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# Lymphoid specific gene expression of the adenovirus early region 3 promoter is mediated by NF-κ B binding motifs

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**A primary site of infection by human adenoviruses is lymphoid cells. However, analysis of the viral control elements and the cellular factors that regulate adenoviral gene expression in lymphocytes has not been reported. The adenovirus early region 3 (E3) gene products are involved in the maintenance of viral persistence by complexing with the class I MHC antigens, thus preventing their cell surface expression with a resultant decrease in host immunologic destruction. To determine whether different cellular factors were involved in E3 regulation in lymphocytes as compared with HeLa cells, both DNA binding and transfection analysis with the E3 promoter in both cell types were performed. These studies detected two novel domains referred to as L1 and L2 with a variety of lymphoid but not HeLa extracts. Each of these domains possessed strong homology to motifs previously found to bind the cellular factor NF-κ B. Transfections of E3 constructs linked to the chloramphenicol acetyltransferase gene revealed that mutagenesis of the distal NF-κ B motif (L2) had minimal effects on promoter expression in HeLa cells, but resulted in dramatic decreases in expression by lymphoid cells. In contrast, mutagenesis of proximal NF-κ B motif (L1) had minimal effects on gene expression in both HeLa cells and lymphoid cells but resulted in a small, but reproducible, increase in gene expression in lymphoid cells when coupled to the L2 mutation. Reversing the position and subsequent mutagenesis of the L1 and L2 domains indicated that the primary sequence of these motifs rather than their position in the E3 promoter was critical for regulating gene expression. These results demonstrate that the E3 promoter contains additional regulatory domains which function to modulate E3 gene expression in a tissue specific manner.**

*Key words:* Adenovirus early promoter/E1A/NF-κ B

## Introduction

Human adenoviruses comprise a number of different serotypes that infect a variety of human cell lines. A natural host for these viruses *in vivo* is lymphoid cells (Horvath *et al.*, 1986; Lavery *et al.*, 1987; Horvath and Weber, 1988). Infection of human peripheral blood lymphocytes usually leads to a low level persistent infection rather than a lytic infection as found in tissue culture with HeLa cells (Horvath *et al.*, 1986; Horvath and Weber, 1988). However, studies of adenoviral gene expression and growth patterns in a variety of T and B lymphoid cell lines indicated characteristic patterns of gene expression which varied depending on the individual cell lines (Lavery *et al.*, 1987). The kinetics of gene expression and growth in most lymphoid cell lines were prolonged compared with expression in HeLa cells (Lavery *et al.* 1987). This suggests that cell-type specific factors may be involved in the regulation of adenovirus early promoters.

A variety of cellular transcription factors have been identified which modulate tissue specific expression in lymphocytes. One of the best studied of these factors is NF-κ B (Sen and Baltimore, 1986a,b; Atchison and Perry, 1987; Kawakami *et al.*, 1988; Lenardo *et al.*, 1987; Nelson *et al.*, 1988; Pierce *et al.*, 1988; Schreck and Baeuerle, 1990). This protein was initially found to be produced in high levels in mature B lymphocytes, but not in immature B cells (Sen and Baltimore, 1986a,b). However, high levels of this protein have also been found to be present in mature T lymphocytes (Wu *et al.*, 1988). This protein was subsequently found to be present in non-lymphoid cell lines where it is presumably complexed in the cytoplasm with a protein known as IkB (Baeuerle and Baltimore, 1988a,b). Upon treatment with phorbol esters, NF-κ B is translocated to the nucleus where it is capable of inducing gene expression (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a,b). Nf-κ B binds to an 11 bp sequence GGGGACTTTCC which is found in a number of cellular and viral regulatory regions (Boshart *et al.*, 1985; Sen and Baltimore, 1986a,b; Davidson *et al.*, 1986; Ondek *et al.*, 1987; Schirm *et al.*, 1987; Nabel and Baltimore, 1987; Wu *et al.*, 1988a). In addition, a variety of other nuclear proteins have been demonstrated to bind to this motif (Yano *et al.*, 1987; Baldwin and Sharp, 1987, 1988; Clark *et al.*, 1988; Wu *et al.*, 1988a,b).

