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DNA Sequences Involved in the Regulation of Human c-MYC Gene Expression by Herpes Simplex Virus Type 1 (HSV-1)

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THESIS APPROVAL

The abstract and thesis of Shanli Ye for the
Master of Science degree in Biology
were presented November 29, 1995 and accepted by the thesis
committee and the department.

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ABSTRACT

An abstract of the thesis of Shanli Ye for the Master of Science in Biology presented November 29, 1995.

Title: DNA Sequences Involved in The Regulation of Human *c-myc* Gene Expression by Herpes Simplex Virus Type 1 (HSV-1).

The human *c-myc* gene is a cellular proto-oncogene composed of three exons and two introns. Transcription of *c-myc* is controlled by two promoters, P1 and P2. The activity of these promoters is regulated by many factors, such as cellular transcription factors E2F, YY1, and HSV-1 immediate-early proteins, ICP0, ICP4.

Many regulatory elements located both upstream of and between P1 and P2 have been identified, and some of these are required for optimum expression of *c-myc*. In this thesis research, a region downstream from P2 in the *c-myc* exon 1 was identified by its response to transactivation by HSV-1 immediate-early proteins, ICP0 and ICP4. The purpose of this research was to examine this region for regulatory sites that respond to HSV-1 infection. I hypothesized that after HSV-1 infection, ICP0 and ICP4 activate *c-myc* expression, in part, through regulatory sequences present in exon 1.

To test for this hypothesis, reporter plasmids containing (i) the *c-myc* promoter (from -101 bp relative to P1) and exon 1 coupled to the bacterial CAT gene were constructed. (ii) The *c-myc* exon sequences used were either intact (wild-type) or they were constructed with various deletions. The activities of these plasmids were examined in transient expression assays. To analyze protein binding, electrophoretic mobility shift assay (EMSA)

and completion EMSAs were carried out. The results from these experiments lead to the following conclusions: (i) ICP4 and ICP0 serve as activators, whereas ICP27 inhibits *c-myc* gene expression. (ii) The region from +332 to +513 within the *c-myc* exon 1 contains an important element required for transactivation of the *c-myc* gene by HSV-1 proteins. (iii) Cellular proteins, including factor YY1, bind to the region from +332 to +513 in the *c-myc* exon 1.

Although the exact mechanism by which HSV-1 immediate-early proteins regulate *c-myc* gene expression is still not clear, it gives rise to a possibility that this regulation is caused by turning on or activation of the cellular regulatory proteins by ICP4 and ICP0. The cellular proteins in turn activate the *c-myc* gene expression by interacting with the *cis*-element downstream from P2.

**DNA SEQUENCES INVOLVED IN THE REGULATION OF
HUMAN *c*-MYC GENE EXPRESSION BY HERPES
SIMPLEX VIRUS TYPE 1 (HSV-1)**

by

SHANLI YE

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE in BIOLOGY

Portland State University
1996

DNA SEQUENCES INVOLVED IN THE REGULATION OF HUMAN *c*-MYC GENE EXPRESSION BY HERPES SIMPLEX VIRUS TYPE 1 (HSV-1)

GENERAL BACKGROUND

1. PREVIOUS STUDIES ON *c*-MYC REGULATION

The *c*-myc gene is the cellular homolog of the *v*-myc oncogene which was first detected as the transforming sequence of the avian myelocytomatosis virus (58). In humans, the *c*-myc gene was mapped to chromosome 8 (8q24) (5) and it is composed of three exons and two introns. Exon 2 and exon 3 contain the coding information for the *c*-myc protein, while exon 1 is an untranslated leader that is highly conserved between human and mouse (2) and entirely absent from *v*-myc (70). Within the exon 1, there are two promoters designated as P1 and P2 which are separated about 170 base pairs (for human *c*-myc gene) (33). The products encoded by *c*-myc gene are two phosphoproteins that are initiated at independent translational sites (29). These phosphoproteins are expressed at a low constitutive level in growing cells and are down-regulated in quiescent and differentiated cells (38). They are thought to function as transcriptional activators and they are involved in the regulation of proliferation, mitogenesis, and differentiation (40). In addition there is evidence that they play a role in programmed cell death (apoptosis) (52) and in the cytotoxicity of TNF- α in tumor cells (37).

Early studies have already revealed that overexpression of *c*-myc (or deregulation of *c*-myc expression) is related to the tumorigenesis of many kinds of human cancers such as Burkitt's lymphoma (BL), colon cancer (15), and breast cancer (16). In BL case, the *c*-myc gene is translocated into first intron of the immunoglobulin (Ig) heavy chain gene located on the chromosome 14 [human, t(8,14)]. The immunoglobulin enhancer element located in the intron

causes the *c-myc* gene to be over-expressed. This suggested that deregulation of *c-myc* expression was important for multistage carcinogenesis.

To understand how *c-myc* functions in these varied processes, it is important to understand what factors, both endogenous and exogenous, are involved in regulating the *c-myc* expression both in the normal cell cycle and in the tumor cells. We already know that *c-myc* expression is dependent on the presence of certain growth factors, e.g. platelet-derived growth factor (PDGF) (38), and on certain transcription regulatory factors such as E2F (14), YY1 (Yin Yang 1) (57), Sp1 (10), etc. In 1983, Kathleen Kelly et al found that *c-myc* mRNA was strongly induced (10- to 40-fold) by mitogens (e.g. lipopolysaccharides or concanavalin A) in lymphocytes and by PDGF in fibroblasts (38). Later Nissim Hay et al demonstrated that the 2.3 kilobase pair (kbp) region upstream from the first transcription start site, P1, contained both positive and negative regulatory elements which were involved in regulating *c-myc* expression by cellular factors (33).

Other previous studies showed that the *c-myc* expression was regulated at the level of elongation of transcription (premature termination of transcription occurs in the first exon of *c-myc* exon) (1, 14, 53). Bentley and Groudine demonstrated that when the HL60 cells (human promyelocytic leukemia cell line) differentiated into granulocytes, by means of treating the HL60 cells with retinoic acid, the *c-myc* mRNA level was reduced 10-fold (1). With the nuclear runoff assays, they showed that this reduction was detected in the transcription of *c-myc* exon 2 but not *c-myc* exon 1. This observation suggested that *c-myc* transcriptional down-regulation was at the level of transcriptional elongation rather than initiation, and this transcriptional elongation block occurred in the first exon of the *c-myc* gene (1).

One of the important findings in the regulation of human *c-myc* regulation came from a report by Hiebert et al. They found that the adenovirus pre-early (or immediate-early) protein E1A was able to activate the endogenous *c-myc* gene. By using site-directed mutagenesis of the E2F site located

at about 60 bp upstream of the P2 in the *c-myc* exon 1, they found that the E1A transactivation was mediated by cellular protein E2F (34). This suggested that E2F might play an important role in activating the *c-myc* gene expression.

Another important observation is the recent report published by DesJardins and Hay. They identified and characterized six potential regulatory sites called CT boxes (i.e. cytosine-thymidine boxes) in the *c-myc* promoter. Five are located at upstream of P1 and one (CT-I₂) is located between P1 and P2 (10). Using a series of deletion mutations along with transfection and electrophoretic mobility shift assays (EMSA), they found that these CT elements were binding sites for Sp1 and Sp1-related zinc-finger regulatory proteins. Sp1 binding was found to be responsible for activating P2-initiated transcription and inhibiting P1-initiated transcription (10), and this promoter usage shift (shift the promoter usage from P1 to P2) was observed in the normal individual.

The early studies described above suggest that human *c-myc* gene expression is regulated at multiple stages and is regulated by multiple factors, including different cellular regulatory proteins, growth factors, viral proteins and certain mitogens. These factors may regulate *c-myc* gene expression by different mechanisms (e.g. by activating initiation of the *c-myc* transcription or blocking elongation of the *c-myc* mRNA) The activation by these factors may cause the overexpression of *c-myc* gene which can lead to multistage carcinogenesis.

2. GENE REGULATION OF HSV-1

In order to understand how herpes simplex virus type 1 (HSV-1) infection may be involved regulation of *c-myc* gene expression, it is necessary to introduce some general aspects of HSV-1 replication. HSV-1 is a member of the viral family Herpesviridae and is composed of four basic structures: an envelope with viral glycoproteins, a tegument, a capsid, and a DNA-containing core. Analysis of the complete sequence of the HSV-1 genome has revealed that its genome has a total

size 152 kbp and contains at least 70 distinct genes (41). Based on the kinetics and characteristics of their expression, these genes can be divided into three main classes: immediate-early genes (IE, or α genes), early genes (E, or β genes), and late genes (L, or γ genes). The expression of these genes is coordinately regulated and sequentially ordered in a cascade fashion: IE $\rightarrow$ E $\rightarrow$ L. The expression of late (or γ) genes requires viral DNA replication. Based on expression time and the degree of requirement of the DNA replication, the γ genes are divided into two subgroups, γ_1 (for example, the major capsid protein, VP5, gene) and γ_2 (for example, the glycoprotein C, gC, gene). The γ_1 genes, also called $\beta\gamma$ or leaky-late genes, are late genes that start their expression before the viral DNA synthesis. However, their full expression needs the viral DNA synthesis. Thus, their expression can still be detected (in a lower amount) in the presence of DNA synthesis inhibitors. On the other hand, γ_2 genes are expressed after viral DNA replication and they have a high stringency for viral DNA synthesis. In the presence of DNA synthesis inhibitors, the expression of γ_2 genes is almost totally blocked. The relationship between the late genes expression and viral genome replication is not clear.

