore, 1999). NF-κB is a striking target for viral pathogens due to various reasons. NF-κB gets activated immediately after being exposed to pertinent inducer without being de novo synthesized and produces a strong transcriptional stimulation of different viral and cellular genes (Hiscott *et al.*, 2001).

The viruses have evolved several different strategies to exploit the NF-κB pathway in order to increase their replication, enhancing the host cell life-span to use it as a reservoir and to escape the immune response. NF-κB could be an attractive target as it controls a number of genes such as growth factors, proto-oncogenes, cytokine and their receptor genes and major factors controlling the host cell cycle. The anti-apoptotic properties of NF-κB are used by the viruses in order to weaken the host defense mechanism, killing of infected cells tends to stop the replication of immune cells. Sometimes they elicit apoptosis to amplify viral spread (Hiscott *et al.*, 2001).

NF-κB controls the expression of cytokines, inducible nitric oxide synthase (iNOS), cyclo-oxgenase-2 (COX-2), growth factors, inhibitors of apoptosis and effector enzymes produced as a result of binding of many receptors engaged in immune responses like including T-cell receptors (TCRs), B-cell receptors (BCRs) and members of the Toll-like receptor/IL-1 (Inter-leukin-1) receptor super family. Furthermore, pathological dysregulation of NF-κB is involved in certain inflammatory and autoimmune diseases as well as cancer. In case of central nervous system NF-κB also play a part in the development and the activity of tissues of the brain (Memet, 2006).

1.1.2.1 Mechanism of activation of NF-κB

A variety of stimuli activate the dormant cytoplasmic NF-κB/IκB complex by phosphorylation that includes viral and bacterial pathogens, cytokines and stress-inducing agents (Pahl, 1999). NF-κB comprises five proteins: c-Rel, Rel A (p65), Rel B, NF-κB1 (p50 and p105) and NF-κB2 (p52), regulated by its inhibitory subunit IκB (Inhibitors of Kappa B) family of anchorin domain-containing proteins (Ghosh *et al.*, 1998).

The inactive NF-κB is confiscated in the cytoplasm in the form of a heterodimer containing p50, p65 and IκB subunits. Apart from viruses, activation signals of NF-κB also includes many carcinogens, inflammatory agents and tumor promoters including cigarette smoke, phorbolester, okadaicacid, H_2O_2 and tumor necrosis factor (TNF). After receiving the activation signal, the IκB undergoes phosphorylation at serine residues 32 and 36, ubiquitinated at lysine residues 21 and 22 and degraded via the proteasomal pathway. This process lead to the exposure of nuclear localization signals on the p50-65 heterodimer. The p65 is subsequently phosphorylated causing nuclear translocation and binding to its specific sequence on DNA leads to gene transcription (Pandey *et al.*, 2007).

NF-κB activation occurs through two types of signaling pathways widely known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway) (Karin 1999; Tergaonkar 2006; Gilmore 2006; Scheidereit 2006). Both these pathways have a regulatory element in common known as IκB kinase (IKK) complex comprising catalytic kinase subunits (IKKα and/or IKKβ) and a regulatory non-enzymatic scaffold protein (NEMO). NF-κB is activated by the IKK-mediated phosphorylation to induce proteosomal degradation, translocating NF-κB transcription factors to the nucleus leading to gene expression (Grey, 2008).

In case of canonical (or classical) pathway (Figure 1.1), the binding of a ligand to the cell surface receptors recruits adaptors which tends to activate the IKK complex to phosphorylate and degrade the IκB inhibitors and releases the inactive NF-κB to the nucleus (Nishikori, 2005). The non-canonical (or alternative) pathway (Figure 1.2) leads to the activation of p100/RelB complexes and it is required for the development of lymphoid organs leading to the generation of B and T lymphocytes. In this pathway an IKK complex is involved that comprises two IKKα subunits, but not NEMO.

In alternative pathway, ligand induced activation causes the activation of NF-κB inducing kinase (NIK), which phosphorylates the IKKα complex, this in turn phosphorylates p100 leads to the liberation of the p52/RelB active heterodimer (Nishikori, 2005).

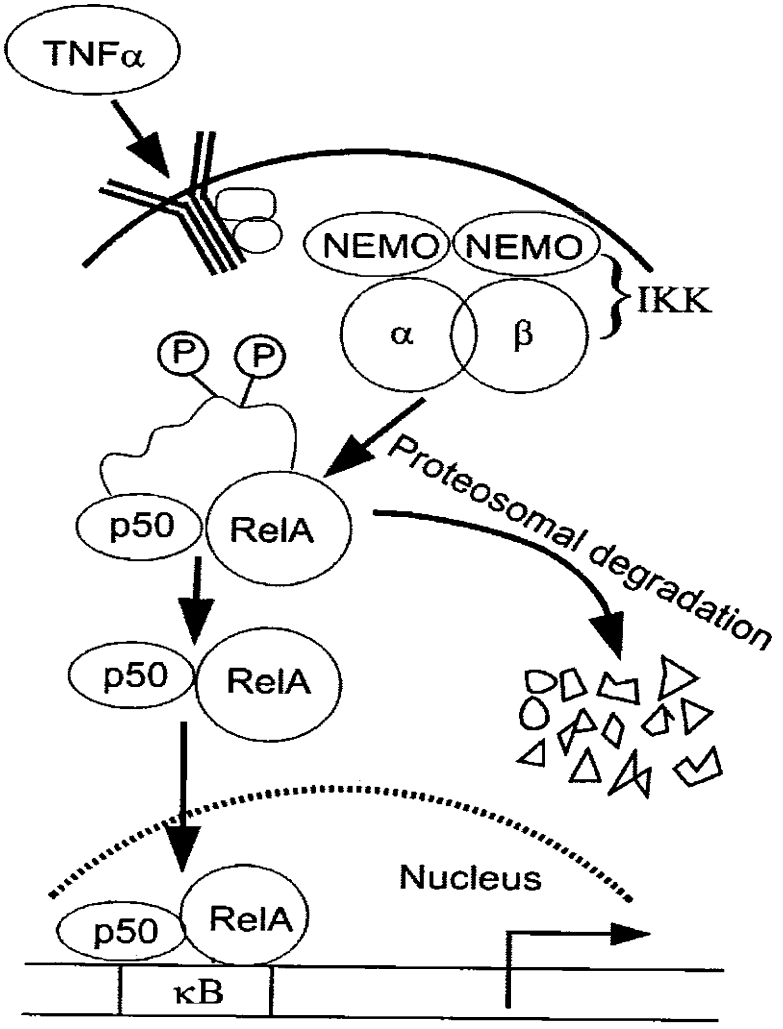


Figure 1.1 Canonical pathway

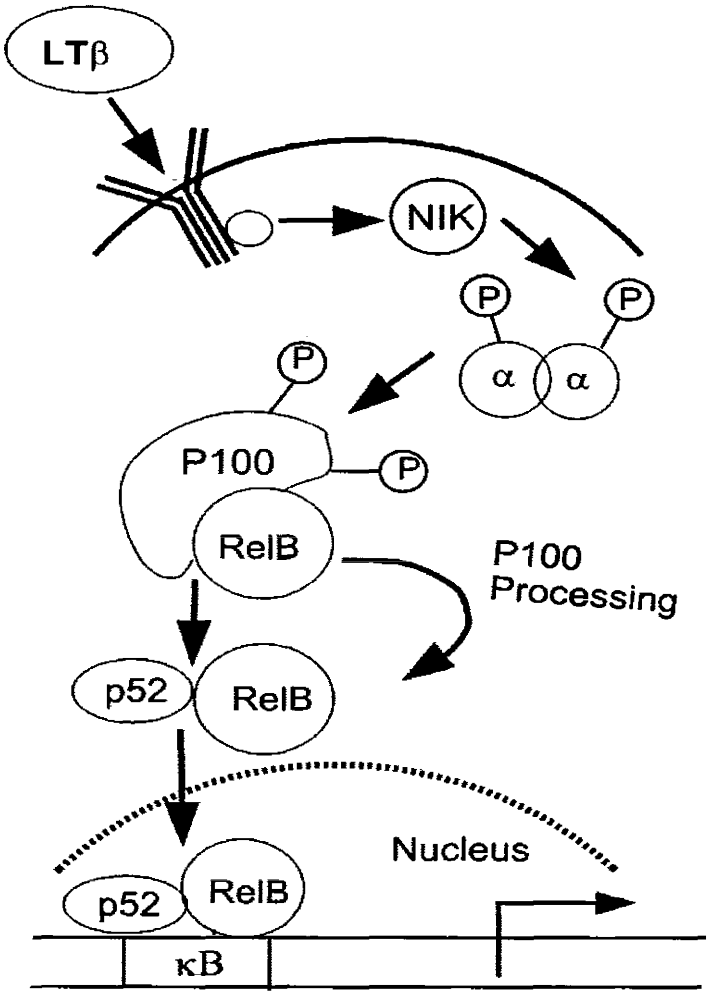


Figure 1.2 Alternative pathway

1.1.3 Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor (TNF) is a cytokine principally known to be associated with immune responses in acute infection as it mediates inflammation. TNF is also called cachectin, in case of malignancies or AIDS it is associated with killing of patients. It has been found in laboratory studies that TNF is associated with increased HIV replication and studies are being done to find it as a possible cofactor in HIV infection. Activated macrophages, lymphocytes and monocytes produce tumor necrosis factor-alpha (TNF-α).

TNF-α elicits transcription and production of the IκB kinase enzyme after getting linkage to corresponding receptors, that produces the transcription factor NF-κB (Vitale and Ribeiro, 2007).

After activation NF-κB tends to induce a bulk of proteins, e.g., cytokines that cause inflammation and various immunological processes responsible for the activity of TNF-α (Bingham *et al.*, 2002).

Two important traits of Human Immunodeficiency virus (HIV) infection are the gradual depletion of both CD4+ and CD8+ T cells and the creation of viral reservoirs (Fauci *et al.*, 1996). Apart from a T helper cells type 1 and 2 (Th1-Th2) switch, AIDS pathogenesis can be described by an immune dysregulation employing proinflammatory cytokines, mainly tumor necrosis factor (TNF) (Herbein and Khan, 2007). Activated macrophages and lymphocytes secrete TNF to stimulate diverse responses, involving inflammation and apoptosis (Legler *et al.*, 2003). There are trans-membrane receptors TNFR1 and TNFR2 for TNF binding to the cell in order to modulate immunity (Herbein and Khan, 2007).

Pathogens activate host responses by various means like increasing TNF (Rahman *et al.*, 2006). The primary source of TNF production is Macrophages which are also important cellular targets in HIV infection. Macrophages serve as a reservoir for the virus, i.e., helps the virus to propagate and comparatively resistant to the damaging effects of HIV infection.

A number of laboratory studies of AIDS patients show that TNF-α specifically kills HIV-infected cells, providing an underlying principle for the early studies where recombinant TNF-α was used to "boost" immune system. While some in vitro studies show it can stimulate HIV messenger RNA production as well. On viral replication the effects of TNF-α may be dose and time dependent. Progressive encephalopathy in persons with AIDS has shown high blood levels of TNF. According to one theory it is suggested that in central nervous system, the microglial cell (equivalent of the macrophage) produces TNF-α in case of HIV infection, leading to the development of AIDS-related dementia. TNF-α can cause neuronal appotosis alone and according to some findings it has been proposed that HIV-1 protein Tat synergizes TNF-α in neuronal death (Kaul *et al.*, 2001).

1.2 Qualitative Modeling

In living organisms gene is the unit of heredity. It is present on a stretch of DNA that codes one of the different types of proteins or for an RNA chain that has a function in the organism. Genes carry the information required to build and maintain an organism's cells and pass genetic traits to the offspring. In this way gene is the basic unit of inheritance.

An mRNA molecule is generally responsible to make a specific protein or set of proteins that performs several different functions in the body. Some of the proteins serve only to activate other genes, are known as the transcription factors. These transcription factors are the main players in regulatory network. They turn them on by binding to the promoter region in the gene and initiate the production of another protein. In some cases they act as inhibitory factors.

Experimental approaches to study living systems behaviors normally focus on various complementary biological components, e.g., a set of genes that encodes a set of proteins. These components interact with each other in the form of a network. The rates at which genes are transcribed into mRNA and the way in which genes interact with each other (through RNA and protein products) and with other substances in the cell is controlled by a gene regulatory network (GRN). A gene regulatory network is the major biological framework for examining dynamical biological behaviors.

Modeling the gene regulatory network behavior was a difficult task due to the presence of large number of unknown biological parameters, i.e., numerical values of dynamical features related to biochemical reactions. Several approaches have been designed to overcome the lack of parameter values by proposing dedicated qualitative modeling approaches. In all these methods the gene interaction was considered as the cornerstone of a biological behavior. From a computational perspective, these modeling approaches employ the structure of the network (e.g., interlocked feedback loops) rather than relying on the numerical values of biological compound concentrations during chemical interactions.

When the qualitative modeling techniques are applied on concrete biological systems, approaches that are based on Piecewise-Affine Differential Equations (PADEs) or the René Thomas's formalism gave astonishing results (De Jong *et al.*, 2004; Thomas and Thieffry, 1995). These techniques can be used for a class of hybrid systems and these powerful techniques can help us in verification and the control of these hybrid systems (Ghosh *et al.*, 2004). Particularly, they facilitate in automatic investigation of qualitative properties of the genetic regulatory networks (Batt *et al.*, 2005).

New experimental techniques like micro-arrays examine the gene expressions over time (Bennett *et al.*, 2009). It drags our attention towards the temporal aspect of a biological phenomenon that takes place in all biological scales. Thus all biological systems modeling must take into account this new parameter that is called 'time delay'. This parameter was often neglected before, although variations of specific products over time were documented.

The existing qualitative models can be refined by the representation of qualitative properties that verify experimental temporal constraints. In this way the time delay represents a unique opportunity to refine existing qualitative models. So it can be stated that for modeling it satisfies both qualitative properties, arisen from the biological network structure, and delays associated with the dynamics of gene or gene products. For this purpose, a new hybrid modeling technique is developed that allows the biological society to directly use the qualitative and partial temporal experimental data. It abstracts the structure of the biological network by positive and negative feedback loops in order to focus on the variation of signs of the gene products as a result of qualitative behavior. In the qualitative abstraction, some constraints or delays are added for the purpose of natural

refinement of the qualitative behavior (Fromentin *et al.*, 2010).

In modern genomic techniques the simultaneous measurement of the expression levels of all genes in an organism has taken a qualitative leap to study the GRNs (Panday and Mann, 2000; Lockhart and Winzeler, 2000). Likewise the formal methods for the modeling and simulation of gene regulation processes will be essential. In most networks many genes are connected through interlocking positive and negative feedback loops, their dynamics is difficult to obtain and may lead to invalid conclusions. Formal modeling and simulation methods supported by computer tools allow one to study the behavior of large and complex networks to be predicted in a systematic way (Mcadams and Arkin, 1998; Endy and Brent, 2001).

Hybrid models allow viewing of both discrete and continuous dynamics (Ahmad *et al.*, 2007). Kinetic logic is a modeling tool that helps to represent qualitative verbal features in a formal mathematical way still preserving the verbal descriptions. It helps to model impact, feedback and temporal evolution of variables. The critical assumptions of kinetic logic are

1) The elements in a system have a little effect on each other before reaching a certain threshold.

2) The effect could reach a plateau at high levels.

On the bases of these assumptions, an element is absent before reaching threshold and is present after that. It should be kept in mind while using kinetic logic that time frame must be specified, i.e., the interactions in biological regulatory network could change with the age of a person. One of the advantages of kinetic logic is that it transfers

a complex dynamical process into a relatively simple model with distinct dynamical patterns and logical attractors (Sulis *et al.*, 1996).

The purpose of the study was to identify the regulatory network associated with NF-κB activation upon entry of HIV-1 in human body. Discrete modeling/Hybrid modeling formalism was then applied on the identified network. Model Checker tool (HYTECH, chapter 2) was used for the Qualitative analysis of the discrete/Hybrid models developed in this research.

Discrete and Hybrid models of the NF-κB associated BRN were developed. Normally dynamic models are based on quantitative differential equations which require very detailed kinetic knowledge. Alternative modeling techniques like constraint-based techniques were therefore applied to available functional genomics data. These qualitative modeling approaches offer formal support in the light of dynamic systems and have proven to be more valid approaches in cell biology in order to check the consistency of molecular networks (Gagneur and Casari, 2004).

An interesting area to study the qualitative behavior of the system is the existence of any steady-state or limit cycle. In a trajectory (oriented curve) the successive time pointing the concentration of each molecular species represents the evolution of the cell. Qualitative characterization of cell behavior can be analyzed from the study of the phase space and informs about the robustness of a particular asymptotic behavior corresponding to variation in the initial conditions (Tyson *et al.*, 2001). Phase space is originally introduced by Poincaré and it represents all possible states (Strogatz, 1994; Gagneur and Casari, 2005). Based on the qualitative characterization of the biological regulatory pathways, Qualitative Models were built. Hybrid model of the system were then

developed using regulation "Delays" (Production/Degradation delays). The behavior of the system was tested for both normal pathways, i.e., oscillation and abnormal (diseased) condition, i.e., deadlock state.

The biological qualitative behavior is the cornerstone of the current modeling formalism which refers to the chronological sequence of ordered concentration peaks (showing timing properties). The main focus was on bi-product peaks, the discrete states, which stand for the time phases separating two such peaks, can be represented by boolean variables as shown in figure 1.3.

Each boolean variable showing the increasing time or decreasing time of its protein production represents the behavior of a given gene, e.g., (x, y) = (-, +) which shows a decrease of the concentration of the product of x and an increase of the concentration of the product of y.

By studying the differential behavior of systems, adjusting similar biological observed characters and performing in-silico experiments, the diversion state of the system from normal to abnormal behavior and the condition(s)/constraint(s) to avoid divergence into abnormal pathway were predicted.

All this helps to identify drug targets in terms of time delays or to find out the role of particular segments within the network (when it is switched on or off). On the basis of the predictions obtained from in-silico experiments, wet-lab experiments can be performed. It will save time and energy.

The main tool employed in the current research for dynamical modeling was GENOTECH. It is used for discrete modeling having a "Qualitative Threshold" and para-

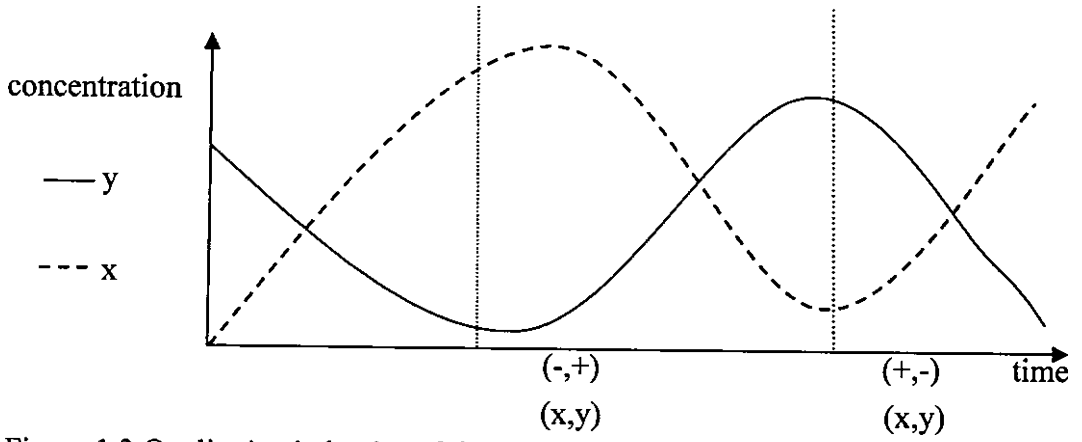


Figure 1.3 Qualitative behavior of the cycle (-, +) → (+, -)

meter in order to be consistent with the observations.

1.2.1 Proposed BRN

Soluble viral proteins such as trans-activator of transcription (Tat) and the glycoprotein gp120 are released from HIV-1 infected macrophages (Rumbaugh and Nath, 2006). Tat interacts and activates surrounding cells including microglia, astrocytes and neurons. These infected microglia and astrocytes become activated to produce the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) to further activate neighboring cells. These cells also produce certain chemokines that attract more inflammatory monocytes and macrophages (D'Aversa *et al.*, 2002). Hence in this vicious cycle, Tat provides the elementary trigger, causing deficits in immune system leading to complete immunosuppression.

HIV-1 infection results in the persistent activation of immune system. HIV-1 proteins such as the (Nef), Tat and (Vpr) have been found in the serum of HIV-1 infected patients released by infected/apoptotic cells. These HIV-1 proteins enter the macrophages and exploit both cellular machinery and viral transcription. It is important to decipher the signaling pathways in HIV infection for a better understanding of AIDS pathogenesis.

For efficient transcription of viral genes and for viral replication of HIV-1, Tat protein is indispensable. It regulates the expression of several cellular genes and interferes with intracellular signaling as well (Chen *et al.*, 1997). The surface receptors required for binding with CD4+ (Cluster of differentiation 4) by HIV-1 protein gp120 on T lymphocytes is CXCR4, while that of macrophage is CCR5.

Nuclear Factor Kappa B (NF-κB) family of transcription factors serves to activate

and regulate genes responsible for the production of many inflammatory molecules, also known as the Rel family. The production of inflammatory molecules is the first line of defense against infection that tends to employ more immune cells to the area of insult (Noonan and Albini, 2000; Minghetti *et al.*, 2004). This shows that this pathway has an important role in both adaptive and innate immunity, and consequently susceptible to HIV-1 infection. Different types of stimuli activate the NF-κB pathway. Cytokine induced activation of NF-κB takes place through Tumor Necrosis Factor (TNF) receptors. These receptors are found in many cell types where they respond to cytokines including TNF-α and IL-1 to enhance innate and adaptive immune systems (Li and Verma, 2002).

The NF-κB family member Rel B has many unique characteristics in contrast to the other NF-κB proteins like its unique amino-terminal leucine zipper motif (Dobrzanski *et al.*, 1993). Experiments strongly reveal that Rel B has anti-inflammatory and cytokine regulating properties. To investigate the anti-inflammatory properties of Rel B in the perspective of HIV-1induced neuro-inflammation, an extended version of the BRN was also proposed (chapter 2).