Adenovirus early promoter gene expression has been extensively studied in HeLa cells (reviewed in Berk, 1986). These promoters are strongly induced by the E1A protein (Berk *et al.*, 1979; Jones and Shenk, 1979). E1A is capable of inducing transcription through a number of different transcription factor binding sites in these promoters. One of the best studied of the early promoters is the early region 3 (E3) promoter (Garcia *et al.*, 1987; Hurst and Jones, 1987; Kornuc *et al.*, 1990). Four regions of the E3 promoter have been identified which serve as binding sites for cellular factors, NF1, AP1, CREB/ATF and TF IID (Garcia *et al.*, 1987; Hurst and Jones, 1987; Kornuc *et al.*, 1990). Mutagenesis of these binding sites indicated that both the AP1 and CREB/ATF sites were critical for both basal and E1A/E1B induced gene expression (Garcia *et al.*, 1987; Kornuc *et al.*, 1990). The E3 promoter regulates the gene expression of a 19 kd glycoprotein which functions in inhibiting the expression of class I MHC antigens (Kvist *et al.*, 1978; Persson *et al.*, 1979, 1980; Signas *et al.*, 1982; Kampe *et al.*, 1983; Andersson *et al.*, 1985; Burgert and

Kvist, 1985; Paabo *et al.*, 1986; Burgert *et al.* 1987). The 19 kd gene product complexes with class I antigens preventing their expression on the cell surface (Kvist *et al.*, 1978; Signas *et al.*, 1982; Kampe *et al.*, 1983; Burgert and Kvist, 1985; Burgert *et al.*, 1987). Thus, E3 is important in decreasing host immunologic response to viral antigens.

To determine whether the pattern of regulation of the adenovirus early region 3 promoter was the same in lymphoid and HeLa cells, DNase I footprinting and gel retardation with these cellular extracts was performed. Two binding sites for Nf-$\varkappa$ B known as L1 and L2, which differ in both their binding affinity and their effects on E3 gene expression, were identified in lymphoid cells. Oligonucleotide-directed mutagenesis of these sites was performed and the mutant promoters were fused to the chloramphenicol acetyltransferase (CAT) gene for analysis of gene expression in both lymphoid and HeLa cells in the presence and absence of the E1A/E1B proteins. The role of the position of the L1 and L2 domains in regulating E3 gene expression was also determined. The results of these studies have defined novel E3 promoter regulatory domains required for expression in lymphoid cells.

## Results

### *Lymphoid specific binding domains in the adenovirus E3 promoters*

Partially purified extracts were prepared from either HeLa cells or a variety of different lymphoid cells including the T cell lines, Jurkat (Gillis and Watson, 1980), HUT 78 (Gootenberg *et al.*, 1981) and a closely related derivative of this line, H9 (Popovic *et al.*, 1984), or the B cell line Raji. DNase I footprinting of the E3 promoter revealed six binding sites with lymphoid extract (Figure 1A). Four of these binding domains, including the TATA, CREB/ATF and NF1 sites, were previously identified using HeLa extract (Garcia *et al.*, 1987; Hurst and Jones, 1987; Kornuc *et al.*, 1990) (Figure 1B). Two previously unreported binding domains designated as L1 and L2, were noted in DNase I footprinting with all of the above mentioned lymphoid extracts. This is illustrated in Figure 1 with the following cellular extracts: A (H9), C (Raji) and D (HUT 78).