The IE class genes are defined as those which can be transcribed in the absence of *de novo* viral protein synthesis, and they code for five proteins (infected cell polypeptides) designated as ICP0, ICP4, ICP22, ICP27, and ICP47. Three of these immediate-early proteins, ICP4, ICP0, and ICP27, are main regulatory proteins for the HSV-1 gene expression (7, 12, 19, 26, 51). ICP4 is a DNA sequence-specific binding protein (11, 20, 44) and a phosphoprotein essential for viral replication. However, the binding of ICP4 to its consensus binding sites may not be required for its activation function (65). The ICP4 regulatory roles in the HSV genes expression have been revealed in early studies: (i) ICP4 is a major transactivator for HSV early and late gene expression; (ii) ICP4 represses its own expression and that of other α genes expression. These ICP4 functions were demonstrated by the study of ICP4 temperature-sensitive (ts) mutants. Dixon and Schaffer

demonstrated that, at non-permissive temperature or in the temperature shift experiments, ICP4 ts mutants exhibited severe impairment in the expression of viral early (VP64) and late (VP100, VP154) genes and the overproduction of the α genes, such as ICP4, ICP22, ICP27 (12). Later, similar results were derived from the studying the regulatory roles of ICP4 by using ICP4 mutants (ts mutants, deletion mutants, and nonsense mutants), assayed by transfection or cotransfection experiments, or by detecting and comparing the different HSV mRNA between wild types ICP4 and different kinds of mutated ICP4 (6, 7, 8). Recently, Gu et al showed that the ICP4 autoregulatory function was related to the cellular protein Sp1 (26).

Another HSV-1 α gene essential for viral replication is the ICP27 gene. By using temperature-sensitive (ts) mutants, Sacks et al showed that the growth of ICP27 ts mutants was markedly impaired at non-permissive temperature, and the expression of three class HSV-1 genes were altered (overexpression some of α genes and underexpression some of late genes) (59). These results indicated that ICP27 played an important regulatory function in HSV replication and it was required for the expression of HSV late genes. Later, the regulatory functions of ICP27 were revealed at both the transcriptional and post-transcriptional level (39, 55, 61). Further study on the post-transcriptional regulatory role of ICP27 revealed that ICP27 could affect mRNA processing (42, 43, 48, 59, 62), because its regulatory activity appeared to be promoter-independent and related to the mRNA processing signals present (62). In a system that used the TK (thymidine kinase) promoter sequence fused to different reporter genes, *lacZ* and CAT, Sandri-Goldin and Mendoza found that the ICP27 activity was related to the reporter gene structure, but not the promoter. They found that pTK-CAT was repressed 12-fold when cotransfected with ICP27, but there was no effect on pTK-*lacZ* (62). In agreement with this observation, Hardwicke and Sandri-Goldin demonstrated that when cells were infected with viral mutants defective in ICP27, the accumulated levels of at least three spliced host mRNAs were higher than those seen with wild-type

infected cells (30). This suggested that the host shut off function of ICP27 might be due to an effect on cellular mRNA splicing.

In contrast to ICP4 and ICP27, ICP0 was demonstrated to be required, but not essential, for viral growth. This was based on the observation that ICP0 mutants of HSV-1 were viable in cell culture, albeit more sluggishly than the wild-type (60). It has been shown in transient expression experiments that ICP0 is the only one of all HSV-1 immediate-early proteins that was able to activate all three classes of HSV-1 genes (15, 16, 22, 19, 54). The activation function of ICP0 can be performed either by itself alone, e.g. in the case of activation of HSV-1 ribonucleotide reductase (RR1) gene (9), or in a synergistic manner with ICP4. However the activation function of ICP0 seems to be paradox, since in ICP4 mutants (wild type ICP0), the expression of HSV-1 early and late genes is not activated (6, 12). This paradox may be explained by the requirement of ICP4 for the nuclear localization of ICP0 (72). In addition, ICP0 has been thought to play an important role in the HSV-1 latency (68). Besides the functions of the three HSV-1 immediate-early proteins described above, these proteins are also involved in regulating the expression of certain cellular genes (4, 30, 62).

3. PREVIOUS WORK ON ACTIVATION OF c-MYC EXPRESSION BY HSV-1

In 1992, Shin Chen et al reported a cellular regulatory protein, leaky-late binding factor (LBF) was involved in the activation of HSV-1 major capsid protein VP5 promoter. By using DNA footprint and sequence analysis, they found that the LBF target sequence, the leaky-late binding sequence (LBS), was very similar to that of a recently described cellular protein, YY1. Using competition electrophoretic mobility shift assay, they revealed that LBF and YY1 might be the same protein (4). Furthermore, using transient expression assays with HSV-1 superinfection or cotransfection of HSV-1 immediate-early proteins, they demonstrated that optimal transactivation of the VP5 promoter depended on an intact YY1 binding site (4). Recently, Lisa Mills et al

confirmed that LBF is the same protein as YY1 (47). These reports suggested that HSV-1 immediate-early proteins could activate HSV-1 leaky-late genes, such as the VP5 and gD genes through cellular factor YY1. On the other hand, YY1 has been shown to be an activator of *c-myc* gene expression (57). From sequence analysis of the *c-myc* gene, several potential YY1 binding sites were detected. One is located at downstream from the second promoter in the first *c-myc* exon (another one is located around -240 bp). These observations led Dr. Millette to propose that HSV-1 infection might involved in activating *c-myc* expression. He and J. Paulson found: (i) The *c-myc* expression was activated by HSV-1 infection. (ii) HSV-1 treated with UV radiation failed to transactivate *c-myc* expression (46). These results suggested that some products produced by HSV-1 had a positive effects on the *c-myc* gene. Using cotransfection of HSV-1 immediate-early proteins, they showed that this transactivation was due to the HSV-1 immediate-early proteins, ICP4 and ICP0. ICP4 appeared to be the major transactivator, since when cotransfected with ICP4 only, the wild type CAT activity increased about 100-fold as compared with those that were non-cotransfected (46). An important observation linking *c-myc* regulation with HSV-1 infection from Dr. Millette laboratory's is that this regulation may be associated with the cellular protein E2F. The maps of the plasmids construction for the wild type (+66 wt-Myc-CAT) and mutant type (+66 mtE2F-Myc-CAT) are shown in Figure 1. In the transient expression assay (CAT assay) for these plasmids, the CAT activity for E2F mutant plasmid decreased about 8.5-fold, comparing with that for the wild type plasmid, when superinfected with HSV-1. These results strongly suggested that E2F played an important role in transactivation of human *c-myc* gene expression by HSV-1 immediate-early proteins. This is in accord with the early observation that E2F plays an important role in the regulation of *c-myc* gene expression (34, 69).

4. SCOPE OF THIS THESIS

Early observations described above showed that (i) cellular regulatory protein YY1, in conjunction with HSV-1 immediate-early proteins, might activate the HSV-1 major capsid protein

VP5 promoter (4); (ii) YY1 is an activator for the *c-myc* gene expression (57); (iii) there is a potential YY1 binding site located at the downstream from the P2. Early studies also revealed that, in certain cases, regulatory elements located downstream from the promoter might be involved in affecting the promoter activity. Huang et al reported that the VP5 leader (the sequence at downstream from the VP5 promoter) is required for full-level expression by means of the series deletion and transient expression assay (CAT assay) (35). Another report made by Guzowski et al also demonstrated that the downstream activation sequence (DAS), a 14 bp element located at between +22 and +33 (from the transcriptional start site) in the 5' untranslated leader region of the U_L38 gene, was required for the full level of transcriptional activity of the U_L38 (late gene) promoter (28). All these data led to the hypothesis that the region at the downstream from P2 in the *c-myc* exon 1 may be also important for the HSV-1 immediate-early protein-mediated *c-myc* regulation.

To test this hypothesis, the region in *c-myc* exon 1 downstream of the P2 was examined. First, the wild type (-101/+513-*c-myc*-CAT) and mutated plasmids (-101/+212-Δexon 1-*c-myc*-CAT and -101/+332-Δexon 1-*c-myc*-CAT) were constructed by following ways: (i) The *c-myc* sequences upstream of -101 (relative to P1), in the *c-myc*-CAT plasmids (in which the *c-myc* exon 1 was fused to the bacterial chloramphenicol acetyltransferase gene, CAT, were cut out by endonuclease digestion. (ii) The desired DNA fragments were isolated and ligated by DNA T4 ligation to generate -101/+513-*c-myc*-CAT and -101/+212-Δexon 1-*c-myc*-CAT. (iii) The sequences from +332 to +513 were cut out by endonuclease digestion of wild type *c-myc* plasmid -101/+513-*c-myc*-CAT. Then the desired large DNA fragment was ligated by using DNA T4 ligase, to generate -101/+332-Δexon 1-*c-myc*-CAT. Second, the regulatory roles of *c-myc* sequences downstream the P2 in exon 1 were assayed by HSV-1 superinfection, transfection of wild type or mutated *c-myc*-CAT plasmids, and cotransfection of the plasmids containing HSV-1 immediate-early proteins

gene. Third, DNA probes were prepared, and electrophoretic mobility shift assay (EMSA) or competition EMSA were performed to investigate the possibility that cellular proteins may be involved in this regulation. The results from these experiments suggested that DNA sequences located downstream from the P2 were important for regulation of *c-myc* gene expression by HSV-1 immediate-early proteins. In this regulation, ICP4 and ICP0 appear to function as activators and ICP27 as an inhibitor of gene expression. These results, combined with the data from early studies in both Dr.Millette's and other laboratories, suggest that the regulation *c-myc* gene by HSV-1 immediate-early proteins is mediated through cellular regulatory protein(s) which interact(s) with a *cis*-regulatory elements located downstream from the P2 start site within exon 1.