When considering a biological network, it is necessary to express some level of abstraction. In case of regulatory networks, the defined genes or their products show activation or inhibition of their targets (Chaouiya, 2008). The kinetic logical approach developed by René Thomas to qualitatively model BRNs, applied on the BRN has been presented in chapter 2. René Thomas devised two rules to represent the dynamical behavior of regulatory networks: positive circuit (having even number of suppressions), a condition crucial for multistationarity and a negative circuit (having odd number of supressions) required for steady oscillation, i.e., homeostasis (Thomas, 1981).

Kinetic logic discretizes the concentration according to their thresholds. René Thomas has demonstrated the logical formalism by applying it on a variety of BRNs, e.g., λ phage infection in E. coli, dorso-ventral pattern formation and Drosophila gap gene control (Thomas and d'Ari, 1990; Sánchez and Thieffry, 2001). Abstraction of flower morphogenesis controlling system in Arabidopsis thaliana is another example, generated six steady states, five of these were observed experimentally while sixth one was not explored hitherto (Mendoza *et al.*, 1999). By examining the state transitions graph it was predicted that transition from nonfloral to floral states requires at least one more regulator. Ahmad *et al.*, 2009 successfully modeled E. coli response to carbon starvation using the kinetic logic and the framework of linear hybrid automata (Ahmad *et al.*, 2009).

Boolean functions are used to represent interactions between the elements of a network to calculate the state of a gene after being activated by other genes (De Jong, 2002). A Boolean network offers global properties of large-scale regulatory systems giving a view of the implications of local properties of the networks for global dynamics (Kauffman, 1991; De Jong, 2002). Continuous and precise modeling of a system based on quantitative methods entails fine-tuning of multiple parameters, not easily available for most biological systems. To analyze a proposed model such methods were not applicable, as the results obtained from experiments only offer qualitative details of description (Schaub *et al.*, 2007) so the current study is focused on the quantitative properties of the BRN in making useful predictions.

Identification of regulatory network associated with HIV-1 activated pathway of NF-κB activation were elucidated further and discrete/hybrid modeling formalism was applied on identified networks. Based on the qualitative characterization of the biological

regulatory pathways, the next step involved building and analysis of Qualitative models; that required model checker tools for the qualitative analysis of the discrete/hybrid models. After the process of model generation, parameter identification was carried out by performing in-silico experiments and adjustment of similar biological observed characters. The research was aimed at the prediction of behavioral pattern of the system, for both normal pathway (i.e., oscillation) and abnormal (diseased) condition (i.e., deadlock state). The second half of the thesis covers the development of the hybrid model of the system using regulation delays (production/degradation delays). The stability behavior of the proteins involved in the BRN was also studied by using in-silico protein knocking out. Precise identification of the way in which the interacting proteins regulate HIV-1 will help in greater understanding of human immune response and subsequently aid the development of a therapeutic drug.

CHAPTER NO. 2

METHODOLOGY

2. Discrete Modeling Formalism

The discrete modeling formalism of René Thomas was used to find the discrete (qualitative) model of the BRN (Thomas and d'Ari, 1990, Thieffry and Thomas, 1998, Ahmad *et al.*, 2008). Following are the main formal definitions which are used to derive a discrete model of a BRN.

2.1 Definitions

2.1.1 Biological Regulatory Network (BRN)

In a directed graph $G = (X, A)$, $G^-(v)$ and $G^+(v)$ represent the set of predecessors and successors of a node $v \in X$ respectively. A BRN is a graph $G = (X, A)$ where X represents the set of nodes (biological entities) and A is the set of edges representing interactions between biological entities. Each edge $a \rightarrow b$ is labeled as (s_{ab}, r_{ab}), where s_{ab} is a positive integer representing a threshold and $r_{ab} \in \{+, -\}$ shows the type of interactions ('+' for activation and '-' for inhibition). There is a limit lm_a for each node a which is equal to the outgoing degree of a, such that $\forall\ b \in G^+(a)$ each $s_{ab} \in \{1, ..., n_a\}$ where $n_a \leq lm_a$. Each entity a carries its abstract concentration in the set $Q_a = \{0, ..., n_a\}$.

To analyze the behavior of a BRN, it is necessary to know all the possible states and transitions between them.

2.1.2 State

A state s of a BRN is a tuple where $s \in \check{S}$, such that

$\check{S} = \Pi_{b \in X} Q_b$.

A vector is normally used to show a qualitative state $(v_b)\ \forall\ b \in X$, where v_b represents the concentration level of the product b.

2.1.3 Resources

A set of resources represents the activators of a variable at any instant. The set of resources R for a variable $a \in X$ at some level y is defined as

$$R_{ya} = \{b \in G^{-}(a) \mid (y_b \geq s_{ba} \text{ and } r_{ba} = \text{'+'} \text{ or } (yb < s_{ba} \text{ and } r_{ba} = \text{'-'})\}$$

From the above definition, it can be inferred that the absence of an inhibitor is considered as an activator. The set of parameters assigned to a biological entity determine the dynamics of a BRN which is defined as:

$$K(G) = \{K_{a,\,Rya} \in \{0, \ldots n_a\} \mid y_a \in Q_a \forall a \in X\}$$

$K_{a,\,Rya}$ gives the level towards which a evolves.

Let y and $K \in Z_{\geq 0}$, the asynchronous evolution operator $\uparrow$ is given as:

$$y \uparrow K = \begin{cases} y + 1 \text{ if } y < K \\ y - 1 \text{ if } y > K \\ y \text{ if } y = K \end{cases}$$

2.1.4 State Graph

If y_x is the level of an entity x in state $s \in \check{S}$, the state graph of a BRN with Transition relation $T \subseteq \check{S} \times \check{S}$ such that

$s \rightarrow s' \in T$ iff:

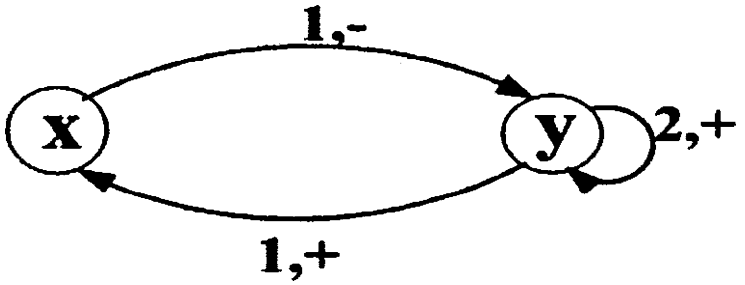


Figure 2.1 The BRN of the activation and inhibition interactions of x and y

x	y	w_x	w_y	$K_{x,wx}$	$K_{y,wy}$
0	0	{}	{x}	0	1
0	1	{y}	{x}	1	1
0	2	{y}	{x,y}	1	2
1	1	{y}	{}	1	0
1	0	{}	{}	0	0
1	2	{y}	{y}	1	2

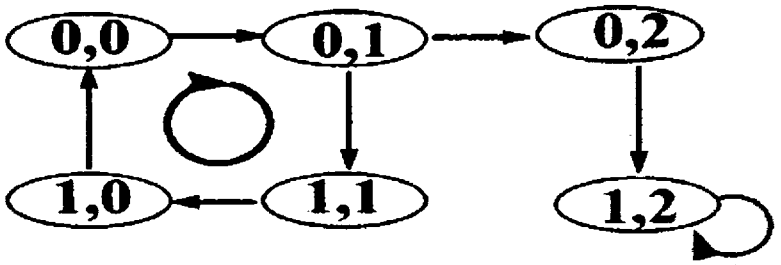


Figure 2.2 State table and state graph of x and y BRN respectively

• There is a unique $a \in X$ such that $s_a \neq s'_a$ and

$$s'_a = s_a \uparrow K_{a, Rya}$$

and

$$s'_b = s_b \forall b \in X_{\setminus\{a\}}.$$

A State graph differs from its successor state by one component only, so if a state s has n elements to be evolved then it will have n successor states.

The discrete modeling formalism can be easily understood by a toy example (Figure 2.1). It consists of two genes x and y (represented by x and y nodes), where y act as an activator of x while y favors its own production and is inhibited by x. The state table (Figure 2.2) shows transition of states from a given state and the state graph shown in figure 2.2 configures the behavior of the BRN by cycle (0, 0), (0, 1), (1, 1), (1, 0) and Stable Steady State (1, 2) .

2.2 Discrete modeling of the NF-κB associated Biological Regulatory Network in case of HIV-1 attack

After having extensive literature survey, discrete modeling formalism of René Thomas was applied on following abstracted BRN of HIV-1 activated NF-κB network.

Viral protein glycoprotein120 (gp120) is essential for HIV-1 viral entry by using the interaction with surface receptors CD4+ (Cluster of differentiation 4) and CXCR4 (CXC Chemokine receptor 4) or CCR5 Co-receptor (Chemokine Receptor 5) (Figure 2.3).

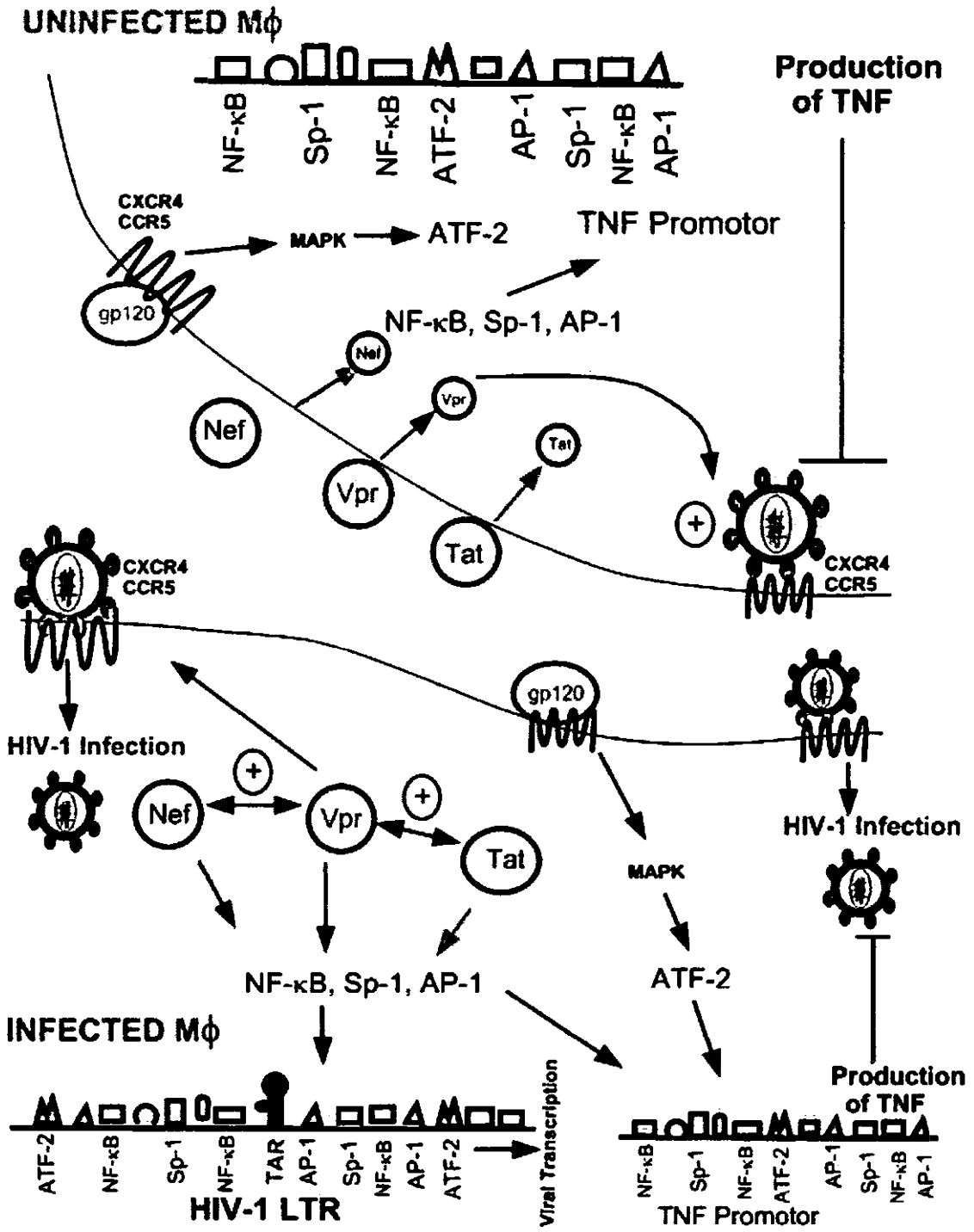


Figure 2.3 HIV signaling and activation of Immune system in Macrophages (M□). HIV proteins modify the signaling pathways in both affected and unaffected cells by activating the transcription factors like NF-κB and AP-1. NF-κB has binding sites for both HIV LTR (long terminal repeat) as well as for TNF-α promoter

The transcription factor NF-κB is involved in both innate and adaptive immune responses as well as in inflammation. When unstimulated (in the absence of foreign particle/antigen) NF-κB dimers are retained in the cytoplasm through the inhibitory action of the inhibitor of kappa B (IκB) molecules (Ghosh and Karin, 2002). The pro-inflammatory cytokines TNF-α and InterLeukin-1b (IL-1β) induce the activation of the NF-κB pathway (Ghosh *et al.*, 1998). Degradation of the inhibitor of Kappa B-alpha (IκB-α) releases NF-κB dimers to the nucleus, where they activate transcription. Production of cytokines (e.g. TNF-α) induced by viral proteins, involve activation of the NF-κB pathway accompanied by rapid degradation of IκB-α in Tat treated cells (Ghosh *et al.*, 1998; Ghosh and Karin, 2002; Sui *et al.*, 2007).

Overexpression of IκB-α can block Tat-induced TNF-α synthesis in macrophages, being resistant to proteasomal degradation, showing the dependency on NF-κB for Tat-induced TNF-α synthesis (Sui *et al.*, 2007). Rel A comprises the classical dimer of NF-κB that is involved in apoptotic gene transcription causing inflammation. To reduce transcription activating complexes of NF-κB, Rel A must be rendered inactive (Ruben *et al.*, 1992). These interactions were presented in the abstract BRN depicted in figure 2.4, showing only the key biological entities involved in the regulation of the immune system.

2.2.1 Discrete modeling of Extended Biological Regulatory Network

The BRN of NF-κB has been extended by including two very important regulators of inflammation, i.e., Rel B and RIP, where Rel B acts to regulate the activity of Rel A by forming inactive complexes with Rel A and receptor interacting protein (RIP) is crucial to initiate the phosphorylation of IKK complexes bound to NF-κB subunits in the cytoplasm. The GENOTECH model of the extended system was shown in figure 2.5.

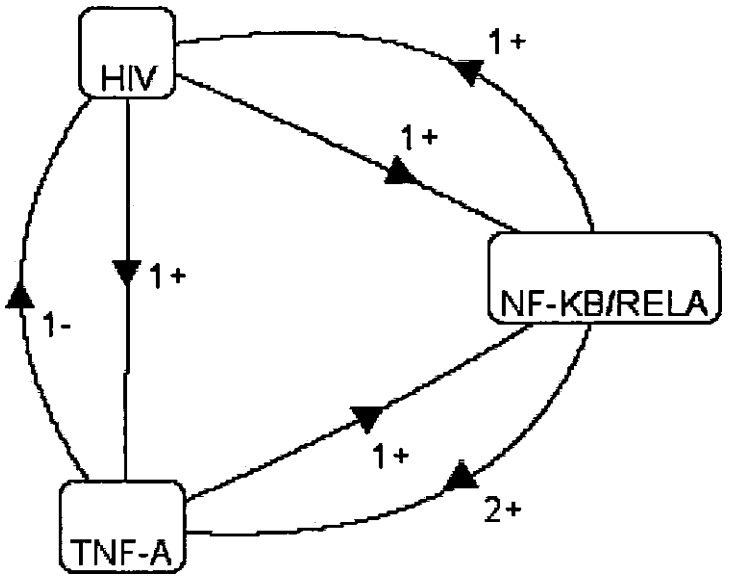


Figure 2.4 BRN of NF-κB responsible for the cytokine production and regulation

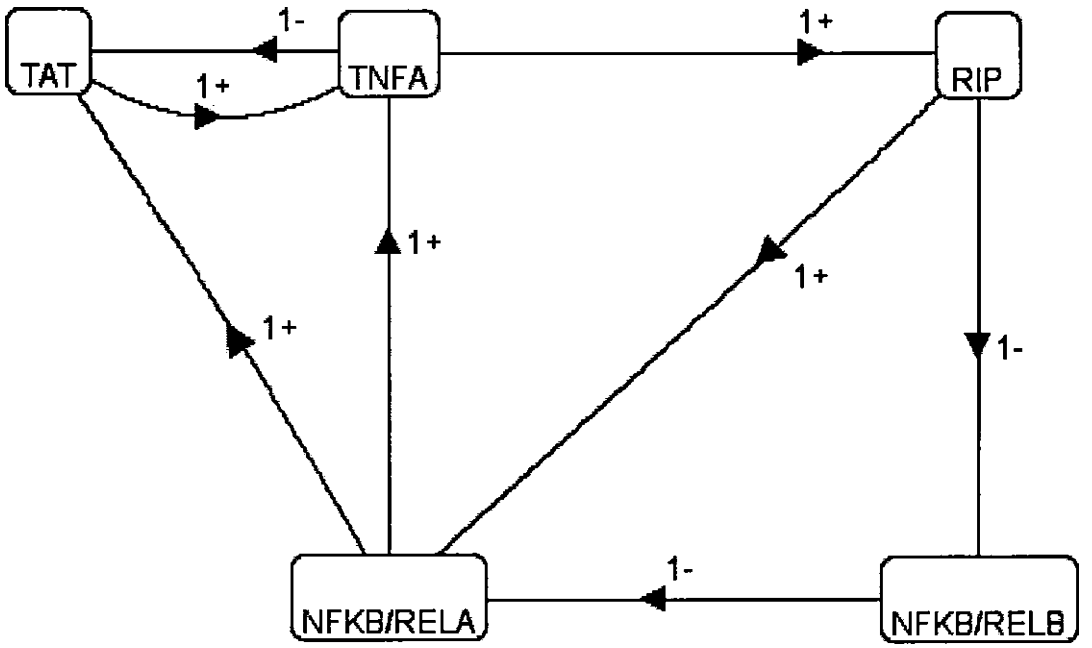


Figure 2.5 BRN of NF-κB Rel Homolgy Domain containing Rel A and Rel B (inhibitor of Rel A)

Table 2.1 Logical parameters of the BRN

Protein	Activator	Inhibitor	Logical parameters
HIV	NF-KB/ RelA	TNF-A	$K_{HIV,\{\}} = 0$ $K_{HIV,\{TNF\text{-}A\}} = 1$ $K_{HIV,\{NF\text{-}KB/RelA\}} = 1$ $K_{HIV,\{TNF\text{-}A,NF\text{-}KB/RelA\}} = 1$
TNF-A	NF-KB / RElA, HIV		$K_{TNF\text{-}A,\{\}} = 0$ $K_{TNF\text{-}A,\{HIV\}} = 1$ $K_{TNF\text{-}A,\{NF\text{-}KB/RelA\}} = 1$ $K_{TNF\text{-}A,\{HIV,NF\text{-}KB/RelA\}} = 1$
NF-KB/ RelA	TNF-A, HIV		$K_{NF\text{-}KB/RelA,\{\}} = 0$ $K_{NF\text{-}KB/RelA,\{HIV\}} = 2$ $K_{NF\text{-}KB/RelA,\{TNF\text{-}A\}} = 2$ $K_{NF\text{-}KB/RelA,\{HIV,TNF\text{-}A\}} = 2$

Table 2.2 Logical parameters of the extended BRN

Protein	**Activator**	**Inhibitor**	**Parameters**
Tat	NF-KB /RelA	TNF-A	$K_{(TAT,\{\})} = 0$ $K_{(TAT,\{NF\text{-}KB/RELA\})} = 1$ $K_{(TAT,\{TNFA,NF\text{-}KB/RELA\})} = 1$
TNF-A	NF-KB /RelA	NF-KB /RelB	$K_{(TNFA,\{\})} = 0$ $K_{(TNFA,\{NF\text{-}KB/RELA\})} = 1$ $K_{(TNFA,\{TAT\})} = 1$ $K_{(TNFA,\{NF\text{-}KB/RELA,NF\text{-}KB/RELB\})} = 1$ $K_{(TNFA,\{TAT,NF\text{-}KB/RELB\})} = 1$ $K_{(TNFA,\{TAT,NF\text{-}KB/RELA\})} = 1$ $K_{(TNFA,\{TAT,NF\text{-}KB/RELA,NF\text{-}KB/RELB\})} = 1$
RIP	TNF-A		$K_{(RIP,\{\})} = 0$ $K_{(RIP,\{TNFA\})} = 1$
NF-KB/RelA	TAT	NF-KB/RelB	$K_{(NF\text{-}KB/RELA,\{\})} = 0$ $K_{(NF\text{-}KB/RELA,\{RIP\})} = 1$ $K_{(NF\text{-}KB/RELA,\{TAT,RIP\})} = 1$ $K_{(NF\text{-}KB/RELA,\{RIP,NF\text{-}KB/RELB\})} = 1$ $K_{(NF\text{-}KB/RELA,\{TAT,RIP,NF\text{-}KB/RELB\})} = 1$
NF-KB/RelB			$K_{(NF\text{-}KB/RELB,\{\})} = 0$ $K_{(NF\text{-}KB/RELB,\{RIP\})} = 1$

After defining the BRN, the discrete formalism has been applied to derive its qualitative model. For this purpose only qualitative data of the network was required, i.e., sign of interactions (+ or -), threshold order and weights for each interaction. In cases when the thresholds were weakly estimated the logical approach could be an alternative (Thieffry and Thomas, 1998). In a feedback circuit only the key players are included and the logical formalism can be applied to observe the behaviors like multistationarity and homeostasis (Gouzé *et al.*, 1998).

2.3 Logical parameters of the Biological Regulatory Networks

To observe the dynamical behavior of the BRN, GENOTECH tool was used for generating steady states (Ahmad, 2009). This tool facilitates the discrete modeling of a BRN. The discrete modeling formalism has been implemented in this tool. Apart from the discrete modeling, this tool also facilitate in the analysis of steady state behaviors (cycles and stable states). BRNs presented in the figures 2.1 and 2.4 contains both positive and negative feedback loops, where positive loops are the necessary conditions for stable states and negative loops are the necessary conditions for homeostasis. The stable steady state behavior was observed for the set of parameters derived in tables 2.1 and 2.2, after performing several experiments.