The sequence of the E3 promoter extending from $-237$ to $+1$ is shown and the position of the binding motifs are indicated (Figure 2A). Examination of these sequences indicate that one of these lymphoid specific sites between $-137$ and $-155$ known as L2 contains complete homology to an NF-$\varkappa$ B binding site found in the immunoglobulin kappa (Sen and Baltimore, 1986a,b), HIV (Nabel and Baltimore, 1987; Wu *et al.*, 1988a; Gaynor *et al.*, 1988), SV 40 (Davidson *et al.*, 1986; Nomiyama *et al.*, 1987; Ondek *et al.*, 1987; Schirm *et al.*, 1987; Kanno *et al.*, 1989; Macchi *et al.*, 1989; and CMV (Boshart *et al.*, 1985) enhancers. The other site known as L1 between $-113$ and $-134$ contains a seven out of ten nucleotide match with the L2 binding motif (Figure 2B). Variations in NF-$\varkappa$ B binding motifs in a variety of cellular and viral promoter elements and their alignment with the L1 and L2 domains are illustrated in Figure 2B (Boshart *et al.*, 1985; Davidson *et al.*, 1987; Nabel and Baltimore, 1987; Schirm *et al.*, 1987; Ballard *et al.*, 1988; Bohnlein *et al.*, 1988; Cross *et al.*, 1989; Schreck and Baeuerle, 1990).

Oligonucleotide mutagenesis of the L1 and L2 binding domains, both individually and together, was performed. The nucleotides changes in each of these constructs are indicated in Figure 2A. Each of these constructs was used in DNase I footprinting with either lymphoid (Figure 1A, C and D) or HeLa extract (Figure 1B). Mutation of either the L1, L2 or L1/L2 binding domains eliminated protection of their respective domains with lymphoid extract, but did not alter the protection of the remaining E3 binding domains observed with either lymphoid or HeLa extract (Figure 1A, C and D). Thus, two domains responsible for lymphoid specific binding domains exist in the E3 promoter with extracts prepared from either T or B lymphocytes.

### *NF-$\varkappa$ B binds to both L1 and L2 domains with different affinities*

The presence of two potential NF-$\varkappa$ B motifs in the E3 promoter was further investigated by gel retardation analysis. Double stranded oligonucleotides corresponding to each of these lymphoid specific binding domains were end-labeled to the same relative specific activity with $\gamma$ATP and used in gel retardation analysis with either lymphoid or HeLa extract. Oligonucleotides corresponding to either the L1 or L2 binding domain resulted in two gel retarded species when using lymphoid extracts (Figure 3A, lanes 2 and 7). Competition analysis indicated that the slower mobility complex was specific to the L1 and L2 binding sites while the faster mobility complex was non-specific. Similar non-specific products using NF-$\varkappa$ B fragments in gel retardation analysis have previously been reported (Macchi *et al.*, 1989). In contrast to the results observed with lymphoid extract, gel retardation analyses of these probes with HeLa extract resulted in only the faster mobility non-specific species (Figure 3A, lanes 11 and 12). This suggested that tissue specific proteins of similar mobility bound to these motifs with lymphoid but not HeLa extracts.

Competition studies using unlabeled double stranded oligonucleotides complementary to the L1 and L2 binding sites were performed to determine if the lymphoid specific binding protein had similar affinity to both sites (Figure 3A). In addition, double stranded oligonucleotides corresponding to the E3 NF1 binding site were also used as a competitor. As shown, competition with either a 35-fold excess of cold L1 or L2 oligonucleotides was capable of markedly competing the slower mobility L1 gel retarded species (Figure 3A, lanes 3 and 4). Competition with a 35-fold excess of NF1 oligonucleotides did not result in significant competition of the slower mobility complex (Figure 3A, lane 5). Likewise, competition with either a 35-fold excess of L1 or L2 oligonucleotides was capable of markedly competing the L2 gel retarded species (Figure 3A, lanes 8 and 9). NF1 oligonucleotides did not result in significant competition of the L2 specific complex (Figure 3A, lane 10). These results suggest that the L1 and L2 sites bound the same lymphoid specific protein.