MATERIALS AND METHODS

1. PLASMID CONSTRUCTION (DELETION)

(A) Construction and subcloning of plasmids -101/+212- Δ exon 1-*c-myc*-CAT and -101/+513-*c-myc*-CAT.

(1) *STRATEGY OF CONSTRUCTION*

The plasmids -2.3- Δ exon 1-*c-myc*-CAT and -1.3-*c-myc*-CAT, kindly provided as a gift from Nissim Hay of the Ben May Institute in Chicago, contain the upstream *c-myc* promoter element from the P1 transcription start site followed by an undeleted exon 1 or a deleted exon 1 linked to a reporter gene called chloramphenicol acetyltransferase (CAT). The plasmid maps and subcloning steps are diagrammed in Figure 2. Two μ g of each of these two plasmids was digested by endonucleases Sma I and Cla I (7 units of each) in a final buffer concentration of 1X KGB [100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 μ g/ μ l bovine serum albumin Fraction V (Sigma), 1 mM β -mercaptoethanol] (61). The larger fragments from these two plasmids were isolated by the DEAE paper electroelution method which has been described previously (13). Briefly, the gel was cut at the site that was 3-4 mm in front of the DNA band, observed under the UV light following ethidium bromide staining. A piece of Whatman DEAE 81 paper that was slightly wider than the DNA band was inserted into the gel at the site that was just in front of the DNA band. The DNAs were electroeluted onto the DEAE 81 paper by running the gel at 200 volts for about 20 minutes. The DEAE paper containing the plasmid DNAs was transferred into a 1 cc syringe which was previously plugged by small amount glasswool. The DNAs on the DEAE paper were washed three times using 200 μ l each time with solution A [0.1 M NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)] by centrifugation each time at 1600 rpm for 30 seconds. Then the DNAs were eluted three times using 120 μ l for each time with solution B [1 M NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)]. Finally, the DNAs were recovered by

means of ethanol precipitation, and dissolved in 1X TE [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA].

In order to generate a blunt end at the Cla I site, 200 ng of each two larger fragments was treated with 1 unit of Klenow fragment in a final buffer concentration of 1.5X KGB in a total volume of 15 μ l (61). Following incubation for 30 minutes at RT, the four deoxyribonucleotides were removed by centrifugation through a 0.9 ml Sephadex G-50 spun column (spun column chromatography). The column prepared in a 1 ml syringe was equilibrated with TEN buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl], and it was prepared according to a procedure described previously (61). After the deoxynucleotides were removed, the fragments were recovered by ethanol precipitation. Finally about 100 ng of each fragment, with blunted end at both ends, was ligated by incubation with 0.5 unit of T4 DNA ligase in 1X T4 DNA ligase buffer [50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000] in a total volume of 20 μ l incubated at 16°C for 24 hours. The purpose was to generate the new subclones, -101/+513-*c-myc*-CAT and -101/+212- Δ exon 1-*c-myc*-CAT, containing full-length or a deletion in exon 1. In generating these two subclones, the Sma I site and Cla I site were removed.

(2) TRANSFORMATION

Competent DM 1 cells, a strain of *E.coli* that lacks the EcoR I and Ava II methylases, were purchased from GIBCO BRL. The competent cells were transformed with the newly ligated plasmids by the method provided by GIBCO BRL. According to this protocol, the transformation was carried out as follows: For each plasmid, 50 μ l DM 1 cells and 3 ng of plasmid DNA were mixed into a 1.5 ml microfuge tube and incubated on ice for 30 minutes. The mixtures were given a heat-shock at 37°C (water bath) for 20 seconds, and were put on ice again for two minutes. Then the mixture were transferred into a clean 15 ml polypropylene tube, and 0.95 ml terrific broth

[1.2% bacto tryptone, 2.4% bacto yeast extract and 0.4% (v/v) glycerol] was added. Then the transformed cells were incubated at 37°C by rotation. Finally, several dilutions of the transformed cells were plated onto LB plates (100 µl of the undiluted culture, 100 µl of a 10 times diluted culture, and 100 µl of a 100 times diluted culture) and incubated at 37°C for 16 to 20 hours.

(3) DNA EXTRACTION--- MINI AND MAXI PLASMID PREPARATION

Mini plasmid preparation. The amplified plasmid DNAs (-101/+513-c-myc-CAT and -101/+212-Δexon 1-c-myc-CAT were first extracted by a mini plasmid preparation method termed the "TENS" method. Briefly, 1.5 ml of overnight culture, grown in the medium of LB containing ampicillin (50 µg/ml), was by sedimented centrifugation at high speed (about 10,000 rpm) at 4°C in a Hermle microcentrifuge. Most of supernatant was poured off (leaving 50 to 100 µl). The transformed cells were lysed by adding 300 µl TENS solution [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.4% NaOH, and 0.5% (w/v) SDS]. Then the lysed cells were neutralized by adding 150 µl 3 M sodium acetate (pH 5.2). The neutralized mixture was centrifuged in Hermle microcentrifuge at high speed (about 10,000 rpm) for 2 minutes at 4°C. The supernatant containing the plasmid DNAs was transferred into a 1.5 ml microfuge tube. The plasmid DNAs were precipitated by adding 0.9 ml 100% ethanol, cooling the mixture to less than -75°C in the Revco -80°C freezer for 20 minutes, and the centrifugation in Hermle microcentrifuge at high speed (about 10,000 rpm) for 5 minutes at 4°C. The supernatant was removed, and the DNA pellet was washed with 500 µl 70% ethanol. Finally, the plasmid DNAs were dissolved in 30 µl of 1X TE [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA] containing 1 unit of RNase A, and the correct subclones were identified by restriction analysis and analytical agarose gel electrophoresis (see below).

Maxi plasmid preparation. All materials including resin columns (Qiagen-tip 500) and solutions were purchased from the Qiagen, Inc. Plasmid DNAs from large culture (500 ml) were

extracted by the Qiagen maxi plasmid preparation method according to the procedures described in the Qiagen plasmid handbook which was provided by Qiagen, Inc. Briefly, 500 ml "Terrific Broth" [1.2% bacto tryptone, 2.4% bacto yeast extract and 0.4% (v/v) glycerol] medium containing ampicillin (50 ng/ml) were inoculated with 5 ml of overnight culture of the transformed *E coli* cells (DM 1 or HB101). These cells were grown with vigorous shaking (280 rpm) at 37°C. When they had reached an absorbency of 1 at 600 nm (for lower copy plasmids), chloramphenicol (170 µg/ml) was added to amplify plasmid DNAs and the culture was further incubated at 37°C with vigorous shaking (280 rpm) for 12 to 16 hours. The cells were sedimented (in a 250 ml bottle) by centrifugation at 6,000 rpm at 4°C for 15 minutes and they were resuspended with 10 ml of P1 solution [100 mM µg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA (pH 8.0)]. Then the cells were lysed by mixing with 10 ml of P2 solution (200 mM NaOH, 1% SDS). After standing 5 minutes on ice, the lysate was neutralized by adding 10 ml of P3 solution [3.0 M KAc (pH 5.5)]. Cellular debris and *E coli* genomic DNA were removed by the centrifugation in the Sorvall centrifuge at 13000 rpm at 4°C for 30 minutes. Plasmid DNAs were separated from proteins and RNAs by passing the supernatant solution through a resin column called Qiagen-tip 500. The DNAs were eluted from the column by adding 15 ml of QF buffer (1.25 mM NaCl, 50 mM Tris/HCl, 15% ethanol.). The eluted DNAs were precipitated with isopropanol (see Qiagen plasmid handbook for detail). Finally, the extracted DNAs were dissolved in 400 to 500 µl of 1X TE and they were analyzed by restriction analysis and agarose gel electrophoresis. The correct subcloned plasmids were kept at 4°C. The concentration and total yields of extracted plasmids were measured by calculating their absorbency at 260 nm, 280 nm, and 320 nm in the Beckman DU-40 spectrophotometer.