2.4 Hybrid Modeling

After obtaining the right set of parameters, the 2nd step of Dynamical Modeling was applied, i.e., Hybrid Modeling.

Hybrid systems bring together both discrete and continuous components normally

controllers having interaction with the physical environment. In Hybrid modeling both discrete and continuous features were employed to study diverse biological properties and to explore several biological properties in detail (Ahmad *et al.*, 2009).

For Hybrid systems specification analysis and verification methods are required as there is a growing mass of embedded applications particularly in safety critical areas, e.g., avionics or automotive electronics. For automatic verification for specific subclasses, Hybrid automata have been designed entailing discrete as well as continuous specification method (Uller and Stauner, 1996).

In order to handle with temporal issues in a biological phenomenon that is a continuous non-linear process in contrast with discrete data, Hybrid modeling has been used. In this modeling formalism the sigmoid-curve is no longer discretised, rather a piece-wise linear curve would be developed. Here the delays required for gene evolution are represented in terms of time intervals and clocks (Ahmad *et al.*, 2009). Hybrid systems are generally known as timed automaton formalism (Alur and Dill, 1994).

2.4.1 Clock

A clock is a vector of continuous variable. A clock is associated with each gene, i.e., synchronous rate of evolution with respect to time for each gene will be recorded. At each transition these clocks record the rate of evolution such that whenever the system passes from one location to the other, clocks will be reset to 0 (Ahmad *et al.*, 2009). In case of Linear Hybrid Automata (LHA) time period is also applied in terms of clock (Henzinger and Ho, 1995). The current value of a clock represents the time elapsed in the discrete space to cover up the latest transition.

2.4.2 Linear Hybrid Automaton

A Linear Hybrid Automata is a subclass of Hybrid Automata, having full automation. A Hybrid Automata X is linear when all the constant and initial conditions are convex linear predicates over A, all the flow conditions are convex over A and all the skip conditions are convex over A U A' , where A' = $\{a_1', \ldots, a_n'\}$. In case a predicate is an inequality having rational coefficients, over variables in a set Q is linear. If a predicate is a finite set of linear predicates then it is a convex linear predicate. This definition shows that a linear differential equation cannot be inevitably translated into a linear flow condition as there are distinctive concepts of differential equations and hybrid automata (Uller and Stauner, 1996).

In HYTECH, model checking algorithm is implemented to analyze a property such that whether it is violated or not in any state. It sorts out the set of states of Linear Hybrid Automaton by repeatedly applying the time and transition steps, which are approachable from the set of initial states. It can also execute backward accessibility analysis by using time and transition steps, of such a path from where the final path is reachable. The algorithm applied in HYTECH may not necessarily be completed as it is semi-decidable process (Uller and Stauner, 1996). HYTECH is used to analyze the models with pre-defined parameters (parameters defined in GENOTECH Model, Table 2.1).

2.4.3 Period

The results obtained from HYTECH analysis requires the definition of full period (denoted by π (p)). It is the sum of all the delays (once at each expression level) that any

gene goes through sequentially. The gene's original round can be greater than the full time period as there can be lazy phases in gene expression (with no increase or decrease) (Ahmad, 2009). The results obtained from HYTECH were expressed as constraints, showing the nature of the cycle.

2.4.4 Invariance Kernel

A set in which all the points starting the trajectory keep the points within the set is called invariant set and the largest of this set is called invariance kernel. Invariance kernel gives information about the behavior of the cycle. It is a set of states which generate a trajectory by primordial permissible command and then remain in it by satisfying the constraints forever.

In a BRN, let all the temporal state space is given by a subset K. When $x \in K$, then set of K is immutable it says that every trajectory or pathway starting in x is feasible and executable in K. The largest unalterable subset of K is known as invariance kernel.

If the system comes outside the kernel then it will move in divergence trajectories leading to stable steady states.

CHAPTER NO. 3

RESULTS

3. RESULTS

The lack of quantitative data to study the behavior of gene regulatory networks is covered by the qualitative characteristics of biological systems using René Thomas logical formalism (Thomas, 1973). To capture the real dynamics of the BRNs "Multivalued levels" of expression was developed from the initial Boolean idealization (Thomas, 1991). Having a finite set of states, the level of precision obtained in resulting dynamics, matches well with the observations that appear mostly qualitative and articulated in natural language. More precise and accurate results were achieved after applying the discrete logical formalism as a first step in model checking.

The results generated by the HYTECH tool have been shown in the form of a discretisation map, representing the main dynamical features of the system. This discretisation of kinetic parameters corresponding to the logical parameters reveals valuable information about the characteristics of the BRN as discussed below with respect to the NF-κB BRN in case of HIV infection.

3.1 State Table

The parameter optimization leads the model to generate a state table as depicted in table 2.1 and 2.2. The state tables illustrated in tables 3.1 and 3.4 in addition to Input and their respective Output states (transition states), also contains Weight (w) and K values. w represents weight of the input state while k shows maximum activation level of a protein, after applying the weight (w). The initial state is given violet colour and the epigenetic state (Deadlock state) was coloured Red in state table (Table 3.1). A state generally has (n-1) output states where 'n' is the number of elements (proteins involved)

Table 3.1 State Transition table

Proteins /Genes			Weights			K parameters			Transition States
HIV	TNF-A	NF-KB	WHIV	WTNF-A	WNF-kB	KHIV	KTNF-A	KNF-kB	
0	0	0	{TNF-A}	{}	{}	1	0	0	[[1,0,0]]
0	0	1	{TNF-A, NF-KB}	{}	{}	1	0	0	[[1,0,1], [0,0,0]]
0	0	2	{TNF-A, NF-KB}	{NF-KB}	{}	1	1	0	[[1,0,2], [0,1,2], [0,0,1]]
0	1	0	{}	{}	{TNF-A}	0	0	2	[[0,0,0], [0,1,1]]
0	1	1	{NF-KB}	{}	{TNF-A}	1	0	2	[[1,1,1], [0,0,1], [0,1,2]]
0	1	2	{NF-KB}	{NF-KB}	{TNF-A}	1	1	2	[[1,1,2]]
1	0	0	{TNF-A }	{HIV}	{HIV}	1	1	2	{[1,1,0], [1,0,1]]
1	0	1	{TNF-A, NF-KB}	{HIV}	{HIV}	1	1	2	[[1,1,1], [1,0,2]]
1	0	2	{TNF-A,NF-KB	{HIV, NF-KB}	{HIV}	1	1	2	[[1,1,2]]
1	1	0	{}	{HIV}	HIV,TNF-A}	0	1	2	[[0,1,0], [1,1,1]]
1	1	1	{NF-KB}	{HIV}	{HIV,TNF-A}	1	1	2	[[1,1,2]]
1	1	2	{NF-KB}	{HIV, NF-KB}	HIV,TNF-A}	1	1	2	[ ]

in the system.

In the BRN abstracted over here, initial state was taken to be (1,0,0) where the immune system gets activated after being attacked by a foreign invader (HIV-1). This leads to the stimulation of transcriptional machinery NF-κB to produce cytokines (TNF-α) and it also assists HIV-1 virus to replicate in affected cells. The state (1,0,0) gave two possible outcomes (1,0,1) and (1,1,0), where (1,0,1) has more chances to take the system towards complete destruction, i.e., (1,1,2). The state (1,1,2) causes hyperactivation of NF-κB that is detrimental and results in the occurrence of AIDS.

3.2 State graph

A state graph gives a straight forward visualization of all states of a system towards subsequent states. By using the parameters given in table 2.1 applied on the qualitative model of NF-κB activation, a state graph was obtained as shown in figure 3.1. In this figure order of proteins was given according to the setting of parameters, highlighted at the top-left corner. The Stable Steady State can be easily identified (coloured in red) having no outgoing while the two cycles were also evident in this state graph. The stable steady state represents diseased condition where no remedy works as in case of AIDS. The cyclic states however maintain some level of viability of patient's existence even after getting affected of HIV-1 virus. In fact, these cyclic states show the periods of clinical latency, the period when virus stimulates the immune system to produce antibodies against HIV-1.

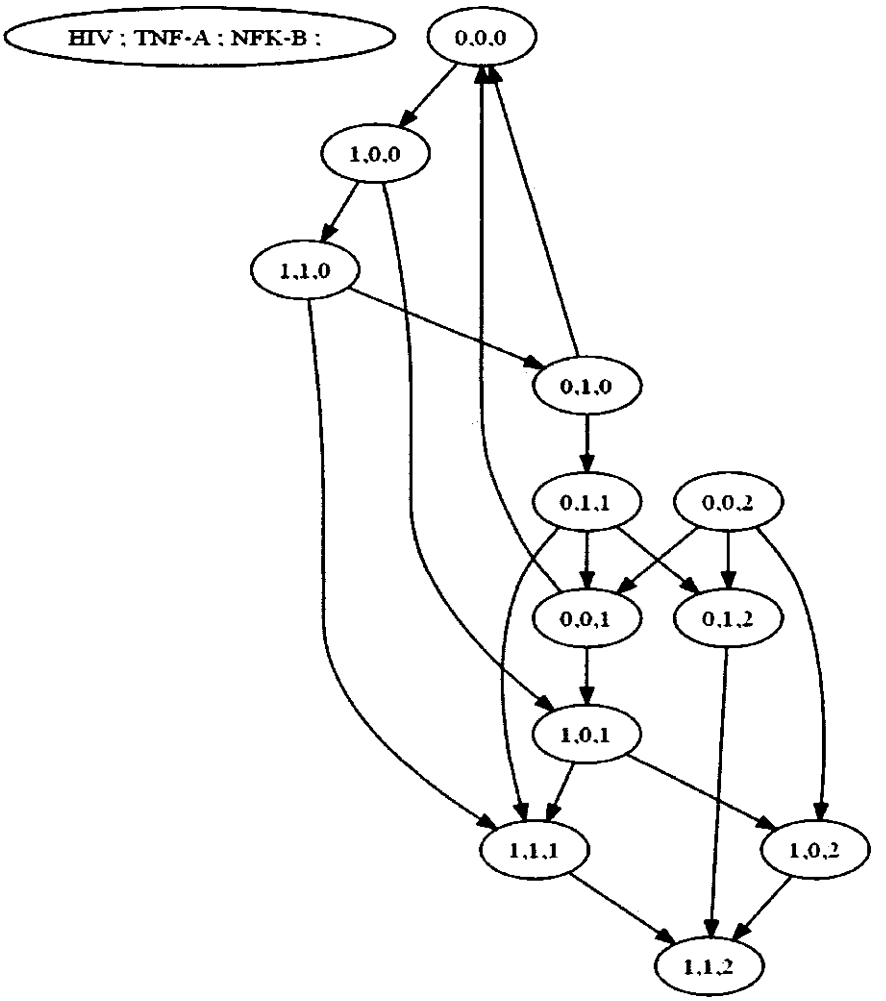


Figure 3.1 States showing all possible paths of NF-κB/RelA and TNF-α and HIV, the deadlock state is represented by the stable steady state (1,1,2)

3.3 Homeostasis and stable steady state of the Biological Regulatory Network

The BRN of T cell activation under the influence of HIV-1 induced activity shows two homeostatic cycles where the HIV-1 infection at early stage is regulated by the production of TNF-α. When the NF-κB Rel A domain is activated by Tat then the uncontrolled production of inflammatory cytokines leads to the vicious cycle. The homeostatic and stable steady state behaviors were shown in figures 3.1, 3.2 and 3.3. Figure 3.1 is the complete discrete model of the BRN while figures 3.2 and 3.3 show only the cyclic behaviors. In these figures, each state defines the discrete concentration of HIV-1, TNF-α and NF-κB respectively.

The oscillating behavior of HIV-1 and TNF-α can be observed, as represented in figure 2.4. It depicts that once HIV-1 enters the cell it starts producing inflammatory cytokine TNF-α (level 1) which is able to kill the virus infected cell or block its entrance into the cell by reducing cell surface receptors CXCR4 and CCR5 (essential for viral entry). It shows the characteristic importance of TNF-α in suppressing viral infection.

The cycle shown in figure 3.3 illustrates that the viral entry into the immune cells is regulated by the subsequent production of inflammatory cytokine TNF-α due to NF-κB activation and makes the cells resistant to further infection of nearby T cells by decreasing the expression of CD4 receptors on T-cells and macrophages. The transcriptional machinery is exploited by the viral RNA for replication which is observed as a stable steady state (1, 1, 2) (figure 3.4).

In the absence of virus or obstruction of viral entry by TNF-α results in regulation

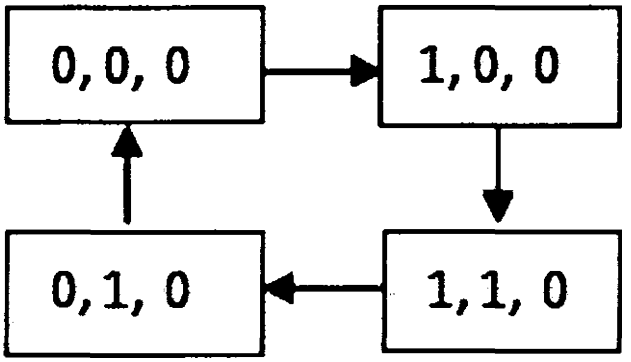


Figure 3.2 Cycle 1 showing oscillating behavior of HIV-1 and TNF-α

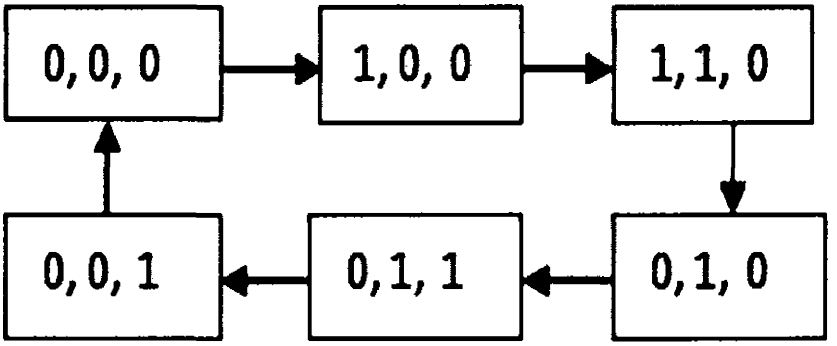


Figure 3.3 Cycle 2 showing Oscillating behavior of HIV-1 and TNF-α

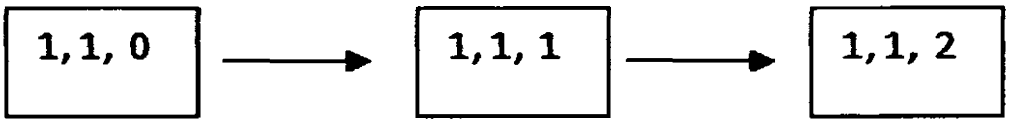


Figure 3.4 Diversion of cyclic behavior (from state (1,1,0)) towards stable steady state (1,1,2)

of immune response which were shown by the cycles in figure 3.2 and 3.3. This response can be generally seen during early periods of HIV-1 entry, this time period is generally required by the virus to infect and kill majority of the immune cells.

The stable steady state (1,1,2) in figure 3.1 depicts that after HIV-1 gets into the macrophage/monocyte, it resides there and uses the host cells as a reservoir and then employs transcription factor NF-κB for replication. The coproduction of TNF-α can deplete the T cells from body and thus any virus can easily invade the immune system which causes death of the patient due to less or no immune response.

From these findings the behavioral pattern of immune system in response to specific antigen like HIV-1 can be easily assessed. Although strong immune response was shown by cyclic behaviors but HIV-1 also overrides the host defense mechanism which is the fundamental aim of HIV by gradual depletion of T cells.

3.4 Pathway leading towards Stable Steady State

A number of pathways lead towards stable steady state (Deadlock state) causing the onset of AIDS, given in table 3.2. These pathways include states emerging from initial state towards the dead end. The cyclic states (those occurring in cycles) were represented in green color and bold red state represents deadlock state. The most fatal pathways include those without cyclic states, i.e., path number 2 and 3 in table 3.2. All other paths have equal chances of entering the homeostatic cycle via the state (1,1,0) and diversion towards stable steady state in case of violation of the conditions (given in Table 3.4) required to be in the cycle has been discussed in HYBRID modeling section in detail.

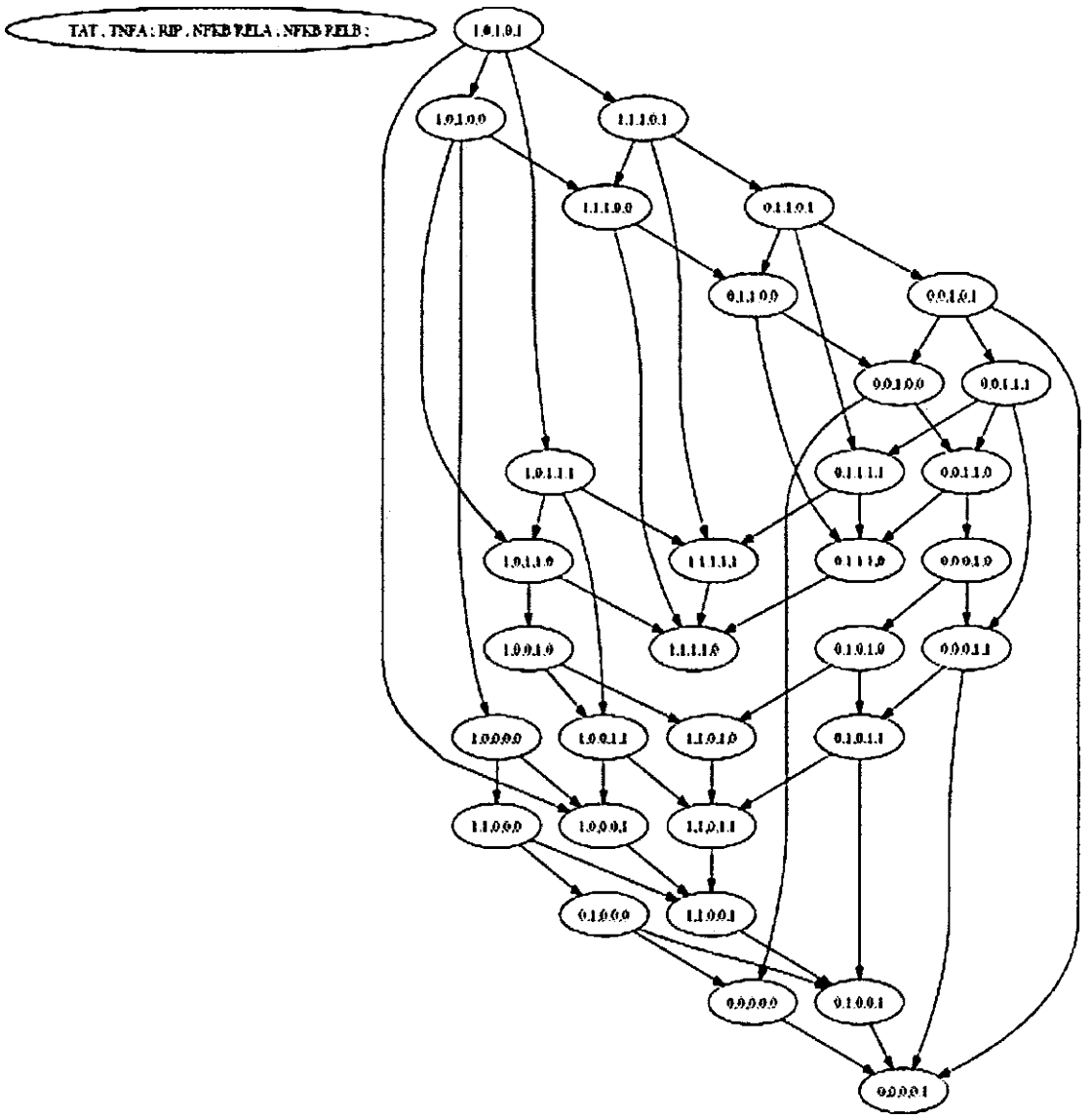


Figure 3.5 Graphical representation of Stable steady states(0,0,0,0,1 and 1,1,1,1,0) of NF-κB with Rel A and Rel B distinct activation (Graphiz model)

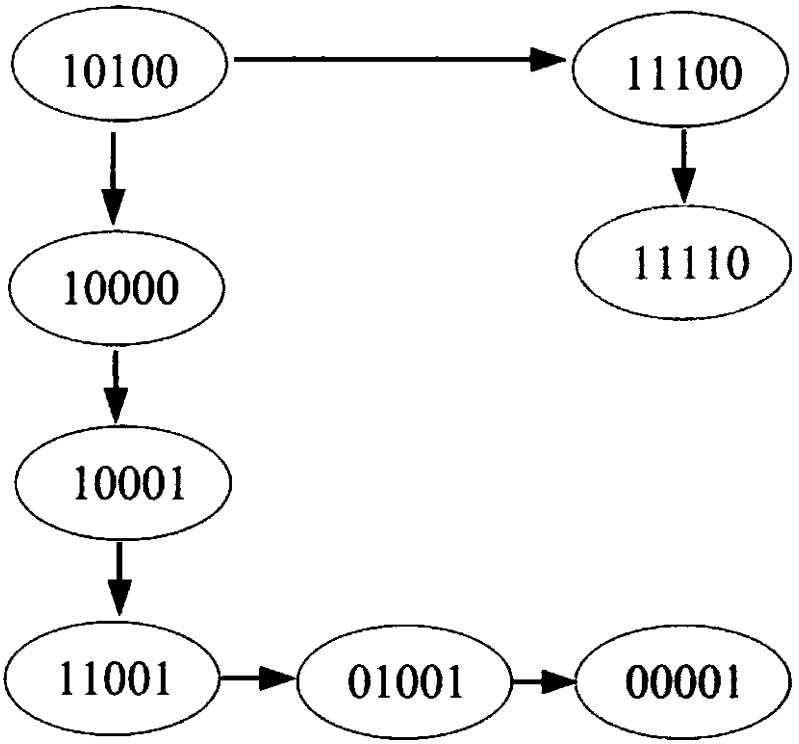


Figure 3.6 Stable steady states of NF-κB with Rel A and Rel B distinctly activated.