Since the intensity of the gel retarded species was stronger with the L2 oligonucleotides as compared with the L1 oligonucleotides, competition of the L2 gel retarded species was performed with carefully quantitated amounts of either unlabeled L1, L2 or NF1 oligonucleotides. As shown in Figure 3B, the L2 gel retarded species was completely competed between a 15 to 30-fold molar excess of L2 oligonucleotides (lanes 9 and 10) whereas these same concentrations of L1 oligonucleotides did not result in complete

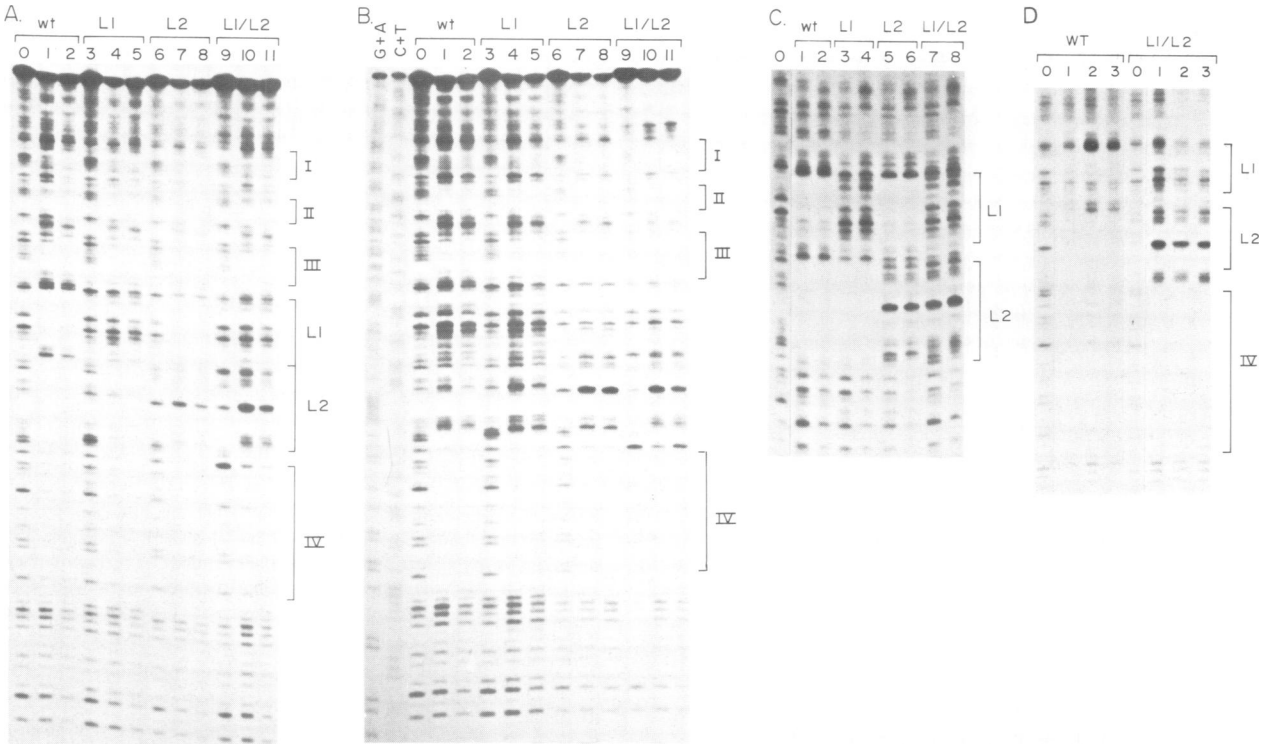
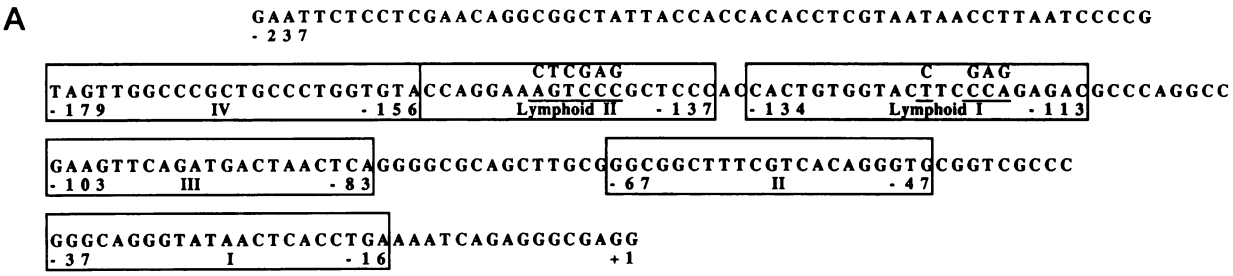