(4) *SELECTING SUBCLONES BY RESTRICTION ANALYSIS*

The enzymes used in the restriction analysis were purchased from GIBCO BRL and New England Biolabs. The extracted subcloned DNAs from both mini and maxi plasmid preparations

were checked by the restriction analysis. Briefly, the plasmid DNAs were cleaved with restriction enzymes (endonucleases) so that the circular plasmid DNAs were cut into one linear fragment, or two or three linear fragments. They were also incubated with enzymes which were not able to cleave this specific kind of the plasmid DNA (e.g. in the subcloned plasmids -101/+513-*c-myc*-CAT and -101/+212- Δ exon 1-*c-myc*-CAT, the Sma I and Cla I sites were removed, so Sma I and Cla I were not able to cleave these two plasmids). These fragments, as well as the uncut plasmid, were electrophoresed in an agarose gel (gel concentrations depended on the sizes of the fragments) using 0.5X TBE (0.54% Tris base, 0.28% boric acid, 1 mM EDTA) as the gel running buffer. DNA fragments with the known sizes, e.g. λ /Hind III, were used as size markers in the same gel (when the bacteriophage λ digested by endonuclease Hind III, several known size DNA fragments, such as 23.1 kbp, 9.4 kbp, 6.68 kbp, 4.36 kbp, 2.3 kbp, 2.1 kbp, and 0.56 kbp, were generated). After electrophoresis, the gel was stained by ethidium bromide (0.5 μ g/ml) for about 16 minutes, and photographed with the Polaroid film No.667 under the transmitted UV light using a UV transilluminator. The distances of the marker fragments run in the agarose gel were measured, and a standard curve, which represented the relationship between fragments sizes and distances, was plotted on semi-log paper according to the sizes and distances of the marker fragments. The sizes of the sample fragments were determined by measuring the distances of the sample fragments run in the gel, and then pointing the distances values on the standard curve.

(B) Subcloning for plasmid -101/+332- Δ exon 1-*c-myc*-CAT.

The plasmid map and subcloning steps are diagrammed in Figure 3. Two μ g of the plasmid -101/+513-*c-myc*-CAT was digested with the endonucleases Hind III and Xho I (each of 7 units) in a final buffer concentration of 1X KGB in a total volume of 30 μ l for 3.5 hours at 37°C. The larger fragment, the Hind III-Xho I fragment which would be used as the vector, was isolated by the electroelution onto a dialysis membrane (dialysis membrane method) (45). Briefly, a slo-

which was slightly wider than the width of the band (DNA fragment being isolated), was made just 3 to 4 mm in front of the band in the ethidium bromide stained agarose gel. The Hind III-Xho I fragment was run onto a piece of nitrocellulose dialysis membrane (whether the DNAs were completely run onto the membrane need to be checked under the UV light) in a 1% agarose gel at 200 volts for about 20 minutes. The fragment was removed from the dialysis membrane by running the gel at 200 volts for about 1.4 minutes in the direction opposite to the original gel running direction. The DNAs in the slots were collected by pipetting them into a 1.5 ml microfuge tube and recovered by ethanol precipitation. Then 100 µg of the plasmid -101/+513-Δexon 1-*c-myc*-CAT were digested again with the endonucleases Hind III and Xho I (each of 350 units) in a final buffer concentration of 1X KGB in a total volume of 550 µl for 4 hours at 37°C. The smaller fragment, the 487 bp Xho I-Hind III fragment (from the Xho I site to Hind III site) was isolated by the dialysis membrane method as described above. This fragment (about 2.5 µg) was further digested by endonuclease of Ava II (10 units) in a final buffer concentration of 1X KGB in a total volume of 30 µl for 3.5 hours at 37°C to generate three fragments: a 266 bp Xho I-Ava II fragment, a 151 bp Ava II-Ava II fragment, and a 71 bp Ava II-Hind III fragment. All three fragments were isolated by means of dialysis membrane method as described above. Then 200 ng of the Hind III-Xho I fragment (as a vector) and 30 ng of the 266 bp Xho I-Hind III fragment were ligated by using 1 unit of T4 DNA ligase. In order to generate the blunt ends at the Ava II and Hind III sites, the ligated mixture was treated with Klenow fragment as described above. After the Klenow fill-in reaction, the deoxynucleotides were removed by passing through a G-50 column as described above (61). The new subclone, -101/+332-Δexon 1-*c-myc*-CAT, was generated by ligating both blunt ends of the fragment with 1 unit of T4 DNA ligase incubated at 37°C for 24 hours. In this subcloned plasmid, both the Ava II site and Hind III site were removed.

The transformation, DNA extraction and restriction analysis processes were the same as described above except that the strain of competent cells used in the transformation was HB 101,

another strain of *E coli*, instead of DM 1, and the endonucleases used in restriction analysis (see table 4).

2. DNA PROBE PREPARATION

Three DNA fragments from *c-myc* exon 1, the 266bp Xho I-Ava II fragment, the 151bp Ava II-Ava II fragment, and the 71bp Ava II-Hind III fragment, were isolated as described above. These three fragments were labeled with α -³²P-dATP and α -³²P-dCTP by means of Klenow fill-in reaction (see Fig.4) which was described previously (61). Briefly, each of being labeled fragment was mixed with radioactive deoxynucleotides (labeled with α -³²P-dATP and α -³²P-dCTP), non-radioactive deoxynucleotides (dGTP and dTTP), and 1 unit of Klenow enzyme. The reaction mixture was incubated for 20 minutes at room temperature (RT). Then the reaction mixture was chased with 1 mM of each four unlabeled deoxynucleotides for 10 minutes at RT. The Klenow fragment activity was inactivated by heating the mixture at 70°C for 10 minutes. The α -³²P-labeled or α -³²P-unlabeled deoxynucleotides were removed by passing the reaction mixture through a G-50 spun column. The α -³²P-labeled fragments (probes) were recovered by ethanol precipitation and were dissolved in 100 μ l 1X TE and stored at -20°C. The radioactivities of the α -³²P-labeled probes were determined in a Beckman LS6500 liquid scintillation spectrometer.

3. CELLS AND VIRUS

Cells and virus used and their propagation were described in the previously published paper by Chen et al (4). Briefly, Hela cells (ATCC CCL2) maintained at 37°C under 5% CO₂ atmosphere in Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratory Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 u/ml), and streptomycin (100 u/ml). The virus, kindly provided by G.S.Read, Loyola, University of Chicago, was the vsh-1 mutant of HSV-1 (KOS) which was defective in the first stage host shutoff function and it was previously propagated in HEP-2 cell monolayers by Dr. Millette in the Biology Department of Portland State

University.

4. TRANSFECTION, COTRANSFECTION AND HSV-1 SUPERINFECTION

The procedures for transfection, cotransfection and HSV-1 superinfection were almost same as described in the previously published article (24, 25, 50) modified by Chen et al (4), except that in the cotransfection assay, the amounts of each plasmid DNA, encoding for HSV-1 immediate-early proteins (ICP0, ICP4, ICP27), were 2 µg instead of 8 µg (the plasmid pUC18 was used to adjust the total amount of DNAs to 16 µg which represented the total amount of different kinds of plasmids in one well). Briefly, Hela cells were seeded in 35-mm well cluster dishes at 3×10^5 cells per well in 3 ml DMEM-10% IFBS-P/S (4), and incubated at 37°C in the presence of 5% CO₂. Twenty hours later, the plasmid DNAs were precipitated by a procedure called calcium phosphate-precipitation. Briefly, for each duplicate, the appropriate amount of plasmid DNAs being transfected or cotransfected was mixed with 0.5 ml of 2X HEPES-buffered saline (HBS) (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM dextrose, 50 mM HEPES) in a 5 ml polypropylene tube. Then 0.5 ml of 0.25 M CaCl₂ was added dropwisely with bubbling and the adding of CaCl₂ took about 15 seconds. The mixture was incubated at RT for 30 minutes. Then 0.5 ml of calcium phosphate-precipitated DNA was transfected (plasmid DNAs containing full length of *c-myc* exon 1 or deletions in the *c-myc* exon 1) or cotransfected (plasmid DNAs containing HSV-1 immediate-early genes) into Hela cells. Four hours after transfection or cotransfection, the medium was removed and the cells were shocked by 1 ml glycerol shock solution [15% (v/v) glycerol in DMEM-10% ICFS] for 1 minute. The cells then were washed 2 times with 3 ml PBS-A (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄) supplemented with 3 ml DMEM-10% IFBS-P/S, and further incubated at 37°C under 5% CO₂. Twenty hours after the transfection or cotransfection, the transfected cells were superinfected with HSV-1 (described in cells and virus) at multiplicity of infection of 3. At the meantime, the mediums for the cotransfected cells were

changed (renewed with the same medium). Both transfected and cotransfected cells were harvested 40-44 hours after the transfection or cotransfection. The cells superinfected by HSV-1 were checked under the microscope, and were harvested when they were approximately 40-50% rounded even the time was less than 40 hours. The harvested cells were lysed by the method performed by Nordeen et al (50). Briefly, the cells in each well were rubbed off from well by using a "rubber policeman", and were sedimented by centrifugation in a Hermle microcentrifuge at lower speed at 4°C for 3 minutes. The supernatant was removed by aspiration. Then the cells were lysed by resuspending them in 100 µl lysis buffer [0.25 M Tris/HCl (pH 7.8), 0.25% (v/v) NP 40, 0.005% (w/v) PMSF], mixing them well, and incubating them on ice for 10 minutes. The mixture was centrifuged in the Hermle microcentrifuge at lower speed at 4°C for 5 minutes. Finally the supernatant (lysate) was transferred into a clean 1.5 ml microfuge tube, and the lysates were kept at -80°C (in Revco) for CAT assay.