3.5 State graph and state table of the Extended Model

The state graph of extended model was given in figure 3.5. The set of transitions that emerge from an initial state and lead towards the deadlock condition were given in figure 3.6. This model has (1,0,1,0,0) as the initial state with Tat (viral protein) and RIP as inducers of immune activation in activated form. It comprises of two stable steady states (0,0,0,0,1) and (1,1,1,1,0) without the existence of any cyclic state, where (0,0,0,0,1) represents normal stable steady state and (1,1,1,1,0) as diseased stable steady state. The state table for the extended model of the BRN has been given in table 3.3, containing the weights for each of the interaction.

3.5.1 Protein Knockout Studies

In order to determine the set of states that are generated by silencing a specific protein (or gene) knock out files have been generated. To knock out Rel B its interactions have been removed, that leads towards three stable steady states (0,0,0,0,0), (1,1,1,1,0) and (1,1,0,1,0). The diseased condition involved inflammatory state of (1,1,1,1,0) with Rel A as inducer of inflammation. It showed that once Rel A gets activated via the RIP it activates the subsequent path of RIP stimulation for IKK phosphorylation which moves NF-κB/RelA subunit into the nucleus to start transcription.

3.5.1.1 Rel B Knock Out Model

To check the inactivation or absence of any gene or protein, knockout files were made. First, Rel B was made inactive by removing all its activation interactions towards other elements of the BRN (Figure 3.7) (if exists). Its state graph both in GENOTECH and Graphiz have been given in figures 3.8 and 3.9 respectively. It confirmed the results

Table 3.2 List of Pathways leading to Stable steady state

1.	100→110→111→**112**
2.	100→101→102→**112**
3.	100→101→111→**112**
4.	100→ 110→ 010→ 011→ 111→ **112**
5.	100→110→010→011→001→101→111→**112**
6.	100→ 110→ 010→ 011→012→**112**

Table 3.3 State Transition table of Extented biological Regulatory Network

Proteins /Genes					**Weights**	**K Parameters**	**Transition States**
TAT	TNF-A	RIP	RELA	RELB	WTAT WTNF-A WRIP WRELA WRELB	KTAT KTNF-A KRIP KRELA KRELB	
0	0	0	0	0	{TNF-A},{},{RIP},{RELB},{RIP}	0 1	[[0,0,0,0,1]]
0	0	0	0	1	{TNF-A},{},{RIP},{},{RIP}	0 0 1	[ ]
0	0	0	1	0	{TNF-A,RELA},{RELA},{RIP},{RELB},{RIP}	1 1 1	[[1,0,0,1,0], [0,1,0,1,0], [0,0,0,1,1]]
0	0	0	1	1	{TNF-A,RELA},{RELA},{RIP},{},{RIP}	1 1 0 1	[[1,0,0,1,1], [0,1,0,1,1], [0,0,0,0,1]]
0	0	1	0	0	{TNF-A},{},{},{RIP,RELB},{RIP}	0 0 1 1	[[0,0,0,0,0], [0,0,1,1,0], [0,0,1,0,1]]
0	0	1	0	1	{TNF-A},{},{},{RIP},{RIP}	0 0 1 1	[[0,0,0,0,1], [0,0,1,1,1]]
0	0	1	1	0	{TNF-A,RELA},{RELA},{},{RIP,RELB},{RIP}	1 1 0 1 1	[[1,0,1,1,0], [0,1,1,1,0], [0,0,0,1,0], [0,0,1,1,1]]
0	0	1	1	1	{TNF-A,RELA},{RELA},{},{RIP},{RIP}	1 1 0 1 1	[[1,0,1,1,1], [0,1,1,1,1],

										[0,0,0,1,1]]
0	0	1	0	0	{TNF-A},{},{},{RIP,RELB},{}	0	0	1	0	[[0,0,1,0,0], [0,0,1,1,0]]
0	0	1	0	1	{TNF-A},{},{},{RIP},{}	0	0	1	0	[[0,0,1,0,1], [0,0,1,1,1], [0,0,1,0,0]]
0	0	1	1	0	{TNF-A,RELA},{RELA},{},{RIP,RELB},{}	1	1	0	1	0 [[1,0,1,1,0], [0,1,1,1,0], [0,0,1,1,0]]
0	0	1	1	1	{TNF-A,RELA},{RELA},{},{RIP},{}	1	1	0	1	0 [[1,0,1,1,1], [0,1,1,1,1], [0,0,1,1,1], [0,0,1,1,0]]
0	1	0	0	0	{},{},{TNF-A,RIP},{RELB},{RIP}	0	0			1 [[0,0,0,0,0], [0,1,0,0,1]]
0	1	0	0	1	{},{},{TNF-A,RIP},{},{RIP}	0	0	0	1	[[0,0,0,0,1]]
0	1	0	1	0	{RELA},{RELA},{TNF-A,RIP},{RELB},{RIP}	1	1			1 [[1,1,0,1,0], [0,1,0,1,1]]
0	1	0	1	1	{RELA},{RELA},{TNF-A,RIP},{},{RIP}	1	1	0	1	[[1,1,0,1,1], [0,1,0,0,1]]
0	1	1	0	0	{},{},{TNF-A},{RIP,RELB},{RIP}	0	0	1	1	1 [[0,0,1,0,0], [0,1,1,0,0], [0,1,1,1,0], [0,1,1,0,1]]

0	1	1	0	1	{},{},{TNF-A},{RIP},{RIP}	0 0 1 1 1	[[0,0,1,0,1], [0,1,1,0,1], [0,1,1,1,1]]
0	1	1	1	0	{RELA},{RELA},{TNF-A},{RIP,RELB},{RIP}	1 1 1 1 1	[[1,1,1,1,0], [0,1,1,1,0], [0,1,1,1,1]]
0	1	1	1	1	{RELA},{RELA},{TNF-A},{RIP},{RIP}	1 1 1 1 1	[[1,1,1,1,1], [0,1,1,1,1]]
0	1	1	0	0	{},{},{TNF-A},{RIP,RELB},{}	0 0 1 1 0	[[0,0,1,0,0], [0,1,1,1,0]]
0	1	1	0	1	{},{},{TNF-A},{RIP},{}	0 0 1 1 0	[[0,0,1,0,1], [0,1,1,1,1], [0,1,1,0,0]]
0	1	1	1	0	{RELA},{RELA},{TNF-A},{RIP,RELB},{}	1 1 1 1 0	[[1,1,1,1,0]]
0	1	1	1	1	{RELA},{RELA},{TNF-A},{RIP},{}	1 1 1 1 0	[[1,1,1,1,1], [0,1,1,1,0]]
1	0	0	0	0	{TNF-A},{TAT},{RIP},{RELB},{RIP}	1 1	[[1,1,0,0,0], [1,0,0,0,1]]
1	0	0	0	1	{TNF-A},{TAT},{RIP},{},{RIP}	1 0 1	[[1,1,0,0,1]]
1	0	0	1	0	{TNF-A ,RELA}, {TAT,RELA}, {RIP}, {RELB}, {RIP}	1 1 1	[[1,1,0,1,0], [1,0,0,1,1]]
1	0	0	1	1	{TNF-A,RELA}, {TAT,RELA}, {RIP}, {}, {RIP}	1 1 0 1	[[1,1,0,1,1], [1,0,0,0,1]]

1	0	1	0	0	{TNF-A},{TAT},{},{RIP,RELB},{RIP}	1 0 1 1	[[1,1,1,0,0], [1,0,0,0,0], [1,0,1,1,0], [1,0,1,0,1]]
1	0	1	0	1	{TNF-A},{TAT},{},{RIP},{RIP}	1 0 1 1	[[1,1,1,0,1], [1,0,0,0,1], [1,0,1,1,1]]
1	0	1	1	0	{TNF-A, RELA}, {TAT,RELA},{},{RIP,RELB}, {RIP}	1 1 0 1 1	[[1,1,1,1,0], [1,0,0,1,0], [1,0,1,1,1]]
1	0	1	1	1	{TNF-A,RELA},{TAT,RELA},{},{RIP},{RIP}	1 1 0 1 1	[[1,1,1,1,1], [1,0,0,1,1]]
1	0	1	0	0	{TNF-A},{TAT},{},{RIP,RELB},{}	1 0 1 0	[[1,1,1,0,0], [1,0,1,0,0], [1,0,1,1,0]]
1	0	1	0	1	{TNF-A},{TAT},{},{RIP},{}	1 0 1 0	[[1,1,1,0,1], [1,0,1,0,1], [1,0,1,1,1], [1,0,1,0,0]]
1	0	1	1	0	{TNF-A,RELA},{TAT,RELA},{},{RIP,RELB},{}	1 1 0 1 0	[[1,1,1,1,0], [1,0,1,1,0]]
1	0	1	1	1	{TNF-A,RELA},{TAT,RELA},{},{RIP},{}	1 1 0 1 0	[[1,1,1,1,1], [1,0,1,1,1], [1,0,1,1,0]]

1	1	0	0	0	{},{TAT},{TNF-A,RIP},{RELB},{RIP}	0 1 1	[[0,1,0,0,0], [1,1,0,0,1]]
1	1	0	0	1	{},{TAT},{TNF-A,RIP},{},{RIP}	0 1 0 1	[[0,1,0,0,1]]
1	1	0	1	0	{RELA},{TAT,RELA},{TNF-A,RIP},{RELB},{RIP}	1 1 1	[[1,1,0,1,1]]
1	1	0	1	1	{RELA},{TAT,RELA},{TNF-A,RIP},{},{RIP}	1 1 0 1	[[1,1,0,0,1]]
1	1	1	0	0	{},{TAT},{TNF-A},{RIP,RELB},{RIP}	0 1 1 1 1	[[0,1,1,0,0], [1,1,1,0,0], [1,1,1,1,0], [1,1,1,0,1]]
1	1	1	0	1	{},{TAT},{TNF-A},{RIP},{RIP}	0 1 1 1 1	[[0,1,1,0,1], [1,1,1,0,1], [1,1,1,1,1]]
1	1	1	1	0	{RELA},{TAT,RELA},{TNF-A}, {RIP,RELB},{RIP}	1 1 1 1 1	[[1,1,1,1,0], [1,1,1,1,1]]
1	1	1	1	1	{RELA},{TAT,RELA},{TNF-A},{RIP},{RIP}	1 1 1 1 1	[[1,1,1,1,1]]
1	1	1	0	0	{},{TAT},{TNF-A},{RIP,RELB},{}	0 1 1 1 0	[[0,1,1,0,0], [1,1,1,1,0]]
1	1	1	0	1	{},{TAT},{TNF-A},{RIP},{}	0 1 1 1 0	[[0,1,1,0,1], [1,1,1,1,1], [1,1,1,0,0]]
1	1	1	1	0	{RELA},{TAT,RELA},{TNF-A},{RIP,RELB},{}	1 1 1 1 0	[ ]
1	1	1	1	1	{RELA},{TAT,RELA},{TNF-A},{RIP},{}	1 1 1 1 0	[[1,1,1,1,0]]

obtained from initial complete model, the absence of Rel B takes the system towards diseased condition (severe inflammation).

10100→11100→11110
↓
10000→11000→01000→00000

3.5.1.2 Rel A knock out Model

Similarly, the absence of Rel A was also confirmed by making it inactive (Figure 3.10). The state graph of Rel A inactivated model in GENOTECH (figure 3.11) and Graphiz (Figure 3.12) represents the whole set of transitions among all the elements of the BRN.

10101→10001→11001→01001→00001

3.6 HYTECH Modeling

The modeling process generated all the possible discrete states and their transitions which give further insights of the steady state behaviors. These steady states (cycles and stable states) are in agreement with the general hypothesis of wet lab experiments. After applying the discrete model of the BRN for NF-κB activation in HIV-1, Hybrid modeling was done.

The BRN can be further extended by adding new biological entities and their interactions. This would lead to analyze the model with new set of logical parameters. Hybrid Modeling is done to analyze the BRN. This would help in computing the necessary and sufficient conditions for the existence of different behaviors. By varying time delays, the speed of concentrations of the entities can help to accurately observe the

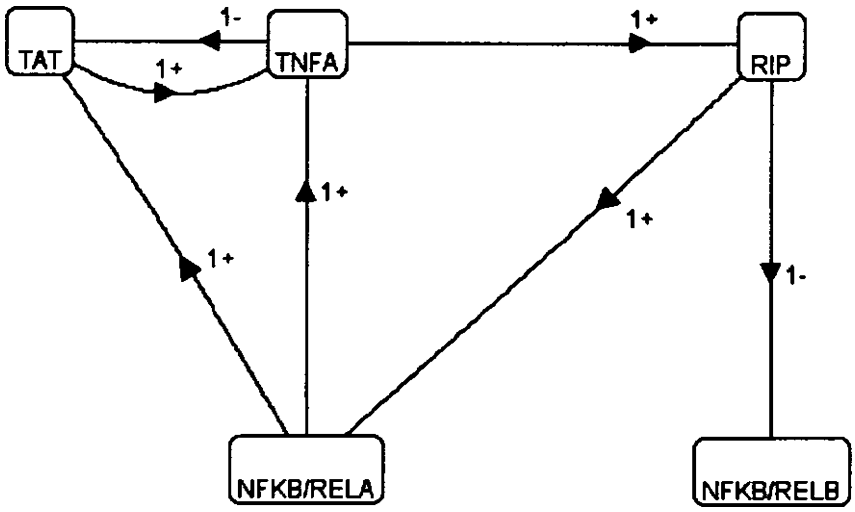


Figure 3.7 GENOTECH Model of Knockout Rel B in extended BRN of NF-κB

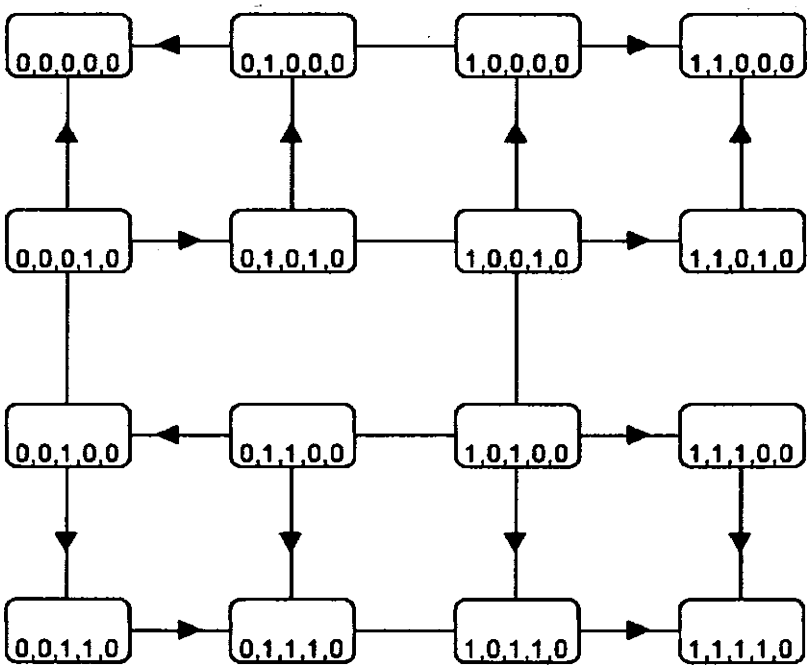


Figure 3.8 State graph of Rel B knockout Model in GENOTECH

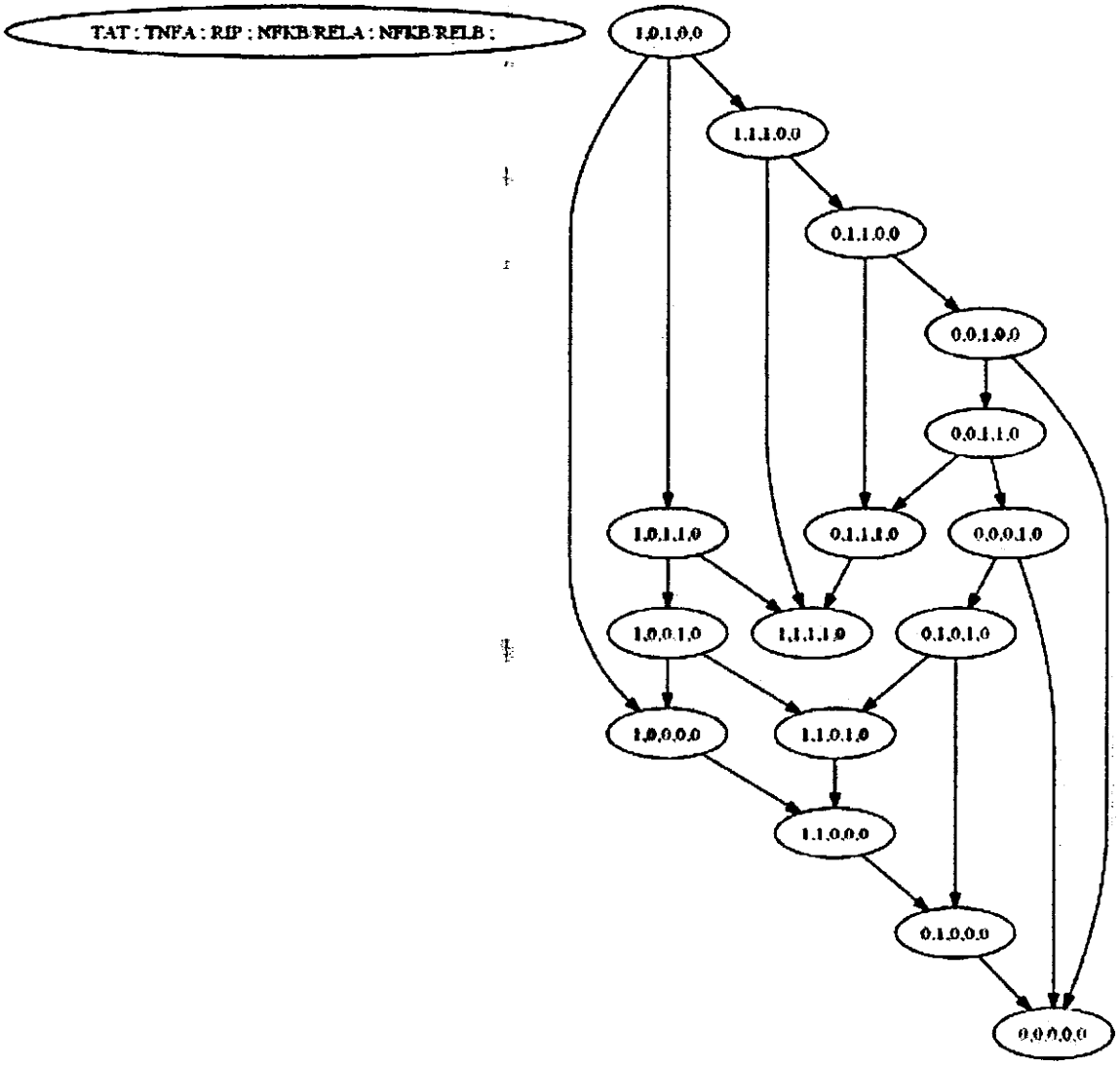


Figure 3.9 State graph of Rel B Knock out of extended BRN (in Graphiz)

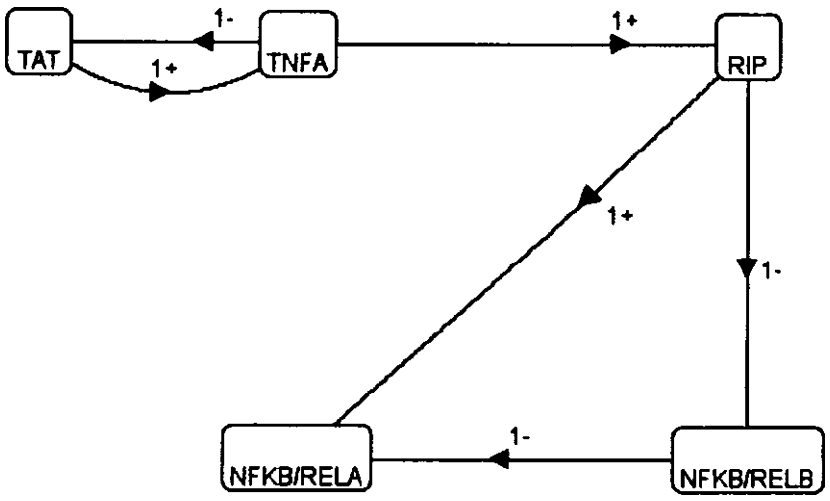


Figure 3.10 GENOTECH Model of Knockout Rel A in extended BRN of NF-κB

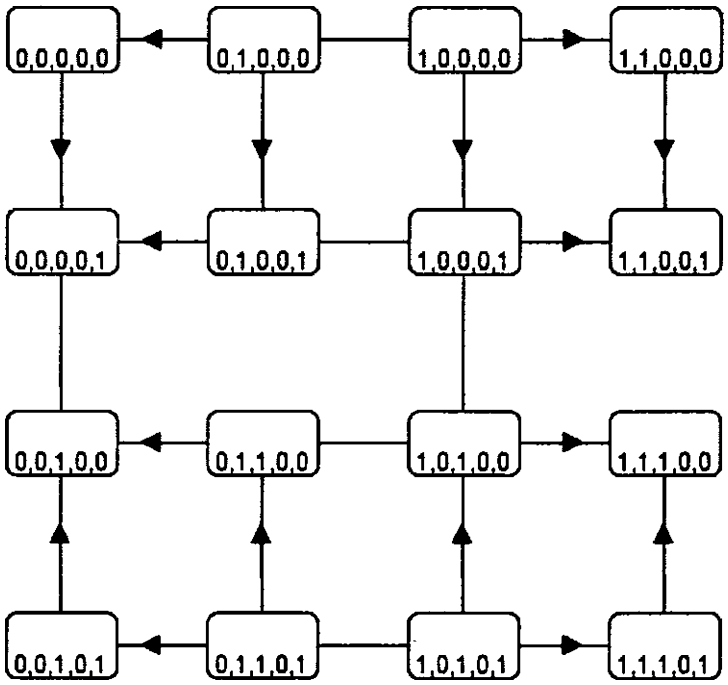


Figure 3.11 State graph of Rel A knockout Model in GENOTECH

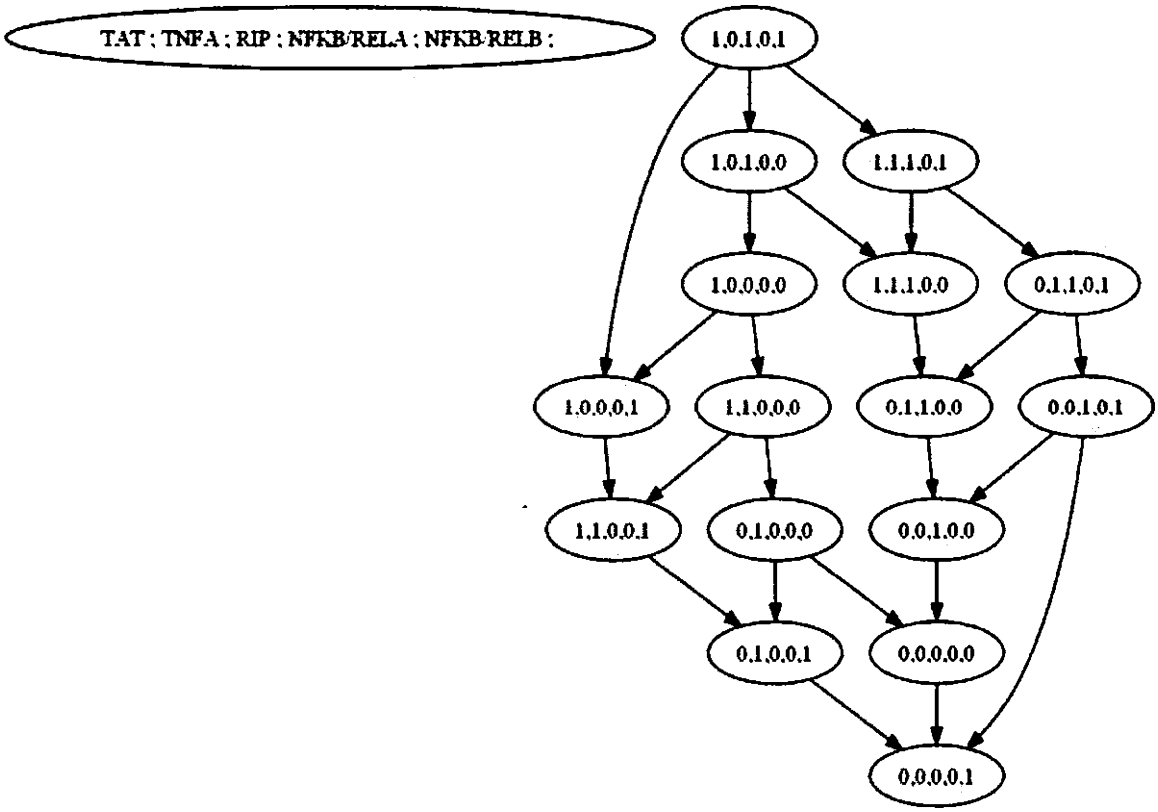


Figure 3.12 State graph of Rel A Knock out of extended BRN (in Graphiz)

system dynamics. This new model was then analyzed by linear hybrid model checking tool HYTECH (Henzinger *et al.*, 1997).