**Fig. 1.** DNase I footprinting of the E3 promoter with HeLa and lymphoid extracts. The coding strand of either the wild-type E3 promoter, or mutations of lymphoid domain 1 (L1), lymphoid domain 2 (L2), or both of these domains (L1/L2) were used in DNase I footprinting with H9 (A), HeLa (B), or Raji (C) or HUT 78 (D) extract. (**A** and **B**) Lanes 0, 3, 6 and 9 contain no extract; lanes 1, 4, 7 and 10 contain 50 $\mu$g of extract; and lanes 2, 5, 8 and 11 contain 100 $\mu$g of extract. (**C**) Lane 0 contains no extract; lanes 1, 3, 5 and 7 contain 50 $\mu$g of extract; and lanes 2, 4, 6 and 8 contain 100 $\mu$g of extract. (**D**) Lane 0 contains no extract; lane 1 contains 50 $\mu$g; lane 2, 100 $\mu$g; and lane 3, 150 $\mu$g of extract respectively. Maxam–Gilbert sequencing of the E3 wild-type promoter shown in (B) was adjacent to panel (A) in the original gel for use in demarcating binding domains.




**B** **NF-kappa B binding domains**

| | |
|---|---|
| Lymphoid 1 | G G G A A G T A C C |
| Lymphoid 2 | G G G A C T T T C C |
| Kappa enhancer, SV 40, HIV, CMV | G G G A C T T T C C |
| IL-2R | G G G A A T C T C C |
| $\beta$-IFN | G G G A A A T T C C |
| IL-2 | G G G A T T T C A C |
| GM-CSF-1 | G G G A A C T A C C |
| GM-CSF-2 | T G G A A T C T C C |
| **Consensus** | G G G A N N Y Y C C |

**Fig. 2.** Schematic diagram of the E3 promoter. (**A**) The nucleotide sequence of the E3 promoter extending from −237 to +1 is indicated. The positions of the binding domains protected with both lymphoid and HeLa extracts are indicated (I–IV) as are the positions of the two domains protected with only lymphoid extracts (Lymphoid I and II). The base pairs altered by oligonucleotide-directed mutagenesis are also indicated. (**B**) The L1 and the L2 binding domains are aligned with NF-$\kappa$ B binding motifs in regulatory elements in the immunoglobulin $\kappa$, SV40, HIV, CMV, IL-2 receptor, $\beta$-interferon, IL-2 and GM-CSF enhancers.