5. CAT ASSAY

The CAT assay was described previously (4, 50), and it consisted of measuring incorporation of ³H-acetate (NEN Research Products, Inc.; 3.3 Ci/mmol, 10 mCi/ml) into acetyl-chloramphenicol by using a coupled reaction with acetyl coenzyme A synthetase (4, 50). Briefly, for each reaction, 43 µl of distilled water, 31.5 µl of 4X CAB [4X CAT assay buffer (400 mM Tris-HCl pH 7.8, 24 mM MgCl₂, and 300 mM KCl)], ³H-labeled acetyl acid (0.78 µCi/µl), 10 µl of 5 mM Coenzyme A, 4 µl of 100 mM ATP, and 0.5 µl of 250 mM chloramphenicol were mixed in a 1.5 ml microfuge tube and pre-incubated for 30 minutes at 37°C. Then 30 µl of each sample lysate was added to each tube and the contents were incubated at 37°C for 2 hours. After the incubation, 1 ml of cold benzene was added to each reaction tube, and the contents were completely mixed by vigorous vortexing and inverting. This mixture was centrifuged in Hermle microcentrifuge at high speed (about 10⁴ rpm) for 2 minutes at 4°C. After centrifugation, 800 µl solution from the benzene phase

was transferred into a scintillation vial and dried by evaporation. Finally, the radioactivities of acetyl chloramphenicol (cpm) was measured in a Beckman liquid scintillation spectrometer. The net cpm of acetyl chloramphenicol for each sample was obtained by subtracting the cpm of the control plasmid (e.g. pUC18) which does not contain the CAT gene.

6. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Both HSV-1 uninfected and HSV-1 infected nuclear extracts (NE) were previously prepared by Dr. Millette (in Biology Department of PSU) according to the procedures performed by J. D. Dignam et al (23). In each reaction, NE containing 2 µg of protein, DNA probe (10^4 cpm), 1-2 mM MgCl₂, and 1 µg polydIdCpolydIdC (double stranded polydIdC) (Pharmacia Biotech) were mixed in 0.65 ml microfuge tube. The binding reaction was taken place at RT for 20 minutes in a total volume of 16 µl. Then 1.6 µl of 10% bromophenol blue (BPB) was added. A 4% polyacrylamide gel (PAGE), which was pre-run at 250 volts at 4°C for about 60 minutes, was used for electrophoresis. The running buffer used in this experiment was 1X NRB [0.3% (v/v) Tris base, 1.43% (w/v) glycine, 10 mM EDTA]. The samples were electrophoresed at 250 volts at 4°C for 130 minutes. Then the gel was transferred onto a piece of 3 mm paper, covered with a piece of plastic food wrap, and put on a gel slab dryer for 2 hours to dry. The dried gel was covered by a RX Fuji film (20.3 x 25.4 cm) which then was exposed for about 13 hours at -80°C. The film was developed and analyzed for protein binding.

7. COMPETITION EMSA

Competition EMSAs were run similarly to the EMSA, except that an additional unlabeled 33 bp dsDNA oligonucleotide, whose sequence is CCAGGATCCAGGGCCATCTTGAATGGATCCTGG, was added. This oligonucleotide was named LBS (4) was synthesized by Vollum Institute, Oregon Health Sciences University. It contained a binding site for nuclear protein YY1, shown in the underlined part of the sequence (4, 47). The control probe used in this assay was the ³²P-

labeled 160 bp DNA fragment from VP5 (HSV-1 major capsid protein) promoter, which was previously shown to contained the sequence for the cellular protein YY1 binding.

RESULTS AND CONCLUSIONS

1. RESULTS

(1) Preparation and analysis of subclones -101/+513-*c-myc*-CAT and -101/+212- Δ exon 1-*c-myc*-CAT.

To determine whether the sequences in the first exon from P2 are important for the activation of *c-myc* gene expression by HSV-1, the plasmids containing deletions in exon 1 of the *c-myc* gene were constructed (see Fig. 2). First, plasmids containing only 101 bp upstream of the *c-myc* promoter were prepared by excising the region from -2.3 kbp to -101 bp in the plasmid -2.3- Δ exon 1-*c-myc*-CAT, and the region from -1.3 to -101 in the plasmid -1.3-*c-myc*-CAT (both plasmids were the gifts from the Nissim Hay in Ben May Institute in Chicago) by digestion with restriction endonucleases *Sma* I and *Cla* I. The large vector fragment was isolated by the DEAE paper electroelution method. The blunt ends were made at the *Cla* I site of the vector fragments by the Klenow fill-in reaction. The two vector fragments were then ligated using T4 DNA ligase to generate plasmids -101/+513-*c-myc*-CAT (used as a wild type control) and -101/+212- Δ exon 1-*c-myc*-CAT. Then the subcloned plasmids were transformed into competent *E coli* cells, screened by mini plasmid preparation. The correct subcloned plasmid DNAs were extracted by maxi plasmid preparation. The total yields from a 500 ml culture was 292 ug for -101/+513-*c-myc*-CAT and 304 ug for -101/+212- Δ exon 1-*c-myc*-CAT.

The resulting plasmids were examined by restriction analysis (see Fig.5 for restriction map). First, both -101/+513-*c-myc*-CAT and -101/+212- Δ exon 1-*c-myc*-CAT were digested by endonuclease *Hind* III, *Hind* III/*Pvu* I, *Eco*R I, and *Sma* I/*Cla* I. The digesting mixtures were run in a 1% mini gel (Fig.10). The mini gel showed that the number of observed bands resulted from this digestion is consistence with the number of expected bands (Table 1), and the sizes of observed

bands are approximately estimated to be correct according to the sizes of marker bands (sizes can not be accurately measured in mini gel) (Fig.10). To confirm the mini gel results, these two subcloned plasmids were examined in two standard agarose gels, 0.9% gel for linear form and 1.2% gel for diagnostic digestion (most important evidence) by EcoR I and EcoR I/Not I (Fig.13 and Table 2). These results indicated that both sizes and numbers of the bands produced coincided with those expected from restriction maps. Therefore they appeared to be the correct subclones. To confirm the deletion part of the subclone -101/+212- Δ exon 1-*c-myc*-CAT, DNA sequencing was carried out. Although part of sequences upstream the *Not* I site (+212 bp) could be read, it failed to show the deletion part. Therefore an alternative experiment, the restriction analysis of a small fragment [from *Not* I site (+22) to *Hind* III site] spanning the deletion part, was performed. Thus the *Not* I-*Hind* III fragment (see Fig.5) was isolated and further analyzed the digestion of endonucleases *Pvu* II, *Alu* I, *Xho* I, *Hinf* I, *Ava* I and *Ava* II. This analysis showed that this fragment was able to be digested by *Xho* I only, and it could not be digested by *Pvu* II, *Alu* I, *Hinf* I, *Ava* I and *Ava* II (Fig.11). This result was consistent with the data shown on the plasmid map (see Fig.5), from Nissim Hay in Ben May Institute in Chicago (32), and confirmed that the -101/+212- Δ exon 1-*c-myc*-CAT was the correct subclone.

(2) Preparation and analysis of subclone -101/+332- Δ exon 1-*c-myc*-CAT

To further determine the more specific region within the *c-myc* exon 1 which was responsible for HSV-1 immediate-early proteins mediated human *c-myc* regulation, the region from +332 to +513 (181 bp), and the adjacent 40 bp up to *Hind* III site in the plasmid -101/+513-*c-myc*-CAT was removed by the endonucleases *Ava* II and *Hind* III as described above. The subclone -101/+332- Δ exon 1-*c-myc*-CAT was generated by first ligating the vector with the 266 bp *Xho* I- *Ava* II fragment by T4 DNA ligase (see Fig. 3), treating Klenow fragment to make both ends blunt, finally ligating both blunt ends together with T4 DNA ligase.

The plasmid DNAs of this subclone were prepared by the Qiagen maxi plasmid preparation method (as described above). The total yield from a 500 ml culture was 360 ug. As with the other subclones, this subclone was examined by restriction analysis with the endonucleases Hind III, EcoR I, Not I, and Xho I (see Fig.5 for the restriction map) first in a 1% mini agarose gel (Fig.12). The mini gel results showed that the number of observed bands is consistent with the number of expected bands (Table 1). In Fig.12, it also showed that the sizes of the bands are approximately measured to be correct according to the sizes of marker bands (the accurate size can not be measured in mini gel). To confirm the mini gel results, this plasmid was examined in two different concentration standard agarose gel, 0.9% for linear form and 1.2% for diagnostic restriction (most important evidence). The standard gel is shown in (Fig.10 and 13) and the sizes of bands in standard gel were measured (Table 2). The results (shown in Table 1, Table 2, Fig.10, Fig.12, and Fig.13) indicate that both sizes and numbers of the bands agree with those predicted from the restriction map. Therefore -101/+332- Δ exon 1-*c-myc*-CAT subclone is correct.

(3) Sequences located at downstream from the P2 in the *c-myc* exon 1 contain the responding elements for the ICP4 and ICP0 mediated activation

To determine whether there is a element located downstream from the start site in the *c-myc* gene, P2, which is activated by HSV-1 immediate-early proteins, the subcloned plasmids -101/+513-*c-myc*-CAT (as wild type control containing -101 to +513 of *c-myc*) and -101/+212- Δ exon I-*c-myc*-CAT (containing -101 to +212 of the *c-myc*) were assayed separately two times in a transient expression assay system (CAT assay). The linear maps are shown in Figure 5. To test their abilities to be transactivated by HSV-1, the HeLa cells were transfected with each of these plasmids, and 20 hours later they were superinfected with HSV-1 at a multiplicity of 3. Parallel control cultures remained HSV-1 unsuperinfected. Twenty to twenty four hours after superinfection the cells were harvested and their lysates were analyzed for CAT enzyme activity.