3.6.1 Time delays of Paths leading towards Stable Steady State

The time constraints of each state towards its output state were (stable steady state) given in table 3.4. The conditions for states colored in green (cyclic states) are essential for the system to be in homeostasis and any violation of these conditions would result in taking the system towards dead end. Not all the states were enlisted here as some of the states were neither involved in the path of cycle nor in the path leading towards deadlock state as traverse from the one initial state. The delays were given in the form of equations in which sigma (δ) represents change of, + or – sign represent activation and degradation respectively and base value of δ represents the element and its location from where it starts evolving.

3.6.2 Real time Modeling results of HIV-1 associated BRN

The BRN generated two cycles in HYTECH model. Both these cycles overlap each other. The presence of equality or inequality determined the instability or stability of the cycle respectfully.

The invariance kernels obtained were given as follows:

Cycle No. 1:

000→100→110→010→011→001→000

dpNFKB010 + dnHIV110 = dpHIV000 + dnNFKB001 + 2dnTNF-A 011 & dpTNF-A 100 + dnHIV110 <= dpHIV000 + dnTNF-A 011 & dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dnHIV110 + dpNFKB110 & dpHIV010 >= 0 & dnTNF-A 010

Table 3.4 List of Pathways with Time delays leading towards Stable steady state

1.	$100 \xrightarrow{dpTNF100 <= dpNFKB100} 110 \xrightarrow{dpNFKB010 <= dnHIV110} 111 \xrightarrow{dpNFKB111 <= dpHIV011} 112$
2.	$100 \xrightarrow{dpNFKB100 <= dpTNFA100} 101 \xrightarrow{dpNFKB101 <= dpTNFA101} 102 \xrightarrow{dpTNFA102 <= dpNFKB101} 112$
3.	$100 \xrightarrow{dpNFKB100 <= dpTNFA100} 101 \xrightarrow{dpNFKB100 <= dpTNFA100} 111 \xrightarrow{dpNFKB111 <= dpHIV011} 112$
4.	$100 \xrightarrow{dpTNF100 <= dpNFKB100} 110 \xrightarrow{dnHIV110 <= dpNFKB010}$ $010 \xrightarrow{dpNFKB010 <= dnHIV110} 011 \xrightarrow{dpHIV011 <= dpNFKB011 \ \& \ dpHIV011 >= dnTNF011}$ $111 \xrightarrow{dpNFKB111 <= dpHIV011} 112$
5.	$100 \xrightarrow{dpTNF100 <= dpNFKB100} 110 \xrightarrow{dnHIV110 <= dpNFKB010} 010 \xrightarrow{dpNFKB010 <= dnHIV110}$ $011 \xrightarrow{dpNFKB101 <= dpTNFA100 \ \& \ dpHIV011 <= dnTNF011}$ $001 \xrightarrow{dpNFKB100 <= dpTNFA100} 101 \xrightarrow{dpNFKB100 <= dpTNFA100} 111 \xrightarrow{dpNFKB111 <= dpHIV011} 112$
6.	$100 \xrightarrow{dpTNF100 <= dpNFKB100} 110 \xrightarrow{dnHIV110 <= dpNFKB010} 010 \xrightarrow{dpNFKB010 <= dnHIV110}$ $011 \xrightarrow{dpNFKB011 >= dnTNF011 \ \& \ dpHIV011 <= dpNFKB011} 012 \xrightarrow{dpHIV012 <= dpNFKB011} 112$

<= 0 & dnTNF-A 011 <= 0 & dpHIV011 + dnTNF-A 011 >= 0 & dnTNF-A 011 + dpNFKB011 >= 0 & dpHIV001 + dnNFKB001 + 2dnTNF-A 011 >= 0 & dpTNF-A 001 + dnNFKB001 + dnTNF-A 011 >= 0 & dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dpNFKB000 & dpHIV000 + dnTNF-A 011 <= dpTNF-A 000 & dnNFKB001 + dnTNF-A 011 <= 0 & dpHIV000 + dnNFKB001 + 2dnTNF-A 011 >= 0 & dnHIV100 + dpTNF-A 100 <= dpHIV000 + dnTNF-A 011 & dnNFKB001 + dnTNF-A 011 + dpTNF-A 100 <= dpNFKB100 & dpHIV000 + dnTNF-A 011 <= dpTNF-A 100

dpNFKB010 + dnHIV110 = dpHIV000 + dnNFKB001 + 2dnTNF-A 011 & dpTNF-A 100 + dnHIV110 <= dpHIV000 + dnTNF-A 011 & dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dnHIV110 + dpNFKB110 & & dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dpNFKB000 & dpHIV000 + dnTNF-A 011 <= dpTNF-A 000 & & dnHIV100 + dpTNF-A 100 <= dpHIV000 + dnTNF-A 011 & dnNFKB001 + dnTNF-A 011 + dpTNF-A 100 <= dpNFKB100 & dpHIV000 + dnTNF-A 011 <= dpTNF-A 100

Cycle No. 2:

000→100→110→010→000

dpHIV010 + dnTNF-A 010 >= 0 & dpHIV000 + dnTNF-A 010 <= dpTNF-A 000 & dpHIV000 + dnTNF-A 010 >= 0 & dnTNF-A 010 <= 0 & dnHIV100 + dpTNF-A 100 <= dpHIV000 + dnTNF-A 010 & dpTNF-A 100 + dnHIV110 <= dpHIV000 + dnTNF-A 010 & dpHIV000 <= dnHIV110 + dpNFKB110 & 2dpHIV000 <= dpNFKB000 + dnHIV110 & 2dpHIV000 + dpNFKB010 <= dpNFKB000 + dnHIV110 & dpHIV000 + dpNFKB010 <= dnHIV110 + dpNFKB110 & 2dpHIV000 + dnHIV100 <= dpNFKB000 + 2dnHIV110 & dpHIV000 + dnHIV100 <= 2dnHIV110 + dpNFKB110 & 2dpHIV000 + dnHIV100 <= dpNFKB100 + 2dnHIV110 & 2dpHIV000 <=

dpNFKB100 + dnHIV110 & dpHIV000 + dnTNF-A 010 <= dpTNF-A 100 & 2dpHIV000 + dpNFKB010 <= dpNFKB100 + dnHIV110

After removing the non-trivial invariance kernels and by re-arranging following constraints were obtained:

Cycle No. 1 000→100→110→010→011→001→000

1. dpNFKB010 + dnHIV110 = dpHIV000 + dnNFKB001 + 2dnTNF-A 011
2. dpTNF-A 100 + dnHIV110 <= dpHIV000 + dnTNF-A 011
3. dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dnHIV110 + dpNFKB110
4. dpHIV000 + dnNFKB001 + 2dnTNF-A 011 <= dpNFKB000
5. dpHIV000 + dnTNF-A 011 <= dpTNF-A 000
6. dnHIV100 + dpTNF-A 100 <= dpHIV000 + dnTNF-A 011
7. dnNFKB001 + dnTNF-A 011 + dpTNF-A 100 <= dpNFKB100
8. dpHIV000 + dnTNF-A 011 <= dpTNF-A 100

Invariance kernel no. 1 and 3 contained quiet similar variables with similar state spaces except those for NF-κB, as discussed below:

1. dpHIV000 + |dnHIV110| = dpNF-KB010 + |dnNF-KB001| +|2dnTNF-A 011|

Ist by ignoring the TNF-α, the equation became,

π (HIV) >= π (NF- κB)

Then by removing NF-κB, the following condition appeared,

π (HIV) >= π (TNF-α)

This constraint captured the general trend of HIV infection and immune stimulation. It showed that sum of positive and negative time delay of HIV is greater than the time delay of TNF-α and NF-κB respectively, as shown graphically in figure 3.13.

2. dpTNF-A 100 + |dn¯ TNF-A 011| >= dpHIV000 + | dn¯ HIV110|

It was graphically shown in figure 3.14. The time delay required for the activation and inhibition (or degradation) of TNF-α at the position 100 and 011 is greater than the activation and inhibition time delay of HIV at position 000 and 011. This condition occurs before the onset of disease in the body.

3. dpHIV000 + | dn¯ HIV110| >= dpNF-KB110 + | dn¯ NF-KB001| + |2dn¯ TNF-A 011|

The time delay of HIV (positive delay at location 000 and negative delay at 110) is greater than that of positive and negative delay of NF-κB (positive delay at 110 and negative delay at 001) and double negative of TNF-α at location 011, shown in figure 3.15. This constraint is an important candidate for keeping the cycle in homeostatic state.

4. dpHIV000 + 2dnTNF-A 011 >= dpNF-KB000 + | dn¯ NF-KB001|

The graph given in figure 3.16 depicted that the activation time period (positive time delay) of HIV at position 000 and double negative time delay or degradation delay of TNF-α at 011 is greater than the period of NF-κB at state space 000 and 001.

5. dpHIV000 >= dpTNF-A 000 + |dn¯ TNF-A 011|

The graph given in figure 3.17 showed that the activation delay of HIV at location 000 takes more time than that of complete period of TNF-α at location 000 and 011. It showed that once HIV gets into the immune system, it propagated the replication

machinery (NF-κB) in order to produce its replicas, the immune system quickly responded to the presence of foreign invader by the production of cytokines (TNF-α). As the time period of HIV is greater than TNF-α, it showed that HIV requires more time to activate and degrade while TNF-α has faster cycle of activation and degradation in this location.

6. dpTNF-A 100 + | dn¯ TNF-A 011| >= dpHIV000 + | dn¯ HIV100|

The graph given in figure 3.18 demonstrates that production rate of HIV at location 000 is faster than that of TNF-α at location 100. It showed that once HIV gets into the immune system, it boosts the replication of HIV and at the same time the system defenders, i.e., the cytokines (TNF-α in BRN here) are also produced to defeat the foreign invaders (HIV here). The time period taken by TNF-α reaches the threshold after the period of HIV. Similarly the degradation time period of TNF-α at position 011 is more than that of HIV at position 100, i.e., TNF-α takes more time to reaches its destination as compared to HIV in order to activate or enhance the immune system stimulation.

7. dnTNF-A 011 + dpTNF-A 100 >= dpNF-KB100 + |dnNF-KB001|

This equation states that the time period of NF-κB at location 100 and 001 is faster than TNF-α at position 100 and 011, given in figure 3.19. The activation of NF-κB is achieved both by HIV and TNF-α, so the functioning of NF-κB should be tuned in a way that utilizes the positive role of NF-κB and evading its role in HIV replication. It could bring an important breakthrough in drug designing.

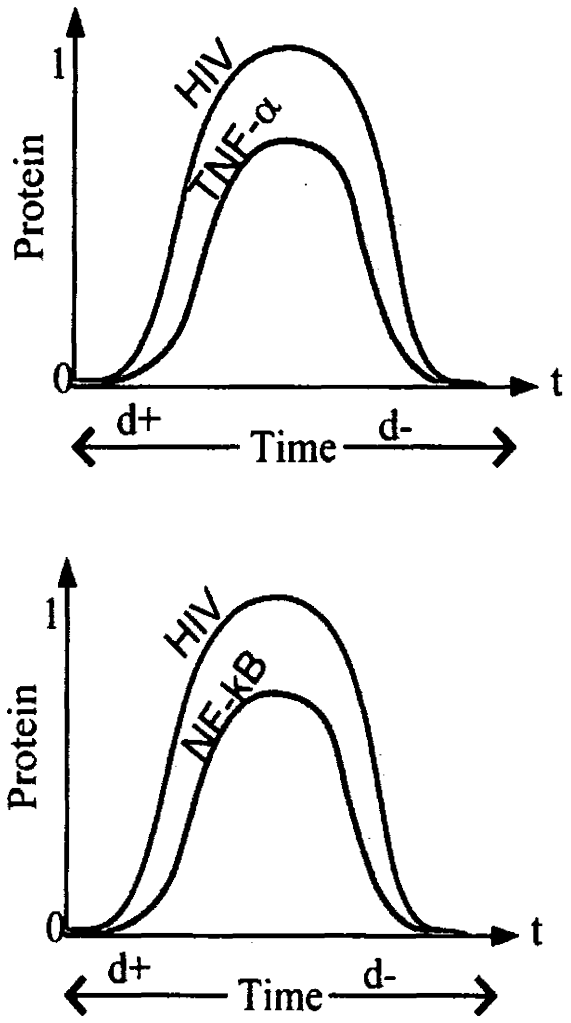


Figure 3.13 Graphical representation of HIV time delay relative to NF-κB and TNF-α (Ist Invariance kernel when π HIV is greater than both NF-κB and TNF-α)

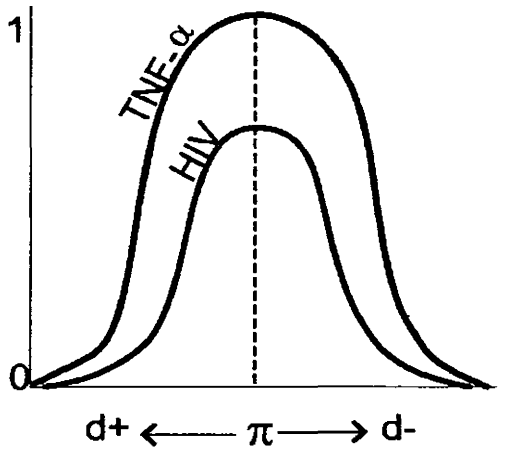


Figure 3.14 Graphical representation of time delay of TNF-α relative to HIV (2nd Invariance kernel)

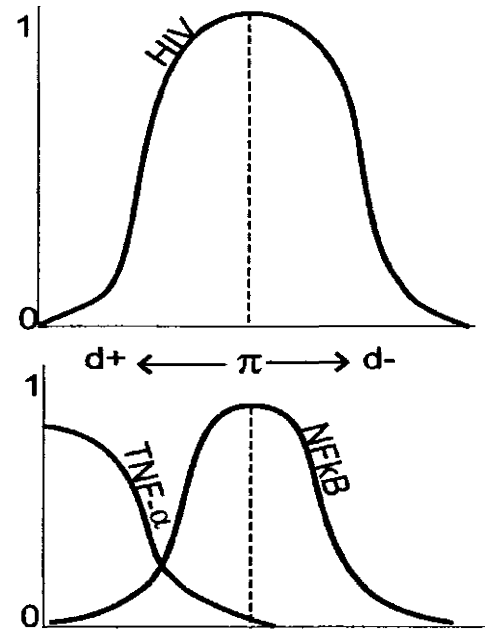


Figure 3.15 Graphical representation of time delay of HIV w.r.t TNF-α and NF-κB (3rd Invariance kernel)

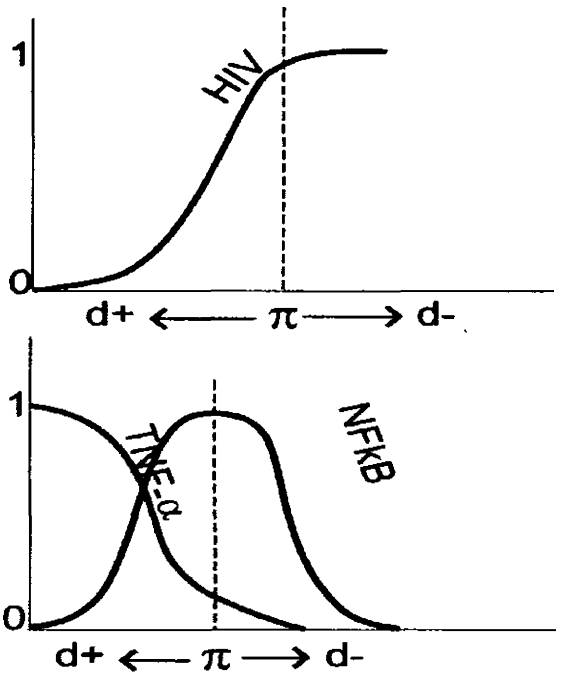


Figure 3.16 Graphical representation of time delay of HIV w.r.t TNF-α and NF-κB (4th Invariance kernel)

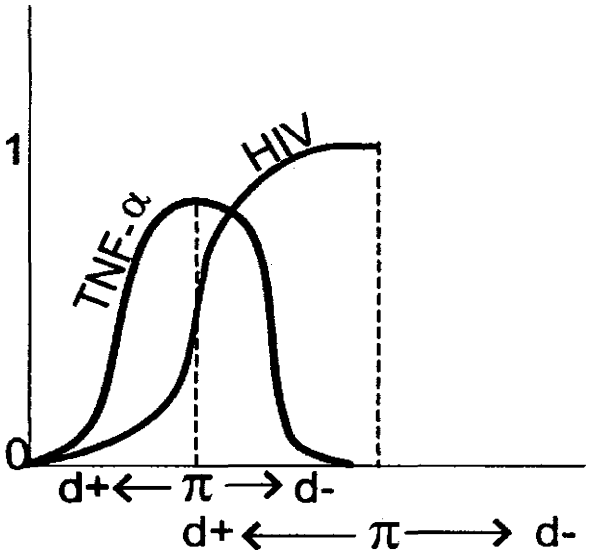


Figure 3.17 Graphical representation of time delay of HIV w.r.t TNF-α (5th Invariance kernel)

8. dpHIV000 >= dpTNF-A 100 + | dn^{-} TNF-A 011|

This condition was similar to the constraint no. 5. It showed that the activation delay of HIV at location 000 takes more time than that of complete period of TNF-α at location 100 and 011, shown in figure 3.20. Both these invariance kernels will be helpful in drawing important predictions.

Cycle No. 2 000→100→110→010→000

1. dpHIV000 + dnTNF-A 010 <= dpTNF-A 000
2. dnHIV100 + dpTNF-A 100 <= dpHIV000 + dnTNF-A 010
3. dpTNF-A 100 + dnHIV110 <= dpHIV000 + dnTNF-A 010
4. dpHIV000 <= dnHIV110 + dpNF-KB110
5. 2dpHIV000 <= dpNF-KB000 + dnHIV110
6. 2dpHIV000 + dpNF-KB010 <= dpNF-KB000 + dnHIV110
7. dpHIV000 + dpNF-KB010 <= dnHIV110 + dpNF-KB110
8. 2dpHIV000 + dnHIV100 <= dpNF-KB000 + 2dnHIV110
9. dpHIV000 + dnHIV100 <= 2dnHIV110 + dpNF-KB110
10. 2dpHIV000 + dnHIV100 <= dpNF-KB100 + 2dnHIV110
11. 2dpHIV000 <= dpNF-KB100 + dnHIV110
12. dpHIV000 + dnTNF-A 010 <= dpTNF-A 100
13. 2dpHIV000 + dpNF-KB010 <= dpNF-KB100 + dnHIV110
14. 2dpHIV000 + dnHIV100 <= dpNF-KB100 + 2dnHIV110

15. 2dpHIV000 <= dpNF-KB100 + dnHIV110

16. dpHIV000 + dnTNF-A 010 <= dpTNF-A 100

17. 2dpHIV000 + dpNF-KB010 <= dpNF-KB100 + dnHIV110

After re-arranging the conditions for cycle 2 following equations were made:

1. dpHIV000 >= dpTNF-A 000 + |dn¯TNF-A 010|

The graph given in figure 3.21 showed that time period of HIV activation at location 000 is slower than that of complete period of TNF-α at location 000 and 010. It showed that once HIV gets into the immune system, it propagates the replication machinery (NF-κB) in order to make replication of HIV, the immune system quickly responds to the presence of foreign by the production of cytokines (TNF-α in our BRN) to defeat the foreign invaders.