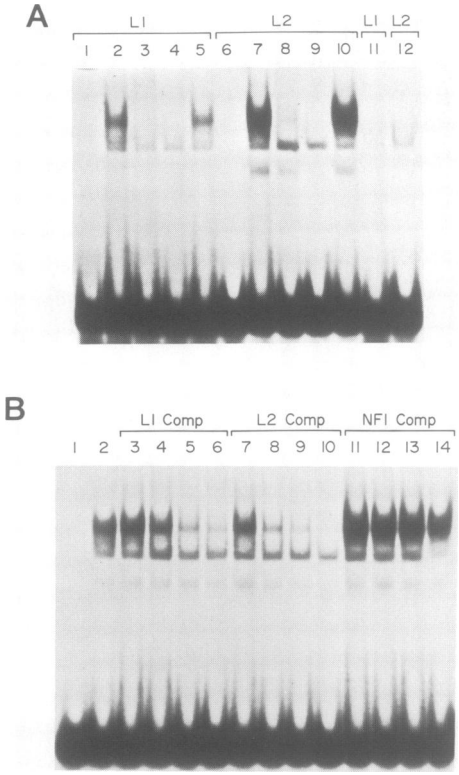



**Fig. 3.** Gel retardation with HeLa and lymphoid extract. (**A**) L1 (lanes 1–4 and 11) and L2 (lanes 6–10 and 12) oligonucleotides were end-labeled with γATP and used in gel retardation with 3 μg of nuclear extract prepared from either HUT 78 (lanes 1–10) or HeLa (lanes 11 and 12) cells. Lanes 1 and 6 contain probe alone, lanes 2 and 7 contain extract with no competition, lanes 3 and 8 contain a 35-fold molar excess of L1 oligonucleotides, lanes 4 and 9 contain a 35-fold molar excess of L2 oligonucleotides, and lanes 5 and 10 contain a 35-fold molar excess of NF1 oligonucleotides. Lanes 11 and 12 contain either L1 or L2 oligonucleotides in the presence of 3 μg of HeLa extract. (**B**) L2 oligonucleotides in the absence of extract (lane 1), or in the presence of HUT 78 extract (lane 2) alone or in the presence of a 2, 6, 15 or 30-fold excess of cold L1 oligonucleotides (lanes 3–6) or similar concentrations of L2 (lanes 7–10) or NF1 oligonucleotides (lanes 11–14).

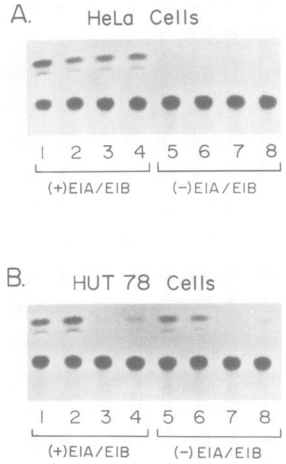



**Fig. 4.** E3 gene expression in HeLa and HUT 78 cells. The E3 promoter containing either wild-type (lanes 1 and 5) or containing mutations of L1 (lanes 2 and 6), L2 (lanes 3 and 7), or both L1 and L2 (lanes 4 and 8) were assayed in either the presence of wild-type adenovirus (lanes 1–4) or dl 434 (lanes 5–8). These constructs were transfected into either HeLa (**A**) or HUT 78 (**B**) and CAT assays performed at 36 h post-transfection.

**Table I.** Gene expression of the E3 promoter in HeLa and HUT 78 cells

| constructs | CAT conversion (%) | |
|---|---|---|
| | (+)E1A/E1B | (−)E1A/E1B |
| HeLa cells | | |
| wild-type | 32.6 ± 8.2 | 1.3 ± 0.4 |
| L1 | 12.8 ± 4.0 | 0.52 ± 0.1 |
| L2 | 18.4 ± 3.8 | 0.52 ± 0.1 |
| L1/L2 | 14.6 ± 2.6 | 0.41 ± 0.03 |
| HUT 78 cells | | |
| wild-type | 34.6 ± 9.5 | 16.0 ± 5.4 |
| L1 | 44.7 ± 10.8 | 25.8 ± 8.9 |
| L2 | 0.94 ± 0.4 | 0.32 ± 0.2 |
| L1/L2 | 3.0 ± 0.9 | 1.0 ± 0.6 |

CAT conversion was determined by scintillation counting of acetylated and unacetylated [$^{14}$C]chloramphenicol for E3 CAT constructs transfected into HeLa or HUT 78 cells in the presence of either wild-type adenovirus [(+) E1A/E1B] or dl 434 [(−) E1A/E1B]. Values and standard deviations were calculated from three independent experiments.

competition of this species. Densitometry of these gel retardations at either a 6 or 15-fold molar excess of competitor indicated that the L2 oligonucleotides were a 3 to 4-fold better competitor than the L1 oligonucleotides (Figure 3B, lanes 4 and 5 as compared with lanes 8 and 9). NF1 oligonucleotides did not result in significant competition at these same concentrations (Figure 3B). These results suggest that the L2 region, which contains a consensus NF-κ B binding sequence, has a higher binding affinity for the lymphoid specific factor than the divergent L1 region.