The results are shown in the Figure 6 and the Table 3. These results showed that the CAT activity increased 5.7-fold for the wild type *c-myc* plasmid -101/+513-*c-myc*-CAT when HSV-1 was superinfected. Similarly, a 3.9-fold and a 1.9-fold increase were observed in the mutant (deletion in the first exon of *c-myc*) *c-myc* plasmids -101/+212- Δ exon 1-*c-myc*-CAT and -101/+332- Δ exon 1-*c-myc*-CAT, respectively. Furthermore, when the CAT activity was compared to that of the wild type -101/+513-*c-myc*-CAT in the presence of HSV-1 superinfection, a 5.2-fold and a 6.2-fold reduction was observed in the cells transfected with the mutant *c-myc* plasmids -101/+212- Δ exon 1-*c-myc*-CAT and -101/+332- Δ exon 1-*c-myc*-CAT, respectively. When compared to the wild-type level set at 100%, the represented relative CAT level of 19.2% and 16.1 to the -101/+212 and -101/+332 deletion respectively (Fig.6, Table 3). These results were accord with the previous data from Dr. Millette's laboratory that *c-myc* gene expression was strongly activated by HSV-1 infection, and they indicated that the sequences within the first exon of *c-myc* gene are important for activation by HSV-1 infection.

According to previous results from this laboratory (46), the major activation is provided by HSV-1 immediate-early proteins ICP4 and ICP0. To investigate the involvement of sequences in the +332 to +513 bp region in this activation, the activity of the subclone -101/+332- Δ exon 1-*c-myc*-CAT was compared with that of the wild-type promoter in two separate cotransfection assays. One reason to speculate the region from +332 to +513 of the first exon of *c-myc* is important for the ICP4 and ICP0 mediated activation is that it contains a potential binding site for the cellular regulatory protein YY1 at about +453 bp. This cellular regulatory protein was previously shown to be involved in activating the murine *c-myc* promoter (57) and the HSV-1 VP5 promoter (4). To investigate this, HeLa cells were transfected with wild type and exon 1 deletions of *c-myc*-CAT, and simultaneously cotransfected with plasmids expressing HSV-1 IE proteins it contains. The results of the cotransfection assays (see Fig.7 and Table 4) demonstrate that, comparing with the

non-cotransfected cells (transfected with -101/+513-*c-myc*-CAT only), the CAT activities of the wild type *c-myc* plasmid -101/+513-*c-myc*-CAT increased 13.7-fold when cotransfected with ICP4, 16.7-fold when cotransfected with ICP4 and ICP0, and it was reduced 1.5-fold when cotransfected with ICP4, ICP0, and ICP27. Relative to the wt (-101/+513) set at 100%, cotransfection of ICP4 alone was 78.3%, and ICP4, 0, and 27 was 5%. On the other hand, the similar comparison in the *c-myc* mutated plasmid showed that the CAT activity of the plasmid -101/+332- Δ exon 1-*c-myc*-CAT increased 2.1-fold when cotransfected with ICP4, and 4.5-fold when cotransfected with ICP0, and it was reduced 1.1-fold when cotransfected with all three HSV-1 immediate-early proteins. Relative to the wt CAT plasmid induced with ICP4/ICP0 (as 100%), this deleted plasmid (-101/+332) gave the CAT level of 2.5%, 16.0%, and 0.9% when cotransfected with ICP4, ICP4/ICP0, and ICP4/ICP0/ICP27, respectively. These results lead to two suggestions: (i) The region from +332 to +513 contains an element required for *c-myc* gene activation by HSV-1 immediate-early proteins. (ii) ICP4+ICP0 together provide maximum transactivation, ICP4 appears to provide the major, single transacting effect, and ICP27 functions to inhibit its expression.

(4) Are there any binding sites for cellular regulatory proteins in the region from +332 to +513 in the first exon of *c-myc* gene?

To determine whether any cellular regulatory protein(s) bind(s) to the *c-myc* exon 1 region between +66 and +513 bp, electrophoretic mobility shift assays were carried out with three *c-myc* fragments spanning this region. These fragments were 266 bp Xho I-Ava II fragment [from +66 bp to +332 bp (see Fig. 5)], 151 bp Ava II-Ava II fragment [from +332 bp to +483 bp (see Fig. 5)], and 71 bp Ava II-Hind III fragment [from +487 bp to +513 bp, and up to Hind III site at the start site of the CAT gene (see Fig. 5)]. Because of the data derived from CAT assay, we emphasized examining the region from +332 to +513 in the *c-myc* exon 1. The results of the

EMSA (Fig. 8) showed: (i) There are two major shift bands observed in the lanes for the 266 bp Xho I-Ava II probe (Fig. 8, lane 11 and 12). (ii) There were three shifted bands (two major bands and one minor band) observed both for the 151 bp Ava II-Ava II probe which contains a potential binding site for transcription factor YY1 (Fig. 8, lane 6 and 7), and 71 bp Ava II-Hind III probe (lane 2 and 3). Previous studies have shown that HeLa cell nuclear extracts form 2 bands with YY1, one representing a YY1 degradation product (4). Therefore two of these bands could be caused by YY1. To determine if any of these bands were formed by YY1, the competition EMSAs were carried out. The results showed (Fig. 8): (i) Positive control probe (VP5) has two clear bands caused by YY1 binding. They appear to be lowered in intensity when the LBS is present. (ii) The bands observed in the 266 bp probe are only slightly competed. (iii) The intensity of these bands did not diminished when the radioactive 151 bp Ava II-Ava II probe was competed with a 200- and 400-fold molar excess of LBS, a unlabeled 33 bp dsDNA fragment containing a YY1 binding site (Fig. 8, lane 8 and 9). This indicated that these shifted bands were not due to the YY1 binding. (iiii) Three bands (two major bands and one minor band) were observed when 71 bp probe was used. The two major shifted bands disappeared when competed with a 400-fold molar excess of LBS, indicating that these bands were due to binding of the cellular regulatory protein, YY1 (Fig. 8, lane 4). This observation was confirmed by early data from competition EMSAs using ³²P-labeled 222 bp Ava II-Hind III fragment (from the Ava II site at +332 within *c-myc* exon 1 to Hind III site, and see Fig. 5 for map). In this experiment, C2, a dsDNA fragment not containing YY1 binding site, was used as a negative control oligonucleotide. The results showed that the two bands supposed to be caused by YY1 binding diminished when the 222 bp Ava II-Hind III probe was competed with LBS, but they did not diminished when the probe was competed with C2 (Fig. 9). Thus, the possibility that the two bands were caused by non-specific proteins binding was excluded. However, from the sequence analysis, there was no YY1 consensus binding sites to be

found in this region, indicating that YY1 was not a factor involved in HSV-1 immediate-early proteins mediated regulation of human *c-myc* gene. This looks like somewhat contradictory. There are two possible explanations: (i) the 40 bp vector sequence (see Fig.5) constructed by Hay contains a YY1 binding site; (ii) the region from +483 to +513 might contain a non-consensus or high degenerated YY1 binding site. According to the second explanation, the role of the cellular protein YY1 in the HSV-1 immediate-early proteins mediated *c-myc* regulation still could not be excluded.

2. CONCLUSION

The results from these studies lead to the following conclusions:

- (1) HSV-1 immediate-early proteins are regulators for human *c-myc* gene expression in transient assays. ICP4 is the major transactivator, ICP0 is a minor transactivator, and ICP27 serves as an inhibitor of the *c-myc* gene expression.
- (2) The region from +332 to +513 in the exon 1 of the *c-myc* gene contains a sequence which is responsible for the ICP4 and ICP0 mediated transactivation of human *c-myc* gene expression [other important regions upstream of the P2, such as E2F site, were also identified in previous studies (46)].
- (3). The data from my EMSA results suggest that at least one cellular regulatory protein is involved in viral regulation of the human *c-myc* gene through sequences in exon 1.
- (4). From the data derived from the EMSA and competition EMSA, I conclude that the cellular regulatory protein YY1 is not likely involved in the HSV-1 mediated regulation of human *c-myc* gene through the exon 1 sequences. However, there is a possibility that a non-consensus YY1 binding site may be involved for the regulation by this region of the *c-myc* gene.

DISCUSSION

Early studies revealed that *c-myc* expression is regulated, either positively or negatively, by many factors at different stages (e.g. it may be regulated at level of initiation of transcription or at level of elongation of transcription). Many target sequences for cellular transcription factors have been identified in the *c-myc* promoter in the region between 2.3 kbp and the transcription start sites, P1 and P2 (10, 33). Recently Dr. Millette and J. Paulson found that HSV-1 immediate-early proteins (mainly ICP4) could activate the expression of *c-myc* gene (46). Their studies further showed that this activation was related to a target sequence for cellular protein E2F binding. This binding site was located between P1 and P2 (around +117) (46). Consistent with the results shown by Millette and Paulson, the experiments presented here also demonstrate that the human *c-myc* gene is regulated by the HSV-1 immediate-early proteins. In these experiments, additional target sequences for HSV-1 immediate-early proteins (ICP4 and ICP0) mediated activation is identified in the region downstream from P2 (between +332 and +513 bp), within the first exon of the *c-myc* gene. The exact mechanism involved in this regulation was not established.