The time period of HIV is greater than TNF-α, showing that HIV requires more time to activate and degrade while TNF-α has faster cycle of activation and degradation. This time delays is important from the prospective of delaying disease progression.

2. dpTNF-A 100 + |dn¯TNF-A 010| >= dpHIV000 + |dn¯HIV100|

This graph given in Figure 3.22 showed that production rate of HIV at location 000 is faster than that of TNF-α at location 100. It showed that once HIV gets into the immune system, it boosts the replication of HIV and at the same time the immune system defenders, i.e., the cytokines (TNF-α in BRN here) are also produced to defeat the foreign invaders (HIV here). The time period taken by TNF-α reaches the threshold after the period of HIV. The time period of degradation of TNF-α at position 010 is less than

that of HIV at position 010, i.e., TNF-α reaches its destination earlier as compared to HIV in order to activate or enhance the immune system activation.

3. dpTNF-A 100 + |dn¯TNF-A 010| >= dpHIV000 + |dn¯HIV110|

This graph given in figure 3.23 showed that activation of HIV is greater than that of TNF-α at location 100. It showed that once HIV gets into the immune system, it boosts the replication of HIV and at the same time the system defenders, i.e., the cytokines (TNF-α in BRN here) are also produced to defeat the foreign invaders (HIV here). The time period taken by TNF-α reaches the threshold after the period of HIV. The time period of degradation of TNF-α at position 010 is greater than that of HIV at position 110, i.e., at this location TNF-α reaches its destination following HIV in order to activate the immune system stimulation.

4. dpHIV000 + |dn¯HIV110| >= dpNF-KB110

The time period for HIV activation and inhibition or degradation rate and activation time of NF-κB is compared. The graph given in figure 3.24 depicts that time period taken by NF-κB in order to get activated at location 110 is less than that of production and degradation rate of HIV at location 000 and 110.

5. 2dpHIV000 + |dn¯HIV110| >= dpNF-KB000

The time period for HIV activation and inhibition or degradation rate and activation time of NF-κB is compared with respect to activation position. The time period taken by NF-κB in order to get activated at location 000 is less than that of production and degradation rate of HIV at location 000 and 110 (figure 3.25).

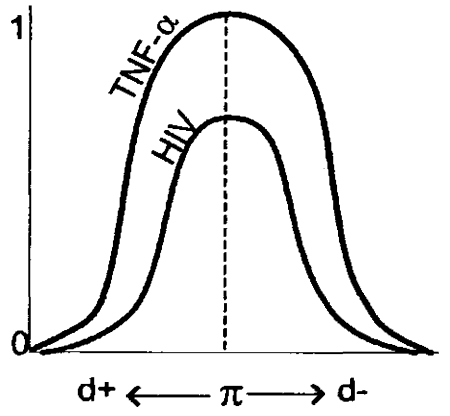


Figure 3.18 Graphical representation of time delay of TNF-α w.r.t HIV (6th Invariance kernel)

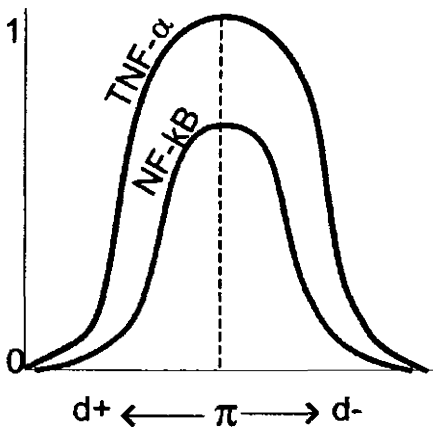


Figure 3.19 Graphical representation of time delay of TNF-α w.r.t NF-κB (7th Invariance kernel)

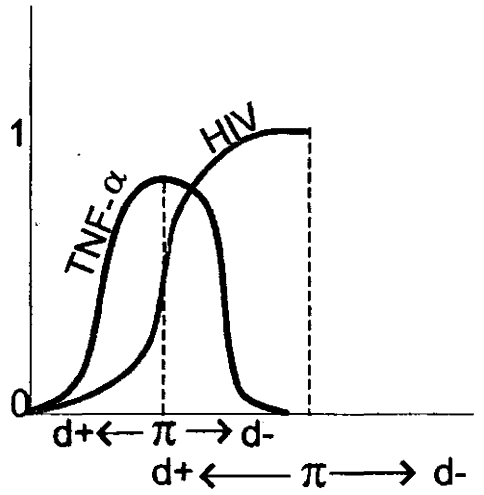


Figure 3.20 Graphical representation of time delay of TNF-α w.r.t HIV (8th Invariance kernel)

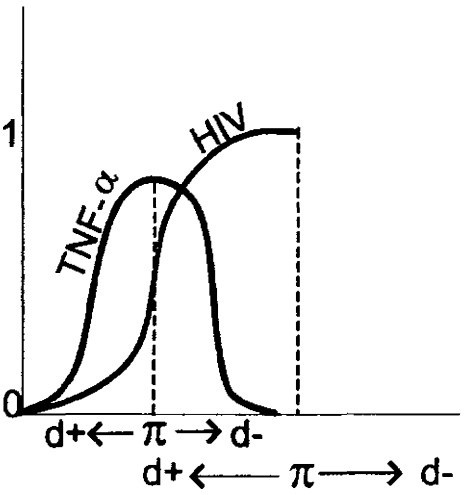


Figure 3.21 Time period of HIV activation at location 000 (I[st] Invariance kernel of cycle 2)

6. 2dpHIV000 + |dn¯HIV110| + dpNF-KB010 >= dpNF-KB000

In graph given in figure 3.26 showed that the period for HIV activation and degradation along with NF-κB activation has shown to be greater the time period required for NF-κB acitvation. As the constraint tells us that production or activation rate of HIV at position 000, its inhibition or degradation at position 110 and activation of NF-κB at location 010 takes more time to reach its threshold than NF-κB at location 000.

7. dpHIV000 + dpNF-KB010 + |dnHIV110| >= dpNF-KB110

This equation resembles the 6^{th} constraint (cycle 2), showing that the period for HIV activation and degradation along with NF-κB activation is greater the time period required of NF-κB activation, as depicted in figure 3.27. This equation reflects that production or activation rate of HIV at position 000, its inhibition or degradation at position 110 and activation of NF-κB at location 010 takes more time to reach its threshold than NF-κB at location 110.

8. 2dpHIV000 + dnHIV100 + |2dn¯HIV110| >= dpNF-KB000

This equation is the representation of strong immune response against HIV, where the period of HIV activation at position 000, degradation at position 100 and double degradation at 110 is exceeding the growth rate of NF-κB at state 000 (figure 3.28). The constraints from 8-10 are similar having a difference in the state of NF-κB activation.

9. dpHIV000 + dnHIV100 + |2dnHIV110| >= dpNF-KB110

It showed a strong immune response shown by the T-cells against HIV, where the period of HIV activation at position 000, degradation at position 100, and double degra-

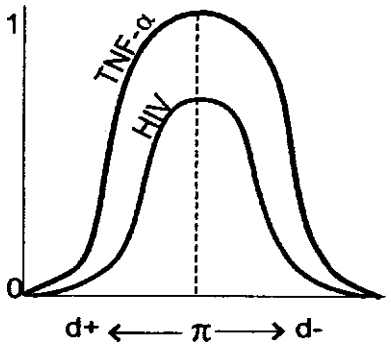


Figure 3.22 Time period of HIV at location 000 is faster than that of TNF-α at location 100 (2nd invariance kernel of cycle 2)

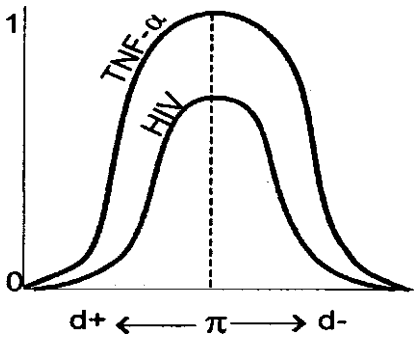


Figure 3.23 Time period of HIV at location 000 is faster than that of TNF-α at location 100 (3rd invariance kernel of cycle 2)

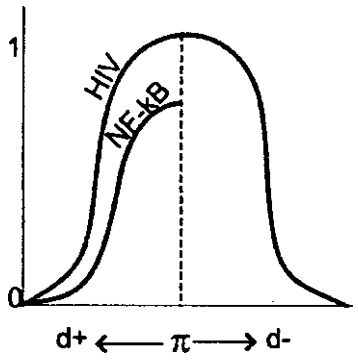


Figure 3.24 Time period of production and degradation rate of HIV at location 000 and 110 w.r.t NF-κB (4th invariance kernel of cycle 2)

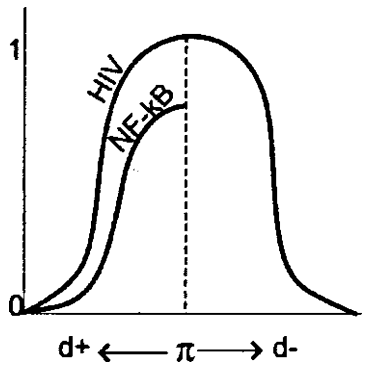


Figure 3.25 Time period of production and degradation rate of HIV at location 000 and 110 w.r.t NF-κB (5th invariance kernel of cycle 2)

dation at 110 is exceeding the growth rate of NF-κB at state 110 (figure 3.29).

10. 2dpHIV000 + dnHIV100 + |2dn¯HIV110| >= dpNF-KB100

Another condition for strong immune response shown by the T-cells against HIV was shown in this graph, in which the time period of HIV activation at position 000, degradation at position 100, and double degradation at 110 is exceeding the growth rate of NF-κB at state 100 (figure 3.30).

11. 2dpHIV000 + |dn¯HIV110| >= dpNF-KB100

The cycle is able to maintain the Homeostatic behavior by keeping the period of HIV activation (replication) and degradation (inhibition) at state 000 and 110 respectively greater than the production rate (activation) of NF-κB at position 100 (figure 3.31). It showed that HIV needs more time to complete its whole cycle of activity while NF-κB responds fastly.

12. dpHIV000 >= dpTNF-A 100 + dnTNF-A 010

In this graphical representation the mode of action of TNF-α at location 100 and 010 has been compared to that of HIV at location 000 (figure 3.32). Here the time delay of HIV is yet again going beyond the time delay required for TNF-α activation and degradation.

13. 2dpHIV000 + |dn¯HIV110| + dpNF-KB010 >= dpNF-KB100

In this graph the period for HIV activation and degradation along with NF-κB activation is greater than the time period required for NF-κB acitvation. This constraint illustrates that the time delay of HIV for its production at position 000, rate of inhibition

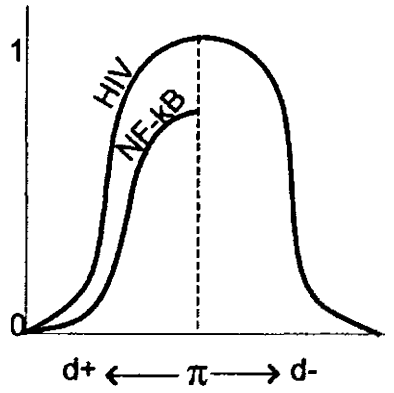


Figure 3.26 Time period of activation and degradation of HIV along with NF-κB activation has shown to be greater the time period required for NF-κB acitvation (6^{th} invariance kernel of cycle 2)

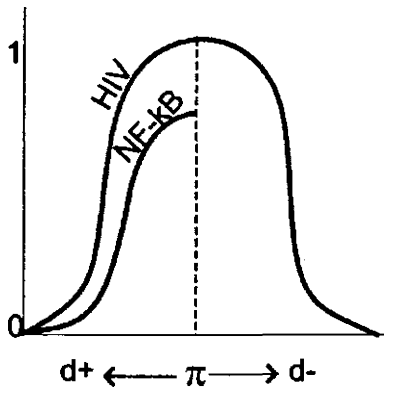


Figure 3.27 Time period of production and degradation rate of HIV at location 000 and 110 w.r.t NF-κB (7^{th} invariance kernel of cycle 2)

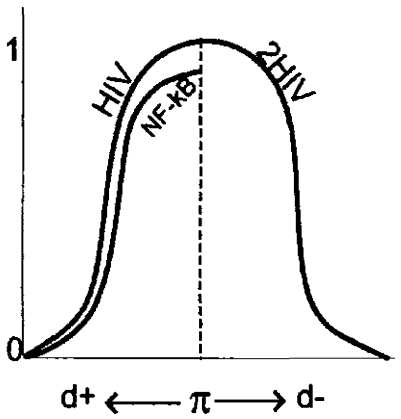


Figure 3.28 Time period of HIV activation at position 000, degradation at position 100 w.r.t NF-κB (8^{th} invariance kernel of cycle 2)

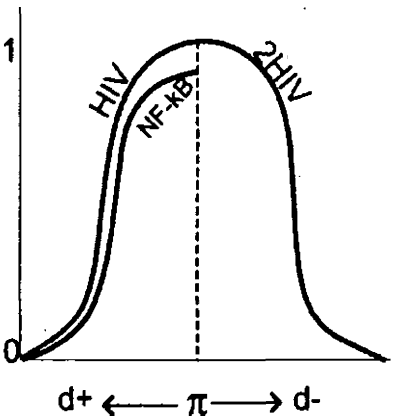


Figure 3.29 Time period of HIV activation at position 000, degradation at position 100 w.r.t NF-κB (9^{th} invariance kernel of cycle 2)

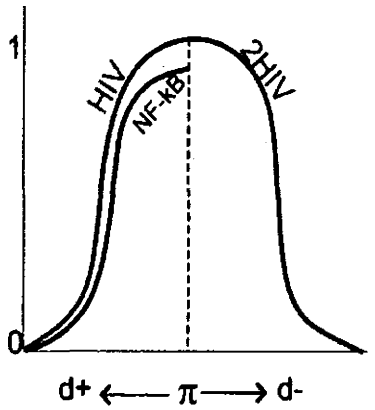


Figure 3.30 Time period of HIV activation at position 000, degradation at position 100 w.r.t NF-κB (10th invariance kernel of cycle 2)

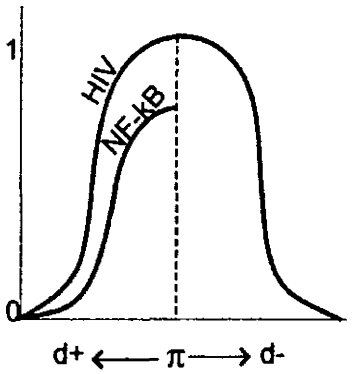


Figure 3.31 Time period of HIV activation at position 000, degradation at position 110 w.r.t NF-κB (11th invariance kernel of cycle 2)

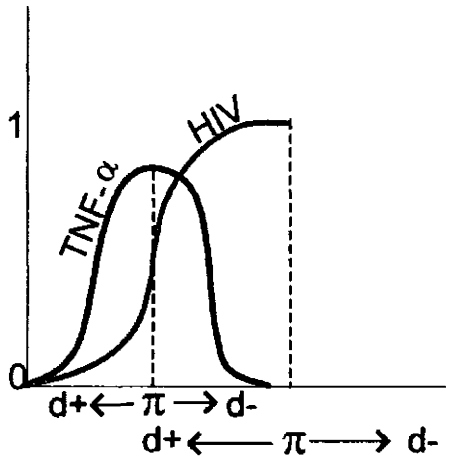


Figure 3.32 Time period of HIV activation w.r.t TNF-α (12th invariance kernel of cycle 2)

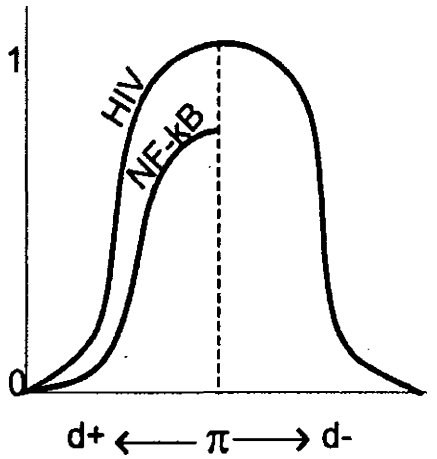


Figure 3.33 Time period of HIV activation w.r.t NF-κB (13th invariance kernel of cycle 2)

or degradation at position 110 along with the activation of NF-κB at the location 010 is greater than the time delay of NF-κB at location 000 to reach its threshold (figure 3.33).

14. 2dpHIV000 + dnHIV100 + |2dnHIV110| >= dpNF-KB100

This equation is similar to the equation no. 10 where a strong immune response was shown by the T-cells against HIV, in which the time period of HIV activation at position 000, degradation at position 100, and double degradation at 110 is exceeding the period required for NF-κB activation at state 100 (figure 3.34).

15. 2dpHIV000 + |dnHIV110| >= dpNF-KB100

The time delay of NF-κB at position 100 for its activation is less than the period of HIV at position 000 and 110 in its activation and inhibition respectively (figure 3.35).

16. dpHIV000 >= dpTNF-A 100 + |dnTNF-A 010|

The mode of action of TNF-α at location 100 and 010 has been compared to that of HIV at location 000 as were shown in the equation No. 12. The graphical representation of this equation (figure 3.36) showed that the time delay of HIV is greater than that of TNF-α for both activation and degradation.

17. 2dpHIV000 + |dnHIV110| + dpNF-KB010 >= dpNF-KB100

It resembles constraint no. 13 except the position for NF-κB activation. In this graph (figure 3.37) the period for HIV activation and degradation is greater than the time period required for NF-κB activation. According to this invariance kernel, positive and negative time delay of HIV at position 000 and 110 respectively along with activation of NF-κB at location 010 is greater than the time delay of NF-κB at location 100 to reach its threshold.

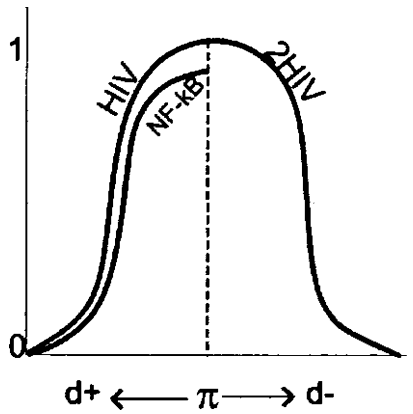


Figure 3.34 Time period of HIV activation w.r.t NF-κB (14th invariance kernel of cycle 2)

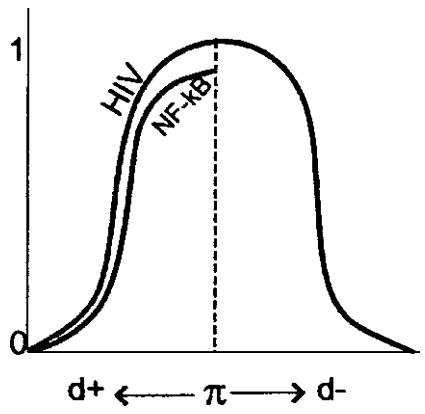


Figure 3.35 Time period of HIV activation w.r.t NF-κB (15th invariance kernel of cycle 2)

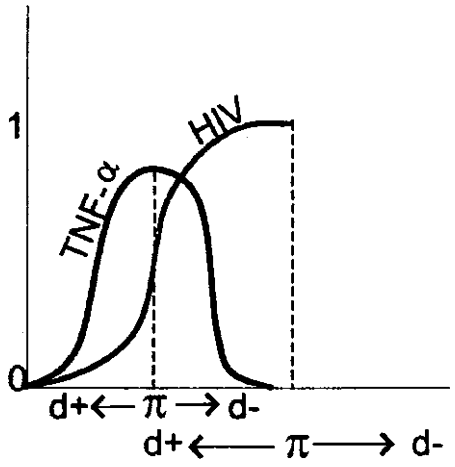


Figure 3.36 Time period of HIV activation w.r.t NF-κB (16th invariance kernel of cycle 2)

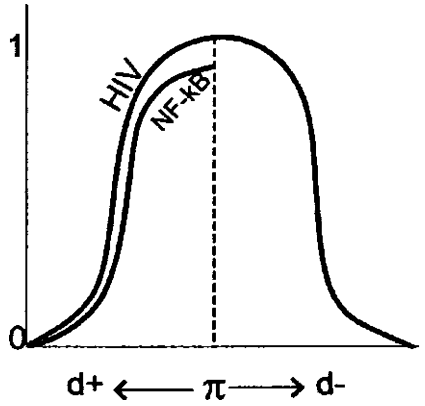


Figure 3.37 Time period of HIV activation w.r.t NF-κB (17th invariance kernel of cycle 2)

CHAPTER NO. 4

DISCUSSION

4. DISCUSSION

Two important relations were derived from the set of constraints (extracted from cycle No. 1, chapter 3) and are stated as follows:

$$\pi \text{ (HIV)} >= \pi \text{ (NF-κB)}$$

$$\pi \text{ (HIV)} >= \pi \text{ (TNF-α)}$$

It shows that period of HIV invasion/infection should be greater than TNF-α and NF-κB in order to maintain the homeostasis of Biological Regulatory Network in case of HIV-1 infection. These relations depict an important behavior among HIV and TNF-α. As the infection of HIV is very slow in comparison to immune attack, the virus normally takes much longer time to infect and override the whole immune system of the infected individual. Production of cytokines by immune system's elementary element NF-κB is rapid and frequent while there is a considerably slow replication and invasion of HIV in the body after entering the cell. This showed frequently oscillating behavior of NF-κB and relatively stable behavior of HIV in comparison to NF-κB and TNF-α. But once this system is violated it could lead towards dead lock state, i.e., Stable Steady State, where no chances of survival exist. So it is inferred that if the system stays in such a state where the period of HIV is greater than NF-κB and TNF-α, survival time of patient can be increased. At initial stage of HIV infection, high-levels of CD8+ T cell frequencies have been seen to be correlated with the control of viral replication (Wilson *et al.,* 2000). From this, it can be concluded that while designing drugs against HIV-1, time delay of HIV-1 must be kept greater than those of NF-κB and TNF-α.