### *L2 is required for efficient E3 gene expression in lymphocytes*

Mutations in either domains L1, L2 or L1/L2 were substituted into the context of the wild-type E3 promoter. These constructs were placed upstream of the chloramphenicol acetyltransferase (CAT) gene. Each of these constructs or the wild-type E3 CAT construct were transfected into either the T lymphocytic leukemia cell line, HUT 78, or HeLa cells in the presence of either wild-type adenovirus or d1 434, an E1A/E1B deletion mutant. Transfections were harvested at between 30–36 h post-transfection and CAT activity determined. Each of these E3 CAT constructs was strongly induced by the E1A/E1B proteins in HeLa cells (Figure 4A, lanes 1–4). Mutations of the L1, L2 or L1/L2 domains (Figure 4A, lanes 2–4) resulted in ~2-fold decreases in gene expression as compared with the wild-type construct (Figure 4A, lane 1). Table I shows quantitation of CAT assays from three independent experiments on HeLa cells. These results suggest that the L1 and L2 binding domains have minimal effects on E3 gene expression on HeLa cells consistent with the fact that HeLa cellular proteins were not detected binding to these motifs.

To determine whether E1A/E1B was capable of activating the E3 promoter to high levels in lymphocytes, we first tested whether adenovirus could infect lymphocytes and produce high levels of E1A mRNA. Cytoplasmic RNA was harvested

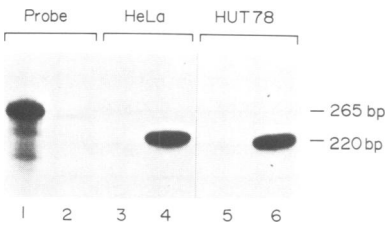



**Fig 5.** RNase protection of E1A mRNA. Either wild-type adenovirus or dl 434 at an m.o.i. of 20 was used to infect HeLa or HUT 78 cells and cytoplasmic RNA was harvested at 36 h. RNase protection with an internally labeled 265 bp probe derived from a *SmaI–Xba* E1A fragment cloned into pGEM 3 was used. Lane 1 contains probe along, lane 2 contains probe and T2 ribonuclease, lanes 3 and 4 contain HeLa RNA from infection with either dl 434 (lane 3) or wild-type adenovirus (lane 4), and lanes 5 and 6 contain HUT 78 RNA from infection with either dl 434 (lane 5) or wild-type adenovirus (lane 6). The positions of the 265 bp E1A probe and the 220 E1A mRNA protected species are indicated.

at 36 h post-infection from HUT 78 cells infected with either wild-type or dl 434 virus and RNase protection analysis performed with an E1A specific probe (Figure 5). HeLa cells were also infected with these viruses and cytoplasmic RNA harvested for RNase protection. As shown in Figure 5, high levels of E1A 13S mRNA was found in both HeLa and HUT 78 cells infected with wild-type adenovirus (lanes 4 and 6), but not dl 434 (lanes 3 and 5) as indicated by the presence of the 220 bp generated E1A specific species. These results indicate that lymphocytes can be infected with adenovirus and produce high levels of E1A mRNA after infection.

Electroporation of either the E3 wild-type, L1, L2 or L1/L2 constructs into virus infected HUT 78 cells was then performed and CAT activity determined. As shown in Figure 4B, the wild-type E3 construct gave high levels of gene expression in the absence of E1A/E1B. Mutations of the L2 domain (Figure 4B, lane 7) which contains a consensus NF-$\varkappa$ B binding sequence resulted in severe decreases in gene expression while mutations of the L1 domain (Figure 4B, lane 6) which diverges from the consensus NF-$\varkappa$ B binding sequence resulted in minimal effects on E3 gene expression. Combining mutations of both L1 and L2 domains (Figure 4B, lane 8) resulted in a slight but reproducible increase in gene expression over that of the L2 mutation alone. These constructs yielded the same pattern of gene expression in the presence of the E1A/E1B proteins except that gene expression of each construct was induced ~2-fold (Figure 4B, lanes 1–4). Quantitation of these CAT assays is shown in Table I.