We have already shown that three immediate-early proteins, ICP4, ICP0, and ICP27 play a role in regulating both viral and cellular genes. All three of the HSV-1 immediate-early proteins are nuclear phosphoproteins that are able to regulate the expression of other genes. ICP4 is the major transcriptional activator of HSV-1, although it represses its own expression (6, 12, 26). It has been reported to bind DNA nonspecifically (21), but it also binds to some of its target sites in a sequence-specific pattern. Its high affinity binding sites have been identified as ATCGT-CNNNNYGRC (where R is purine, Y is pyrimidine, N is any base (20, 44), and all of its binding sites conform to the consensus sequence RTCGTCNNYNYSG (where R is purine, Y is pyrimidine, S is C or G, and N is any base) (11). Early studies showed that the activation function of ICP4 is independent on the specific binding. Imbalzano suggested that ICP4 may activate the

expression of HSV-1 early and late genes by a mechanism that does not require sequence-specific binding (36). Similar to this suggestion, other laboratories demonstrated that the mutations in either the ICP4 target sequence or in the ICP4 binding domain did not affect its transactivation function (64, 65).

Early studies and my data showed: (i) ICP4 binding consensus binding sites were not found in the region from +332 bp to +513 bp within the exon 1 (J. Paulson, personal communication), and the binding of ICP4 to its target sequence might not be required for its activation function. (ii) The data derived from transient expression (CAT assay) showed that the region from +332 bp to +513 bp in the first exon of *c-myc* was important for the regulation of *c-myc* gene expression by HSV-1 infection. (iii) The data from EMSA and competition EMSA demonstrated that some cellular proteins bound to the DNA sequences in the region from +332 bp to +513 bp. (iiii) The early data from this laboratory (46) and the data in this paper showed that the ICP27 inhibition of *c-myc* expression seemed mainly to inhibit the ICP4 mediated transactivation. Early studies also revealed that ICP27 was involved in the secondary host shutoff process. Taken together these observations, it is reasonable to speculate that cellular factor(s) is/are involved in the ICP4 mediated activation of human *c-myc* gene expression at the level of transcription. This speculation may also be supported by the early studies that human *c-myc* expression depends on the presence of cellular proteins. However, the previous studies, described above (40, 71, 72), might also give rise to the possibility that ICP4 might bind to a site which was of the low affinity or high degeneracy type, and the human *c-myc* gene expression might be activated by this binding. Therefore the possibility that ICP4 directly transactivates human *c-myc* gene expression still can not be excluded.

In contrast, among these three immediate-early proteins, ICP0 is the only one that activates all three kinetic classes (α , β , γ) of HSV genes, without apparent DNA sequence specificity (17, 18, 49, 51, 54). It is able to further enhance the expression of any gene that exhibits a basal level of

transcription (71). There are many reports which showed that ICP0 exhibits a synergistic transactivation with ICP4 (71, 72), and our cotransfection assay results are concordant with these reports. This synergistic transactivation may require the physical interaction between ICP0 and ICP4 (71). Recent study also revealed that ICP4 appeared to be required for the nuclear localization of ICP0 (72)

ICP27 is another HSV-1 immediate-early protein involved in the regulation of *c-myc* gene expression. Although early showed that ICP27 alone was able to inhibit the expression of *c-myc* gene, early data (46) and my experiments demonstrated that ICP27 mainly inhibited ICP4 mediated *c-myc* activation. Previous study revealed that ICP27 is involved in the secondary stage shutoff of the host proteins synthesis (31). When cells were infected with HSV-1 mutants defective ICP27, the shutoff host protein synthesis was greatly reduced.(59). Other studies showed that ICP27 regulated cellular genes expressions post-transcriptionally (31, 62, 67), and that regulation (both activation and repression) was not dependent on the target gene promoters, but on their processing signals (62). For the inhibitory activity of ICP27, the presence of introns in the target genes were required. (62). These observations suggest that the inhibitory function of ICP27 we have observed in the regulation of human *c-myc* gene might also occur at the post-transcriptional level. If this is true, it is reasonable to consider the possibility that the mechanism of ICP27 repression of *c-myc* gene expression might be due to inhibition of action of certain cellular proteins. These proteins, activated by ICP4, possibly and/or ICP0 might be required for activating human *c-myc* gene by binding to the region from +332 to +513 within exon 1. These possibilities support the speculation that cellular regulatory factor(s) is/are involved HSV-1 mediated regulation of human *c-myc* gene expression. ICP27 can also inhibit the nuclear localization of ICP0 (72), and reduce the ICP4-ICP0 synergy. This would therefore inhibit the ICP4-ICP0 synergistic activation.

In summary, my experiments demonstrate that the HSV-1 immediate-early proteins, ICP0,

ICP4, ICP27 are the regulators of the human *c-myc* gene. The results of cotransfection experiment showed that the relative CAT activity observed for wild type *c-myc* plasmid is 16% for non-cotransfection, 78.3% for the cotransfection of ICP4 alone, 100% for the cotransfection of ICP4/ICP0, and 5% for the cotransfection of ICP4/ICP0/ICP27. The relative CAT activity for the mutated *c-myc* plasmid -101/+332- Δ exon 1-*c-myc*-CAT is 1.7% for non-cotransfection, 2.5% for cotransfection of ICP4 alone, 16% for cotransfection of ICP4/ICP0, and 0.9% for cotransfection of ICP4/ICP0/ICP27 (the CAT activity observed in wild type *c-myc* plasmid cotransfected with ICP4/ICP0 is set as 100%). Therefore ICP4 is the major transactivator whereas ICP27 inhibits the expression of the *c-myc* gene. This inhibition was observed either at basal level of *c-myc* expression (minor effect) (46) or in the ICP4 mediated *c-myc* activation (major effect). This implied that cellular proteins may be involved, because of its host shutoff function. The cotransfection results, together with the HSV-1 superinfection results, also suggest that one of the responding element in the *c-myc* exon 1 is located at the region from +332 bp to +513 bp downstream of the P2. In addition, the results from the EMSA and competition EMSA show that some cellular proteins bound to this region (Picture 1). This also implies that cellular proteins may be involved in this regulation.

Taken together with the observations from Dr. Millette's previous studies (46) and earlier studies on the HSV-1 immediate-early proteins (31, 36, 46, 59, 62, 65), my results suggest that cellular regulatory factor(s) may be involved in the ICP4 mediated human *c-myc* gene activation. Hypothetically, ICP4 may activates cellular protein(s) which in turn bind(s) to their target sequences in the *c-myc* gene and activate(s) *c-myc* gene expression. The exact mechanism of HSV-1 immediate-early proteins mediated regulation of *c-myc* gene is still unclear and it needs further investigation.

In my opinion, Further study will include DNA footprinting. This experiment will give infor-

mation of the exact cellular proteins binding sites. From the data derived from the DNA footprint, these binding sites could be used to compare with the binding sites of known proteins. Thus the possible kinds of proteins bound to the *c-myc* gene may be obtained.

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Subclones	-101/+513-c-Myc-CAT			101/+212- Δ exon 1-c-Myc-CAT			-101/+332- Δ exon 1-c-Myc-CAT			
Enzyme	Hind III	Hind III/ Pvu I	Sma I/ Cla I	Hind III	Hind III/ Pvu I	Sma I/ Cla I	Hind III	Xho I	Pst I	Xho I/ Pst I
Number of observed bands	1	2	no cut	1	2	no cut	no cut	1	1	2
Number of expected bands	1	2	no cut	1	2	no cut	no cut	1	1	2

Table 1. Results of restriction analysis in mini-gels for the subclones -101/+513-c-Myc-CAT, -101/+212- Δ exon 1-c-Myc-CAT, and -101/+212- Δ exon 1-c-Myc-CAT. The results indicate that the number of observed bands are consistence with that of the expected bands. See Fig.10, and Fig.12 for the gel.

Subclone	-101/+513-c-Myc-CAT			-101/+212- Δ exon 1-c-Myc-CAT			-101/+332- Δ exon 1-c-Myc-CAT		
Enzyme	Pst I	EcoR I	EcoR I/ Not I	Pst I	EcoR I	EcoR I/ Not I	Pst I	EcoR I	EcoR I/ Not I
Number of observed bands	1	2	2	1	2	2	1	2	2
Number of expected bands	1	2	3 (see ^{**})	1	2	3 (see ^{**})	1	2	3 (see ^{**})
size of observed bands	6.3 kbp	0.94 kbp and a large size band	0.80 kbp and a large size band	6.0 kbp	0.61 kbp and a large size band	0.46 kbp and a large size band	6.10 kbp	0.71 kbp and a large size band	0.55 kbp and a large size band
size of expected bands	6.3 kbp	0.93 kbp and 5.37 kbp	146 bp, 0.79kbp, and 5.37 kbp	6.0 kbp	0.63 kbp and 5.37 kbp	146 bp, 0.484 kbp, and 5.37 kbp	6.08 kbp	0.71 kbp and 5.37 kbp	146 bp, 0.564 kbp, and 5.37 kbp.
conclusion	all subcloned plasmids are correct								

Table 2. Results of the restriction analysis of the subcloned c-myc plasmids -101/+513-c-Myc-CAT, -101/+212- Δ exon 1-c-Myc-CAT, and -101/+332- Δ exon 1-c-Myc-CAT in two different concentrations of agarose gel (0.9% and 1.2%). The results indicate that both the number and sizes of observed bands are same or very close to those of the expected bands. The most convincing evidence that they are correct plasmids is derived from the measurement and comparison of the diagnostic bands. The size differences in these bands represent the deletion parts in the exon 1. The gels are shown in Fig.10 (0.9% gel) and Fig.13 (1.2% gel for the diagnostic digestion by EcoR I and EcoR I/Not I).