When the time period for HIV's activation is smaller, indicating that the system is

attainting the stage of viral setpoint. At this stage TNF-α decrease in T lymphocytes occurs and this can be seen by the shifting of the initial steady state of TNF-α (cytokines) to a new equilibrium value. This new equilibrium value is lower than the preinfection value (Fauci, 1993). This is a stage of exponential viral increase, reaching a peak and finally declining to the steady state level also called viral setpoint (Kaufmann et al., 1998; Lindback et al., 2000). HIV setpoint is a predicator of AIDS and it gets stabilizes after a period of acute HIV infection (Burg et al., 2009).

In vitro studies have shown that HIV-infected CD4+ T cells are lysed by HIV and by mathematical modeling the primary HIV infection kinetics, it has been revealed that the viral-induced cytopathicity and susceptible T cells (also called the 'target-cell-limited model') controls the infection (Somasundaran and Robinson, 1987; Phillips, 1996).

By observing the behavioral patterns of HIV, TNF-α and NF-κB with respect to time, it can be seen that it depends upon the time delays taken by these key players to incite or repress a specific response. The constraints followed in the cycle No. 2 varies in conditions with respect to locations. The I^{st} constraint shows that the time period required for activation or production of HIV at location 000 is greater than TNF-α at location 000 and 010. This condition locates an important position for HIV inhibition, i.e., TNF-α tends to be faster than HIV to complete its period at this location (specified in the constraint). By following this constraint one can limit or inhibit period of HIV. Similarly the 2^{nd} constraint shows that the period of TNF-α could exceed HIV at location 100 and 010 for TNF-α and 000 and 100 for HIV. At this point the disease can lead towards AIDS as the time period of HIV becomes faster than the immune defense system. It normally

happens during the later stages of HIV infection, so should be controlled earlier in infection. The cyclic conditions 5 and 8 of cycle No. 1 represent conditions to enhance the immune activation by fastly producing the cytokines.

The cycle is able to maintain the Homeostatic behviour by keeping the period of HIV activation (replication) and degradation (inhibition) at state 000 and 110 respectively greater than the production rate (activation) of NF-κB at position 100 (observed in 11^{th} constraint of cycle No. 2). In this condition it renders the T-cells resistant to fight the foreign invader to achieve viral load by keeping the time period greater for HIV activation and degradation. In this way the immune cells can suppress the viral infected cells or kill these by speeding up the production of cytokines, chemokines and several other growth factors essential for immune stimulation. Another cyclic condition shown in constraint 12 (chapter 3) represents the homeostatic behavior of T-cells after HIV infection, where the time delay of TNF-α activation and degradation at state 100 and 010 respectively is smaller than that of HIV activation period.

The extended model of HIV-1 infection of immune system is currently under investigation and is being tuned on the bases of recent findings. The involvement of Rel Homology domain with discriminatory domains (specially Rel A and Rel B) having contrasting functions make NF-κB a very interesting candidate for drug designing. The contrasting role of Rel B in anti-inflammation as compared to Rel A (causing inflammation) revealed astonishing results in initial findings. Its GENOTECH model showed that suppression of Rel A domain by introducing Rel B externally (or making it active by other means) tends to slow down the disease progression in the body and helps to inhibit inflammation caused by Rel A.

By adding more entities in the models discussed in this study, in future exact therapeutic targets will be found by docking and simulation studies of the interacting proteins. These findings lead us towards drug designing. On the basis of modeling techniques, a computer aided tool for rapid immunological analysis can also be developed. Real time modeling and its applications can help in predicting the preventive medicines, which will give the advancement in medicine developed for the treatment of AIDS.

CHAPTER NO. 5

REFERENCES

5. REFERENCES

- Ahmad J., (2009). Modélisation hybride et analyse des dynamiques des réseaux dé régulations biologiques en tenant compte des délais, *Thesis, Ecole Centrale de Nantes, France.*
- Ahmad J., Bernot G., Comet J. P., Lime D., and Roux O., (2007). Hybrid Modelling and Dynamical Analysis of Gene Regulatory Networks with Delays, *ComPlexUs*, 3, p. 231–251.
- Ahmad J., Bourdon J., Eveillard D., Fromentin J., Roux O., and Sinoquet C., (2009). Temporal constraints of a gene regulatory network: Refining a qualitative simulation, *BioSystems*, 98, p. 149–159.
- Ahmad J., Roux O., Bernot G., Comet J. P., and Richard A., (2008). Analysing formal models of genetic regulatory networks with delays, *Int. J. Bioinformatics Research and Application.*, 4, p. 240–262.
- Albini A., Benelli R., Giunciuglio D., Cai T., Mariani G., Ferrini S., and Noonan D. M., (1998). Identification of a novel domain of HIV tat involved in monocyte chemotaxis, *J Biol Chem*, 273, p. 15895-15900.
- Alimonti J. B., Ball T. B., and Keith R., (2003). Fowke Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS, *Journal of General Virology*, 84, p. 1649–1661.
- Altes H. K., Wodarz D., and Jansen V. A. A., (2002). The dual role of CD4 T helper cells in the infection dynamics of HIV and their importance for vaccination, *J Theor Biol*, 214, p. 633–46.

- Barkett M., and Gilmore T. D., (1999). Control of apoptosis by Rel/NF-kappaB transcription factors, *Oncogene*, 18, p. 6910–6924.
- Batt G., Ropers D., De Jong H., Geiselmann J., and Page M., (2005). Qualitative analysis and verification of hybrid models of genetic regulatory networks, *International Workshop on Hybrid Systems:Computation and Control*, p. 134-150.
- Batt G., Ropers D., De Jong H., Geiselmann J., Mateescu R., Page M., and Schneider D., (2005). Validation of qualitative models of genetic regulatory networks by model checking: analysis of the nutritional stress response in Escherichia coli, *Bioinformatics*, 21, p. 19-28.
- Bennett M., and Hasty J., (2009). Microfluidic devices for measuring gene network dynamics in single cells, *Nature reviews Genetics*, 10, p. 628-638.
- Bingham C. O., (2002). The pathogenesis of rheumatoid arthritis: pivotal citokynes involved in bone degradation and inflammation, *J Reum*, 29, p. 3-9.
- Bonizzi G., and Karin M., (2004). The two NF-κB activation pathways and their role in innate and adaptive immunity, *Trends Immunol.*, 25, p. 280-288.
- Burg D., Rong L., Neumann A. U., and Dahari H., (2009). Mathematical modeling of viral kinetics under immune control during primary HIV-1infection, *Journal of Theoretical Biology*, 259, p. 751–759.
- Cantrell D. A., (2002). T-cell antigen receptor signal transduction, *Immunology*, 105, p. 369–374.

- Cecilia D., Kleeberger C., Munoz A., Giorgi J. V., and Zolla-Pazner S., (1999). A longitudinal study of neutralizing antibodies and disease progression in HIV-1 infected subjects, *J Infect Dis*, 179, p. 1365–74.
- Chang H. K., Gallo R. C., and Ensoli B., (1995). Regulation of Cellular Gene Expression and Function by the Human Immunodeficiency Virus Type 1 Tat Protein, *J Biomed Sci*, 2, p. 189-202.
- Chaouiya C., (2008). Qualitative Modelling of Biological Regulatory Networks Combininga Logical Multi-Valued Formalism and Petri nets, *International Workshop on Discrete Event Systems Göteborg, Sweden*, p. 263.
- Chen P., Mayne M., Power C., and Nath A., (1997). The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases, *J Biol Chem.*, 272, p. 22385–22388.
- Cohen M. S., Hellmann N. and Levy J. A. (2008). The spread, treatment, and prevention of HIV-1: evolution of a global pandemic, *J Clin Invest.* 118, p. 1244-1254.
- Coleman C. M., and Wu L., (2009). HIV interactions with monocytes and dendritic cells: viral latency and reservoirs, *Retrovirology*, 6, p. 2752-2761.
- D'Aversa T. G., Weidenheim K. M., and J.W. Berman., (2002). CD40-CD40L interactions induce chemokine expression by human microglia: implications for human immunodeficiency virus encephalitis and multiple sclerosis, *Am J Pathol*, 160, p. 559–567.
- De Boer R. J., and Boer lijst M. C., (1994). Diversity and virulence thresholds in AIDS. *Proc Natl Acad Sci USA*, 94, p. 544–8.

- De Cock K. M., and Weiss H. A., (2000). The global epidemiology of HIV/AIDS, *Tropical Medicine and International Health*, 5, p. 3-6.
- De Jong H., (2002). Modeling and simulation of genetic regulatory systems: a literature review, *J. Comput. Biol.*, 9, p. 67–103.
- De Jong H., Geiselmann J., Hernandez C., and Page M., (2001). Genetic Network Analyzer: A Tool for the Qualitative Simulation of Genetic Regulatory Networks, *Rapport de recherché*, 4262-4278.
- De Jong H., Gouzé J. L., Hernandez C., Page M., Sari T., and Geiselmann J., (2004). Qualitative simulation of genetic regulatory networks using piecewise-linear models, *Bull Math Biol*, 66, p. 301-40.
- Dobrzanski P., Ryseck R. P., and Bravo R. (1993). Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipperlike motif, *Mol Cell Biol*, 13, p. 1572–1582.
- Endy D., and Brent R., (2001). Modeling cellular behavior, *Nature*, 409, p. 391-395.
- Everett H., and McFadden G., (1999). Apoptosis: an innate immune response to virus infection, *Trends Microbiol*, 7, p. 160–165.
- Fauci, A.S., (1996). Host factors and the pathogenesis of HIV-induced disease, *Nature*, 384, p. 529–534.
- Fromentin J., Eveillard D., and Roux O., (2010). Hybrid modeling of biological networks: mixing temporal and qualitative biological properties, *BMC Syst Biol*, 4, p. 79-90.

- Gagneur J., and Casari G., (2005). From molecular networks to qualitative cell behavior, *FEBS Lett*, 579, p. 1867-1871.
- Galvani A. P., (2005). The role of mutation accumulation in HIV progression, *Proc Roy Soc Lond B*, 272, p. 1851–8.
- Gautier V. W., Gu L., O' Donoghue N., Pennington S., Sheehy N., and Hall W. W., (2009). In vitro nuclear interactome of the HIV-1 Tat protein, *Retrovirology*, 6.
- Gayet-Ageron A., Baratin D., Marceillac E., Allard R., Peyramond D., and Chidiac C., (2000). The AIDS epidemicin Lyon: patient characteristics and defining illnesses between 1985 and 2000, *HIV Med*, 5, p. 163–70.
- Ghosh R., and Tomlin C., (2004). Symbolic reachable set computation of piecewise affine hybrid automata and its application to biological modelling Delta Notch protein signaling, *Syst Biol IEE Proceedings*, 1, p. 170-183.
- Ghosh S., and Karin M., (2002). Missing pieces in the NF-kappa B puzzle, *Cell*, 109, p. 81–96.
- Ghosh S., May M. J., and Kopp E. B., (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses, *Annu Rev Immunol.*, 16, p. 225–260.
- Golstein P., (1998). Cell death in us and others, *Science*, 281, p. 1283.
- Gouzé J. L., (1998). Positive circuits and multistationarity in dynamical systems, *J. Biol. Systems.*, 6, p.11-15.
- Grey S. T. (2008). Regulating inflammation: The ying and yang of NF-κB activation, *Immunology and Cell Biology*, 86, p. 299–300.

- Henzinger T. A., Ho P. H., and Toi H. W., (1997). HYTECH: a model checker for hybrid systems, *Int J STTT*, 1, p. 110-122.
- Herbein G., and Khan K. A., (2007). Is HIV infection a TNF receptor signalling-driven disease?, *Trends in Immunology*, 29, p. 61-67.
- Herbein G., Gras G., Khan K. A., and Abbas W., (2010). Macrophage signaling in HIV-1 infection, *Retrovirology*, 7:34.
- Hiscott J., Kwon H., and Génin P., (2001). Hostile takeovers: viral appropriation of the NF-κB pathway, *The Journal of Clinical Investigation*, 107, p. 143-151.
- Hogue I. B., Bajaria S .H., Fallert B. A., Qin S., Reinhart T. A., and Kirschner D. E., (2008). The dual role of dendritic cells in the immuneresponse to humanimmuno deficiency virus type 1 infection, *J Gen Virol*, 89, p. 2228–39.
- Iwami S., Nakaoka S., and Takeuchi Y., (2008). Viral diversity limits immune diversity in asymptomatic phase of HIV infection, *Theor Pop Biol*, 73, p. 332–41.
- Iwami S., Nakaokab S., Takeuchi Y., Miurad Y., and Miura T., (2009). Immune impairment thresholds in HIV infection, *Immunology Letters*, 123, p. 149–154.
- Janeway C. A., Travers P., Walport M., and Shlomchik M. J., (2001). Immunobiology: the immune System in health and disease, *Garland Science*, ISBN-10: 0-8153-3642-X.
- Karin M., (1999). How NF-κB is activated: the role of the IkB kinase (IKK) complex, *Oncogene*. 18, p. 6867-6874.
- Kauffman S. A., (1991). Antichaos and adaptation, *Sci. Am.*, 265, p. 78–84.
- Kaufmann G. R., Cunningham P., Kelleher A. D., Zaunders J., Carr A., Vizzard J., Law M., and Cooper D. A., (1998). Patterns of viral dynamics during primary

human immunodeficiency virus type 1 infection. The Sydney Primary HIV Infection Study Group, *J. Infect. Dis.*, 178, p. 1812–1815.

- Kaul M., Garden G.A., and Lipton S.A., (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia, *Nature*, 410, p. 988-994.
- Legler D. F., (2003). Recruitment of TNF receptor1 to lipid rafts is essential for TNFa-mediated NF-κB activation, *Immunity*, 18, p. 655–664.
- Li Q., and Verma I. M., (2002). NF-κB regulation in the immune system, *Nature Rev. Immunol.*, 2, p. 725-734.
- Lindback S., Karlsson A. C., Mittler J., Blaxhult A., Carlsson M., Briheim G., Sonnerborg A., and Gaines H., (2000). Viral dynamics in primary HIV-1 infection. Karolinska Institute Primary HIV Infection Study Group, *AIDS* 14, p. 2283–2291.
- Lockhart D. J., and Winzeler E. A., (2000). Genomics, gene expression and DNA arrays, *Nature*, 405, p. 827-836.
- Mcadams H. H., and Arkin A., (1998). Simiulation of prokaryotic genetic circuits, *Annual Rewiew of biophysics and biomolecular structure*, 27, p. 199-224.
- Meissnera E. G., Duus K. M., Gao F., Yu X-F., and Su L., (2004). Characterization of athymus-tropic HIV-1 isolate from a rapid progressor: role of the envelope, *Virology*, 328, p. 74–88.
- Mellors J. W., (1998). Viral-load tests provide valuable answers, *Sci Am*, 279, p. 90-93.

- Memet S., (2006). NF-κB functions in the nervous system: From development to disease, *Biochem. Pharmacol.* 72, p. 1180-1195.
- Mendoza L., Thieffry D., and Alvarez-Buylla E. R., (1999). Genetic control of flower morphogenesis in Arabidopsis thaliana: A logical analysis, *Bioinformatics*, 15, p. 593–606.
- Minghetti L., Visentin S., Patrizio M., Franchini L., Ajmone-Cat M. A., and Levi G., (2004). Multiple Actions of the Human Immunodeficiency Virus Type-1 Tat Protein on Microglial Cell Function, *Neurochem. Res.*, 29, p. 965-978.
- Nishikori M., (2005). Classical and Alternative NF-κB Activation Pathways and Their Roles in Lymphoid Malignancies, *J. Clin. Exp. Hematopathol*, 45.
- Noonan D., and A. Albini., (2000). From the outside in: extracellular activities of HIV Tat, *Adv Pharmacol.*, 48, p. 229-250.
- Nowak M. A., (1992). Variability in HIV infections, *J Theor Biol*, 155, p. 1–20.
- Nowak M. A., (2006). Evolutionary dynamics, *Harvard University Press*, p. 384 ISBN 9780674023383.
- Nowak M. A., and May R. M., (1993). AIDS pathogenesis: mathematical models of HIV and SIV infection, *AIDS*, 7, p. 3–18.
- Nowak M. A., and May R. M., (2000).Virusdynamics, *Oxford University Press.*
- Nowak M. A., Anderson R. M., McLean A. R., Wolfs T. F., Goudsmit J., and May R. M., (1991). Antigen diversity thresholds and the development of AIDS, *Science*, 15, p. 963–9.
- Pahl H. L., (1999). Activators and target genes of Rel/NF-kappaB transcription factors, *Oncogene*, 18, p. 6853–6866.

- Panday A., and Mann M., (2000). Proteomics to study genes and genomics, *Nature*, 405, p. 837-846.
- Pandey M. K., Sung B., Ahn K. S., Kunnumakkara A. B., Chaturvedi M. M., and Bharat Aggarwal B. B., (2007). Gambogicacid, anovellig and fortransferrin receptor, potentiates TNF-induced apoptosis through modulation of the nuclear factor-kB signaling pathway, *Blood*, 110, p. 3517-3525 .
- Peters P. J., Kilmarx P. H. and Mastro T. D. (2008). AIDS: Global Epidemiology, *Encyclopedia of virology*, 1, p. 58-68.
- Phenix B. N., and Badley A. D., (2002). Influence of mitochondrial control of apoptosis on the pathogenesis, complications and treatment of HIV infection, *Biochimie* 84, p. 251–264.
- Rahman M. M., and McFadden G., (2006). Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog.*, 2.
- Richard A., and Comet J., (2007). Necessary conditions for multistationarity in discrete dynamical systems, *Discrete Applied Mathematics*, 155, p. 2403-2413.
- Rosenberg E. S., and Walker, B. D., (1998). HIV type 1-specific helper T cells: a critical host defense, *AIDS Res Hum Retroviruses*, 14, p. 143–147.
- Rosenberg E. S., Billingsley J. M., Caliendo A. M., Boswell S. L., Sax P. E., Kalams, S. A., and Walker, B. D., (1997). Vigorous HIV-1- specific CD4+ T cell responses associated with control of viremia, *Science*, 278, p. 1447–1450.
- Roulston A., Lin R., Beauparlant P., Wainberg M. A., and Hiscott J., (1995). Regulation of HIV-1 and cytokine gene expression in myeloid cells by NF-κB/Rel

transcription factors, *Microbiol Rev*, 59, p. 481-505.

- Ruben S. M., Klement J. F., Coleman T. A., Maher M., Chen C., and Rosen C. A., (1992). IRel: a novel *rel*-related protein that inhibits NF-κB transcriptional activity, *Genes Dev*, 6, p. 745-760.
- Rumbaugh J. A., and A. Nath., (2006). Developments in HIV neuropathogenesis, *Curr Pharm Des*, 12, p. 1023–1044.
- Sánchez L., and Thieffry D., (2001). A logical analysis of the Drosophila gap gene, *J. Theor . Biol.*, 211, p. 115–141.
- Schaub M. A., Henzinger T. A., and Fisher J., (2007). Qualitative networks: Asymbolic approach to analyze biological signaling networks, *BMC Systems Biology*, 1, p. 4-24.
- Scheidereit C., (2006). IkB kinase complexes: gateways to NF-κB activation and transcription, *Oncogene*, 25, p. 6685-6705.
- Somasundaran M., and Robinson H. L., (1987). A major mechanism of human immune deficiency virus-induced cell killing does not involve cellfusion, *J. Virol.*, 61, p. 3114–3119.
- Strogatz S. H., (1994). Nonlinear Dynamics and Chaos, *Addison-Wesley Co., Reading, MA.*
- Sui Z., Sniderhan L. F., Schifitto G., Phipps R. P., and Gelbard H. A., (2007). Functional synergy between CD40 ligand and HIV-1 Tat contributes to inflammation:implications in HIV type 1 dementia, *J Immunol*, 178, p. 3226–3236.
- Sulis W. H., and Combs A., (1996). Nonlinear Dynamics in human behavior. *By studies of nonlinear phenomena life sciences*, 5, p. 161-190.

- Tassie J. M., Grabar S., Lancar R., Deloumeaux J., Bentata M., and Costagliola D., (2002). Time to AIDS from 199 2 to 1999 in HIV-1-infected subjectswith known date of infection, *JAIDS*, 30, p. 81–87.
- Tergaonkar V., (2006). NF-κB pathway:A good signaling paradigm and therapeutic target, *Int J. Biochem Cell Biol.*, 38, p. 1647-1653.
- Thieffry D., and Thomas R., (1998). Qualitative analysis of gene networks, *Pac Symp Biocomput*, p.77-88.
- Thomas A., Henzingery T. A., Preuigz J., and Wong-Toi H., (2001). Some Lessons from the HyTech Experience, *Proceedings of the 40th Annual IEEE Conference on Decision and Control CDC*. p. 2887-2892.
- Thomas R., (1973). Boolean formalization of genetic control circuits, *J Theor . Biol.*, 42, p. 563–585.
- Thomas R., (1981). On the relation between the logical structure of systems and their ability to generate multiple steady states and sustained oscillations, *Ser. Synergetics*, 9, p. 180–93.
- Thomas R., (1991). Regulatory networks seen as asynchronous automata: A logical description, *J. Theor. Biol.*, 153, p. 1–23.
- Thomas R., and d'Ari R., (1990). Biological Feedback, *Boca Raton, Florida, U.S.A: CRC Press.*
- Thomas R., Thieffry D., and Kaufman M., (1995). Dynamical behaviour of biological regulatory networks--I. Biological role of feedback loops and practical use of the concept of the loop-characteristic state, *Bull Math Biol.*, 57, p. 247-76.

- Tunetsugu-Yokota Y., (2005). How does HIV infection destroy the host immune system?, *JAIDS Res*, 7, p. 171–9.
- Tyson J. J, Chen K., and Novak B., (2001). Network dynamics and cell physiology, *Nat. Rev. Mol. Cell Biol*, 2, p. 908–916.
- Uller O., and Staunery T., (1996). Modelling and Verification using Linear Hybrid Automata a Case Study, *Mathematical Modelling of Systems*, 1, p. 000-111.
- UNAIDS (2000). Report on the global HIV/AIDS epidemic: *UNAIDS*, Geneva, Switzerland.
- Vitale R. F., and Ribeiro F. de A. Q., (2007). The role of tumor necrosis factor-alpha (TNF-a) in bone resorption present in middle ear cholesteatoma, *Braz J Otorhinolaryngol*, 73, p. 117-21.
- Whiteside A., (2008). HIV/AIDS: a very short introduction, *The emergence and state of the HIV/AIDS epidemic*, p. 1-22, ISBN 978-0-19-280692-5.
- WHO, UNAIDS, UNICEF (2008). Towards Universal Scaling Up Priority HIV/AIDS Interventions in the Health Sector: *Progress Report 2008*. Geneva, Switzerland: World Health Organization.
- Xiao H., Neuveut C., Tiffany H. L., Benkirane M., Rich E. A., Murphy P. M., and Jeang K. T., (2000). Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1, *Proc Natl Acad Sci USA*, 97, p. 66-114.

ANNEXURE

```
-- Fichier Hyfile1

var
hHIV,hTNFA,hNFKB :analog;
k,n: discrete;
dpHIV000,dpTNFA000,dpNFKB000, dpHIV001, dpTNFA001, dnNFKB001,
dpHIV002, dpTNFA002,dnNFKB002, dpHIV010,
dnTNFA010,dpNFKB010,dpHIV011, dnTNFA011,dpNFKB011, dpHIV012,
dnHIV100, dpTNFA100,dpNFKB100, dpTNFA101, dpNFKB101, dpTNFA102,
dnHIV110, dnTNFA110, dpNFKB110, dpNFKB111,dpHIV, dnHIV, dpTNFA,
dnTNFA, dpNFKB, dnNFKB:parameter;
automaton auto
synclabs: ;
-- gène n°0 = HIV
-- gène n°1 = TNFA
-- gène n°2 = NFKB
initially loc_000;
-- pour la configuration 0,0,0
loc loc_000: while hHIV <= dpHIV000 & hTNFA <= dpTNFA000 & hNFKB
<= dpNFKB000 wait {dhHIV=1,dhTNFA=1,dhNFKB=1}
when hHIV=dpHIV000 do {hHIV'=0, k'=k+1} goto loc_100;
--when hTNFA=dpTNFA000 do {hTNFA'=0, k'=k+1} goto loc_010;
--when hNFKB=dpNFKB000 do {hNFKB'=0, k'=k+1} goto loc_001;


-- pour la configuration 0,0,1
loc loc_001: while hHIV <= dpHIV001 & hTNFA <= dpTNFA001 & hNFKB
>= dnNFKB001 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV001 do {hHIV' =0, k'=k+1} goto loc_101;
--when hTNFA=dpTNFA001 do {hTNFA' =0, k'=k+1} goto loc_011;
when hNFKB=dnNFKB001 do {hNFKB' =0, k'=k+1} goto loc_000;

-- pour la configuration 0,0,2
loc loc_002: while hHIV <= dpHIV002 & hTNFA <= dpTNFA002 & hNFKB
>= dnNFKB002 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV002 do{hHIV'=0, k'=k+1} goto loc_102;
when hTNFA=dpTNFA002 do {hTNFA' =0, k'=k+1} goto loc_012;
when hNFKB=dnNFKB002 do {hNFKB' =0, k'=k+1} goto loc_001;

-- pour la configuration 0,1,0
loc loc_010: while hHIV <= dpHIV010 & hTNFA >= dnTNFA010 & hNFKB
>= dpNFKB010 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
--when hHIV=dpHIV010 do{hHIV'=0, k'=k+1} goto loc_110;
when hTNFA=dnTNFA010 do {hTNFA' =0, k'=k+1} goto loc_000;
--when hNFKB=dpNFKB010 do {hNFKB' =0, k'=k+1} goto loc_011;

-- pour la configuration 0,1,1
```

```
loc loc_011: while hHIV <= dpHIV011 & hTNFA >= dnTNFA011 & hNFKB
<= dpNFKB011 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
when hHIV=dpHIV011 do{hHIV'=0, k'=k+1} goto loc_111;
when hTNFA=dnTNFA011 do {hTNFA' =0, k'=k+1} goto loc_001;
when hNFKB=dpNFKB011 do {hNFKB' =0, k'=k+1} goto loc_012;

-- pour la configuration 0,1,2
loc loc_012: while hHIV <= dpHIV012 wait
{dhHIV=1,dhTNFA=0,dhNFKB=0}
when hHIV=dpHIV012 do {hHIV' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,0,0
loc loc_100: while hHIV >= dnHIV100 & hTNFA <= dpTNFA100 & hNFKB
<= dpNFKB100 wait {dhHIV=-1,dhTNFA=1,dhNFKB=1}
--when hHIV=dnHIV100 do {hHIV' =0, k'=k+1} goto loc_000;
when hTNFA=dpTNFA100 do {hTNFA' =0, k'=k+1} goto loc_110;
when hNFKB=dpNFKB100 do {hNFKB' =0, k'=k+1} goto loc_101;

-- pour la configuration 1,0,1
loc loc_101: while hTNFA <= dpTNFA101 & hNFKB <= dpNFKB101 wait
{dhHIV=0,dhTNFA=1,dhNFKB=1}
when hTNFA=dpTNFA101 do {hTNFA' =0, k'=k+1} goto loc_111;
when hNFKB=dpNFKB101 do {hNFKB' =0, k'=k+1} goto loc_102;

-- pour la configuration 1,0,2
loc loc_102: while  hTNFA <= dpTNFA102 wait
{dhHIV=0,dhTNFA=1,dhNFKB=0}
when hTNFA=dpTNFA102 do {hTNFA'=0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,0
loc loc_110: while hHIV >= dnHIV110 & hNFKB <= dpNFKB110 wait
{dhHIV=-1,dhTNFA=0,dhNFKB=1}
when hHIV=dnHIV110 do {hHIV' =0, k'=k+1} goto loc_010;
when hNFKB=dpNFKB110 do {hNFKB' =0, k'=k+1} goto loc_111;

-- pour la configuration 1,1,1
loc loc_111: while hNFKB <= dpNFKB111 wait
{dhHIV=0,dhTNFA=0,dhNFKB=1}
when hNFKB=dpNFKB111 do {hNFKB' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,2
loc loc_112: while True wait {dhHIV=0,dhTNFA=0,dhNFKB=0}

end
var
   init_reg, acces,
portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r_ol
d,r_new,r_acc: region;

--var
  -- init_reg, acces : region;
```

```
init_reg := loc[auto] = loc_100 & hTNFA>=0 & hNFKB>=0  & hTNFA <=
dpTNFA100 & hNFKB <= dpNFKB100;

-- -------------------Les variables-----------------------------
----------
-- Analysis commands
r_ini:= loc[auto] =  loc_100 & hTNFA>=0 & hNFKB>=0  & hTNFA <=
dpTNFA100 & hNFKB <= dpNFKB100;
--r_ini:= loc[auto] = loc_100 & hMDM2>=0 & hMDM2 <= dpMDM2100;
-- hP53>=0  & hMDM2>=0 &  hP53 <= dpP53100 &  hMDM2 <= dpMDM2100;
r_new:=hide k,n in hull (post(r_ini & k=n) & ~k=n) endhide;
r_old:=r_ini & ~r_ini;

while not empty(r_new) and empty(r_new & r_ini) do
r_old:=r_new;
r_new:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
-- To verify that the initial zone is accessible from itself
if not empty (r_new & r_ini) then
-- if accessible
r_acc:=hide k,n in hull(post(r_new & k=n) &~k=n) endhide;
r_old:=r_ini & ~r_ini; --empty region initialization
while not empty(r_acc) and not r_new<=r_old do
r_old:=r_new;
while not empty(r_acc) and empty(r_acc & r_ini) do
r_acc:= hide k,n in hull(post(r_acc & k=n) &~k=n) endhide;
endwhile;
r_acc:=hull(r_acc & r_ini);
r_new:=hull(r_acc & r_new);
r_acc:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
if not empty(r_new) then
prints
"============================================================";
prints "Constrained region of the Invariance Kernel in the
zone:";
--print hide h in r_new endhide;
prints
"============================================================";
prints
"============================================================";
prints "Delay constraintes:";
print  hide hHIV,hTNFA,hNFKB, dpTNFA000, dpTNFA001, dpTNFA002,
dnTNFA010,dnTNFA011, dpTNFA100, dpTNFA101,  dpTNFA102, dnTNFA110,
dpTNFA, dnTNFA in r_new endhide;
prints
"============================================================";
else
prints "Invariance kernel does not exist from the initial
region";
endif;
```

```
else
-- if not accessible
prints "The initial region is not accessible from itself hence";
prints "there is no initial condition that leads to an invariance
kernel.";
endif;

-- Fichier Hyfile1

var
hHIV,hTNFA,hNFKB :analog;
k,n: discrete;
dpHIV000,dpTNFA000,dpNFKB000, dpHIV001, dpTNFA001, dnNFKB001,
dpHIV002, dpTNFA002,dnNFKB002, dpHIV010,
dnTNFA010,dpNFKB010,dpHIV011, dnTNFA011,dpNFKB011, dpHIV012,
dnHIV100, dpTNFA100,dpNFKB100, dpTNFA101, dpNFKB101, dpTNFA102,
dnHIV110, dnTNFA110, dpNFKB110, dpNFKB111,dpHIV, dnHIV, dpTNFA,
dnTNFA, dpNFKB, dnNFKB:parameter;
automaton auto
synclabs: ;
-- gène n°0 = HIV
-- gène n°1 = TNFA
-- gène n°2 = NFKB
initially loc_000;
-- pour la configuration 0,0,0
loc loc_000: while hHIV <= dpHIV000 & hTNFA <= dpTNFA000 & hNFKB
<= dpNFKB000 wait {dhHIV=1,dhTNFA=1,dhNFKB=1}
when hHIV=dpHIV000 do {hHIV'=0, k'=k+1} goto loc_100;
--when hTNFA=dpTNFA000 do {hTNFA'=0, k'=k+1} goto loc_010;
--when hNFKB=dpNFKB000 do {hNFKB'=0, k'=k+1} goto loc_001;


-- pour la configuration 0,0,1
loc loc_001: while hHIV <= dpHIV001 & hTNFA <= dpTNFA001 & hNFKB
>= dnNFKB001 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV001 do {hHIV' =0, k'=k+1} goto loc_101;
--when hTNFA=dpTNFA001 do {hTNFA' =0, k'=k+1} goto loc_011;
when hNFKB=dnNFKB001 do {hNFKB' =0, k'=k+1} goto loc_000;

-- pour la configuration 0,0,2
loc loc_002: while hHIV <= dpHIV002 & hTNFA <= dpTNFA002 & hNFKB
>= dnNFKB002 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV002 do{hHIV'=0, k'=k+1} goto loc_102;
when hTNFA=dpTNFA002 do {hTNFA' =0, k'=k+1} goto loc_012;
when hNFKB=dnNFKB002 do {hNFKB' =0, k'=k+1} goto loc_001;

-- pour la configuration 0,1,0
loc loc_010: while hHIV <= dpHIV010 & hTNFA >= dnTNFA010 & hNFKB
>= dpNFKB010 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
--when hHIV=dpHIV010 do{hHIV'=0, k'=k+1} goto loc_110;
when hTNFA=dnTNFA010 do {hTNFA' =0, k'=k+1} goto loc_000;
```

```
--when hNFKB=dpNFKB010 do {hNFKB' =0, k'=k+1} goto loc_011;

-- pour la configuration 0,1,1
loc loc_011: while hHIV <= dpHIV011 & hTNFA >= dnTNFA011 & hNFKB
<= dpNFKB011 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
when hHIV=dpHIV011 do{hHIV'=0, k'=k+1} goto loc_111;
when hTNFA=dnTNFA011 do {hTNFA' =0, k'=k+1} goto loc_001;
when hNFKB=dpNFKB011 do {hNFKB' =0, k'=k+1} goto loc_012;

-- pour la configuration 0,1,2
loc loc_012: while hHIV <= dpHIV012 wait
{dhHIV=1,dhTNFA=0,dhNFKB=0}
when hHIV=dpHIV012 do {hHIV' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,0,0
loc loc_100: while hHIV >= dnHIV100 & hTNFA <= dpTNFA100 & hNFKB
<= dpNFKB100 wait {dhHIV=-1,dhTNFA=1,dhNFKB=1}
--when hHIV=dnHIV100 do {hHIV' =0, k'=k+1} goto loc_000;
when hTNFA=dpTNFA100 do {hTNFA' =0, k'=k+1} goto loc_110;
when hNFKB=dpNFKB100 do {hNFKB' =0, k'=k+1} goto loc_101;

-- pour la configuration 1,0,1
loc loc_101: while hTNFA <= dpTNFA101 & hNFKB <= dpNFKB101 wait
{dhHIV=0,dhTNFA=1,dhNFKB=1}
when hTNFA=dpTNFA101 do {hTNFA' =0, k'=k+1} goto loc_111;
when hNFKB=dpNFKB101 do {hNFKB' =0, k'=k+1} goto loc_102;

-- pour la configuration 1,0,2
loc loc_102: while  hTNFA <= dpTNFA102 wait
{dhHIV=0,dhTNFA=1,dhNFKB=0}
when hTNFA=dpTNFA102 do {hTNFA'=0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,0
loc loc_110: while hHIV >= dnHIV110 & hNFKB <= dpNFKB110 wait
{dhHIV=-1,dhTNFA=0,dhNFKB=1}
when hHIV=dnHIV110 do {hHIV' =0, k'=k+1} goto loc_010;
when hNFKB=dpNFKB110 do {hNFKB' =0, k'=k+1} goto loc_111;

-- pour la configuration 1,1,1
loc loc_111: while hNFKB <= dpNFKB111 wait
{dhHIV=0,dhTNFA=0,dhNFKB=1}
when hNFKB=dpNFKB111 do {hNFKB' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,2
loc loc_112: while True wait {dhHIV=0,dhTNFA=0,dhNFKB=0}

end
var
   init_reg, acces,
portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r_ol
d,r_new,r_acc: region;
```

```
--var
  -- init_reg, acces : region;
init_reg := loc[auto] = loc_100 & hTNFA>=0 & hNFKB>=0  & hTNFA <=
dpTNFA100 & hNFKB <= dpNFKB100;

-- -------------------Les variables-----------------------------
----------
-- Analysis commands
r_ini:= loc[auto] =  loc_100 & hTNFA>=0 & hNFKB>=0  & hTNFA <=
dpTNFA100 & hNFKB <= dpNFKB100;
--r_ini:= loc[auto] = loc_100 & hMDM2>=0 & hMDM2 <= dpMDM2100;
-- hP53>=0  & hMDM2>=0 &  hP53 <= dpP53100 &  hMDM2 <= dpMDM2100;
r_new:=hide k,n in hull (post(r_ini & k=n) & ~k=n) endhide;
r_old:=r_ini & ~r_ini;

while not empty(r_new) and empty(r_new & r_ini) do
r_old:=r_new;
r_new:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
-- To verify that the initial zone is accessible from itself
if not empty (r_new & r_ini) then
-- if accessible
r_acc:=hide k,n in hull(post(r_new & k=n) &~k=n) endhide;
r_old:=r_ini & ~r_ini; --empty region initialization
while not empty(r_acc) and not r_new<=r_old do
r_old:=r_new;
while not empty(r_acc) and empty(r_acc & r_ini) do
r_acc:= hide k,n in hull(post(r_acc & k=n) &~k=n) endhide;
endwhile;
r_acc:=hull(r_acc & r_ini);
r_new:=hull(r_acc & r_new);
r_acc:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
if not empty(r_new) then
prints
"============================================================";
prints "Constrained region of the Invariance Kernel in the
zone:";
--print hide h in r_new endhide;
prints
"============================================================";
prints
"============================================================";
prints "Delay constraintes:";
print  hide hHIV,hTNFA,hNFKB, dpTNFA000, dpTNFA001, dpTNFA002,
dnTNFA010,dnTNFA011, dpTNFA100, dpTNFA101,  dpTNFA102, dnTNFA110,
dpTNFA, dnTNFA in r_new endhide;
prints
"============================================================";
else
```

```
prints "Invariance kernel does not exist from the initial
region";
endif;
else
-- if not accessible
prints "The initial region is not accessible from itself hence";
prints "there is no initial condition that leads to an invariance
kernel.";
endif;

-- Fichier

var
hHIV,hTNFA,hNFKB :analog;
k,n: discrete;
dpHIV000,dpTNFA000,dpNFKB000, dpHIV001, dpTNFA001, dnNFKB001,
dpHIV002, dpTNFA002,dnNFKB002, dpHIV010,
dnTNFA010,dpNFKB010,dpHIV011, dnTNFA011,dpNFKB011, dpHIV012,
dnHIV100, dpTNFA100,dpNFKB100, dpTNFA101, dpNFKB101, dpNFKB102,
dnHIV110, dnTNFA110, dpNFKB110, dpNFKB111,dpHIV, dnHIV, dpTNFA,
dnTNFA, dpNFKB, dnNFKB:parameter;
automaton auto
synclabs: ;
-- gÃ¨ne nÂ°0 = HIV
-- gÃ¨ne nÂ°1 = TNFA
-- gÃ¨ne nÂ°2 = NFKB
initially loc_000;
-- pour la configuration 0,0,0
loc loc_000: while hHIV <= dpHIV000 & hTNFA <= dpTNFA000 & hNFKB
<= dpNFKB000 wait {dhHIV=1,dhTNFA=1,dhNFKB=1}
when hHIV=dpHIV000 do {hHIV'=0, k'=k+1} goto loc_100;
when hTNFA=dpTNFA000 do {hTNFA'=0, k'=k+1} goto loc_010;
when hNFKB=dpNFKB000 do {hNFKB'=0, k'=k+1} goto loc_001;


-- pour la configuration 0,0,1
loc loc_001: while hHIV <= dpHIV001 & hTNFA <= dpTNFA001 & hNFKB
>= dnNFKB001 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV001 do {hHIV' =0, k'=k+1} goto loc_101;
when hTNFA=dpTNFA001 do {hTNFA' =0, k'=k+1} goto loc_011;
when hNFKB=dnNFKB001 do {hNFKB' =0, k'=k+1} goto loc_000;

-- pour la configuration 0,0,2
loc loc_002: while hHIV <= dpHIV002 & hTNFA <= dpTNFA002 & hNFKB
>= dnNFKB002 wait {dhHIV=1,dhTNFA=1,dhNFKB=-1}
when hHIV=dpHIV002 do{hHIV'=0, k'=k+1} goto loc_102;
when hTNFA=dpTNFA002 do {hTNFA' =0, k'=k+1} goto loc_012;
when hNFKB=dnNFKB002 do {hNFKB' =0, k'=k+1} goto loc_001;

-- pour la configuration 0,1,0
```

```
loc loc_010: while hHIV <= dpHIV010 & hTNFA >= dnTNFA010 & hNFKB
>= dpNFKB010 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
when hHIV=dpHIV010 do{hHIV'=0, k'=k+1} goto loc_110;
when hTNFA=dnTNFA010 do {hTNFA' =0, k'=k+1} goto loc_000;
when hNFKB=dpNFKB010 do {hNFKB' =0, k'=k+1} goto loc_011;

-- pour la configuration 0,1,1
loc loc_011: while hHIV <= dpHIV011 & hTNFA >= dnTNFA011 & hNFKB
<= dpNFKB011 wait {dhHIV=1,dhTNFA=-1,dhNFKB=1}
when hHIV=dpHIV011 do{hHIV'=0, k'=k+1} goto loc_111;
when hTNFA=dnTNFA011 do {hTNFA' =0, k'=k+1} goto loc_001;
when hNFKB=dpNFKB011 do {hNFKB' =0, k'=k+1} goto loc_012;

-- pour la configuration 0,1,2
loc loc_012: while hHIV <= dpHIV012 wait
{dhHIV=1,dhTNFA=0,dhNFKB=0}
when hHIV=dpHIV012 do {hHIV' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,0,0
loc loc_100: while hHIV >= dnHIV100 & hTNFA <= dpTNFA100 & hNFKB
<= dpNFKB100 wait {dhHIV=-1,dhTNFA=1,dhNFKB=1}
when hHIV=dnHIV100 do {hHIV' =0, k'=k+1} goto loc_000;
when hTNFA=dpTNFA100 do {hTNFA' =0, k'=k+1} goto loc_110;
when hNFKB=dpNFKB100 do {hNFKB' =0, k'=k+1} goto loc_101;

-- pour la configuration 1,0,1
loc loc_101: while hTNFA <= dpTNFA101 & hNFKB <= dpNFKB101 wait
{dhHIV=0,dhTNFA=1,dhNFKB=1}
when hTNFA=dpTNFA101 do {hTNFA' =0, k'=k+1} goto loc_111;
when hNFKB=dpNFKB101 do {hNFKB' =0, k'=k+1} goto loc_102;

-- pour la configuration 1,0,2
loc loc_102: while  hNFKB <= dpNFKB102 wait
{dhHIV=0,dhTNFA=1,dhNFKB=0}
when hNFKB=dpNFKB102 do {hNFKB'=0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,0
loc loc_110: while hHIV >= dnHIV110 & hNFKB <= dpNFKB110 wait
{dhHIV=-1,dhTNFA=0,dhNFKB=1}
when hHIV=dnHIV110 do {hHIV' =0, k'=k+1} goto loc_010;
when hNFKB=dpNFKB110 do {hNFKB' =0, k'=k+1} goto loc_111;
!
-- pour la configuration 1,1,1
loc loc_111: while hNFKB <= dpNFKB111 wait
{dhHIV=0,dhTNFA=0,dhNFKB=1}
when hNFKB=dpNFKB111 do {hNFKB' =0, k'=k+1} goto loc_112;

-- pour la configuration 1,1,2
loc loc_112: while True wait {dhHIV=0,dhTNFA=0,dhNFKB=0}

end
```

```
var
   init_reg, acces,
portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r_ol
d,r_new,r_acc: region;

--var
  -- init_reg, acces : region;
init_reg := loc[auto] = loc_100 & hTNFA>=0 & hNFKB>=0  & hTNFA <=
dpTNFA100 & hNFKB <= dpNFKB100;

-- --------------------Les variables------------------------------
----------

acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;

print hide hHIV,hTNFA,hNFKB  in acces endhide;

--init_reg:=hull(acces) & init_reg;

acces:=  post(post(post(init_reg ))) ;
init_reg:=hull(acces) & init_reg;
prints "Etats accessibles";
print hide hHIV,hTNFA,hNFKB  in init_reg endhide;

acces:=  post(post(post(init_reg ))) ;
init_reg:=hull(acces) & init_reg;
prints "Etats accessibles";
print hide hHIV,hTNFA,hNFKB  in init_reg endhide;
```