*The sizes of the larger fragments in Fig.13 can not be measured because of the concentration of the agarose gel (1.2%). The larger marker DNA fragments can not be separated well at the higher concentration gel.

**The 146 bp fragment in Fig.13 is too small to be detected. The reason to examine the EcoR I/Not I band is that this band is really important for the diagnostic purpose.

Plasmids	HSV-1	CAT Activity (pmol ³ H-OAc Chloramphenicol/ mg Protein/h	percentage of induced CAT activity of the wild type <i>c-myc</i> plasmid (%)
-101/+513- <i>c-myc</i> -CAT	-	5.8 x 10 ³	14.9
	+	39.0 x 10 ³	100.0
-101/+212-Δexon 1- <i>c-myc</i> -CAT	-	1.9 x 10 ³	4.9
	+	7.5 x 10 ³	19.2
-101/+513- <i>c-myc</i> -CAT	-	7.2 x 10 ²	23.2
	+	31.0 x 10 ³	100.0
-101/+332-Δexon 1- <i>c-myc</i> -CAT	-	3.2 x 10 ²	10.3
	+	5.1 x 10 ²	16.1

Table 3. Relative CAT activities for the the wild type and mutated *c-myc* plasmids -101/+513-*c-myc*-CAT, -101/+212Δexon 1-*c-myc*-CAT, and -101/+332-Δexon 1-*c-myc*-CAT in two separate HSV-1 superinfections. The CAT activity represents pmol chloramphenicol acetylated/mg protein/h. The CAT activity of wild type -101/+513-*c-myc*-CAT superinfected by HSV-1 is set as 100%.

Transfected plasmid	Cotransfected plasmid	pmol 3H OAC Chloramphenicol/mg Protein/h	percentage of induced CAT activity of the wild type <i>c-myc</i> plasmid (%)
	ICP4	9.4X10 ³	78.3
-101/+513- <i>c-Myc</i> -CAT	ICP4/ICP0	1.2X10 ⁴	100.0
	ICP4/ICP0/ICP27	6.0X10 ²	5.0
	ICP4	3.0X10 ²	2.5
-101/+332-Δexon 1- <i>c-Myc</i> -CAT	ICP4/ICP0	2.0X10 ³	16.0
	ICP4/ICP0/ICP27	1.1X10 ²	0.9

Table 4. Relative CAT activities for the the wild type and mutated *c-myc* plasmids -101/+513-*c-Myc*-CAT and -101/+332-Δexon 1-*c-myc*-CAT in two separate cotransfection experiments. The CAT activity represents pmol chloramphenicol acetylated/mg protein/h. The CAT activity of wild type -101/+513-*c-myc*-CAT cotransfected with ICP4/ICP0 is set as 100%.

Fig.1. The maps of the wild type plasmid of +66-*c-myc*-CAT and the E2F-site mutated plasmid, *c-myc*+66 mtE2F-CAT.

Fig.2 Map and steps in constructing wild type plasmids -101/+513-c-myc-CAT and -101/+212-Δexon I-c-myc-CAT. The vector plasmid is pBR322.

Fig.3 Map and steps in constructing the subcloned plasmid **-101/+332-Δexon 1-c-myc -CAT**. The vector plasmid is pBR322. The fragments used as probes in electrophoretic mobility shift assay (EMSA) and in competition EMSA are also shown.

Fig.4. Diagram of the DNA probes prepared. * represents α -³²P-labeled deoxynucleotides. All three probes were labeled by Klenow fill-in method.

Fig.5. The restriction maps of the three subcloned *c-myc* plasmids, -101/+513-*c-myc*-CAT, -101/+212-Δ-exon 1-*c-myc*-CAT, and -101/+332-Δ-exon 1-*c-myc*-CAT. The deleted parts of the two deletion mutated *c-myc* plasmids are also shown in this figure.

Fig.6. The relative CAT activities (use the CAT activity of the wild-type c-myc plasmid super-infected with HSV-1 as 100%) of the three subclones, -101/+513-c-myc-CAT, -101/+212-Δexon 1-c-myc-CAT, and -101/+332-Δexon 1-c-myc-CAT in two separate HSV-1 infections. The CAT activity of 100% represents pmol chloramphenicol acetylated/mg protein/h.

Fig.7. The relative CAT activities in two separate cotransfection assays (use wild type c-myc plasmid cotransfected with ICP0/ICP4 as 100%) for subclones -101/+513-c-myc-CAT and -101/+332-Δexon 1-c-myc-CAT. The CAT activity of 100% represents pmol chloramphenicol acetylated/mg protein/h.

Fig.8. Results of the electrophoretic mobility shift assay (EMSA) and competition EMSA. The probes used in this experiment are 71 bp Ava II-Hind III fragment, 151 bp Ava II-Ava II fragment, and 266 bp Xho I-Ava II fragment, and a 164 bp fragment from VP5 promoter is used as the positive control. A non-radiolabeled oligonucleotide (LBS) containing YY1 binding site is used to compete with radiolabeled probes. All probes are bound with proteins in the EMSA, but only 71 bp probe is competed with LBS (lane 4). P represents free probe.

Fig.9. Result of the competition electrophoretic mobility shift assay using unlabeled oligonucleotide C₂ as a negative control. The probe used in this experiment is a 222 bp Ava II-Hind III fragment, spanning from the Ava II site at +332 to the Hind III site (see Fig.5). The result shows that the binding of protein (YY1) to this probe is competed by unlabeled oligonucleotide LBS, but it is not competed by unlabeled oligonucleotide C₂ (See lane 2, 4, 6, 8,). P represents the free probe. VP5 probe is used as a control.

Fig.10. Two separate agarose gels for the restriction analysis of the wild type *c-myc* plasmid -101/+513-*c-myc*-CAT and mutated *c-myc* plasmid -101/+212- Δ exon 1-*c-myc*-CAT. (A) Pst I digestion to generate linear form of these two subclones (lane 4 for wild type *c-myc* plasmid -101/+513-*c-myc*-CAT and lane 5 for mutated *c-myc* plasmid -101/+212- Δ exon 1-*c-myc*-CAT). Lane 3 is the Pst I digestion of -101/+212- Δ exon 1-*c-myc*-CAT (the size is shown in Table 4). The gel concentration is 0.9% and the running buffer is 0.5X TBE. (B) The digestion of these two subclones by different endonuclease enzymes (lanes 2 to 7 for -101/+212- Δ exon 1-*c-myc*-CAT and lanes 8 to 13 for -101/+513-*c-myc*-CAT). Lanes 3 and 9 show the linearization of these two plasmids by the Hind III digestion. Lanes 4, 5, 6, 10, 11, and 12 show the Pvu I/Hind III and EcoR I digestion to produce two bands. Lanes 7 and 13 show that the endonucleases Sma I and Cla I can not digest these two subclones. The gel concentration is 1% and the running buffer is 0.5X TBE.

Fig.11. The Not I-Hind III fragment is analyzed by the endonucleases Xho I, Pvu II, Alu I, Ava II, and Hinf I. The gel concentration is 4% and the running buffer is 0.5X TBE. The undigested Not I-Hind III fragment (the size of this fragment was previously proven to be correct) is used as size marker. The picture shows that this fragment is only digested by Xho I to generate a 40 bp smaller fragment (lane 2).

Fig.12. Photograph of the mini agarose gel for the restriction analysis of the subclone -101/+332- Δ exon 1-*c-myc*-CAT. The gel concentration is 1% and the running buffer is 0.5X TBE. Lane 1 is the uncut control. Lane 2 shows that this subclone can not be digested by Hind III. Lanes 3, 4, 6, and 8 show the linearization of this subclone by Xho I (lane 3), Pst I (lane 4), Hind III/Xho I (lane 6) and Hind III/Pst I. Lane 5 shows that the Xho I/Pst I digestion generates two bands (size shown in Table 4). Lane 10 shows the two bands generated by Xho I/Pst I digestion. Lane 12 is the digestion by EcoR I (clearer photo is shown in Fig.3). Lanes 5, 9, 11, and 13 are the digestion of wild-type *c-myc* plasmid by Hind III (lane 5), Hind III/Pst I (lane 9), Xho I/Pst I (lane 11), and EcoR I (lane 13).

λ 1 2 3 λ 4 5 6

Fig.13. Photograph of the agarose gel for the diagnostic restriction analysis of the subcloned plasmids - 101/+513-*c-myc*-CAT, 101/+212- Δ exon 1-*c-myc*-CAT, 101/+332- Δ exon 1-*c-myc*-CAT. The gel concentration is 1.2% and the running buffer is 0.5X TBE. Lanes 1, 2, and 3 show the EcoR I digestion of these three plasmids. Lanes 4, 5, and 6 show the EcoR I/Not I digestion of these three plasmids.

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