### *L2 regulation of the E3 promoter is independent of positional effects*

We also wished to determine whether the position of the L1 and L2 domains in the E3 promoter were critical in activation of the E3 promoter. As shown in Figure 6A, oligonucleotides corresponding to the L1 and L2 domains were synthesized that reversed the positions of these domains in the E3 promoter. The wild-type construct designated WT′ contains the L2 sequence in the position of Lymphoid Domain 1 and the L1 sequence in the position of Lymphoid Domain 2. Construct L1′ mutates the L2 sequences in Lymphoid Domain 1, construct L2′ mutates the L1 sequence in Lymphoid Domain 2 and construct L12′ mutates both L1 and L2 sequences. Each of these constructs in the context of the wild-type E3 promoter was fused to the CAT gene and introduced into both HeLa and HUT 78 cells.

In the electroporation studies into HUT 78 cells shown in Figure 6B, expression from the WT′ construct (lane 2) was similar to that of the parental E3 construct (lane 1). Mutation of the L2 sequences in Lymphoid Domain (L1′) resulted in marked decreases in E3 gene expression (Figure 6B, lane 3) while mutation of the L1 sequences in Lymphoid Domain 2 (L2′) resulted in minimal changes in E3 gene expression (Figure 6B, lane 4). Mutation of both the L1 and L2 sequences (L12′) resulted in severe decreases in E3 gene expression (Figure 6B, lane 5). However, in several other experiments there was a 2- to 3-fold increase of E3 gene expression with the L12′ as compared with the L1′ construct, again indicating a potential negative regulatory role for the L1 domain. All constructs exhibited low expression in uninfected HeLa cells but their expression was markedly induced in adenovirus infected HeLa cells (data not shown). These results are consistent with a significant position independent role for the L1 and L2 sequences in regulating E3 gene expression in lymphoid cells.

## Discussion

Adenovirus infection of lymphocytes is a primary site of viral infection in humans. The adenovirus genome and its gene products can be detected in human peripheral blood lymphocytes (Horvath *et al.*, 1986; Horvath and Weber, 1988). However, the mechanisms by which these adenovirus infected lymphocytes escape host immunologic destruction are unknown. In this study, we determined the gene expression of the early region 3 promoter in both HeLa cells and the lymphoid cell line HUT 78. E3 expression in lymphoid cells was dependent on the presence of two NF-$\varkappa$ B motifs in the E3 promoter. Both of these sites served as binding sites for cellular factors found in a variety of lymphoid, but not HeLa extracts. These motifs were not critical for expression in HeLa cells. E1A induction of the E3 promoter was minimal in lymphocytes as compared with HeLa cells, although the same overall expression of the E3 promoter was seen in wild-type adenovirus infected HeLa cells and lymphocytes. These results suggest that in lymphoid cells, the NF-$\varkappa$ B motifs diminish the need for E1A to maintain high levels of E3 gene expression.

What role does this high level of E3 promoter gene expression in lymphocytes have on the biology of adenovirus infection? The E3 gene has been shown to encode a 19 kd glycoprotein which is capable of complexing with class I MHC antigens (Kvist *et al.*, 1978; Persson *et al.*, 1979, 1980; Signas *et al.*, 1982; Kampe *et al.*, 1983; Andersson *et al.*, 1985; Burgert and Kvist, 1985; Paabo *et al.*, 1986; Burgert *et al.*, 1987). Inhibition of class I MHC expression on the surface of cells is associated with ability of cells to escape immunologic surveillance. Adenovirus has been shown to infect lymphocytes without establishing a lytic infection. Thus, the high level of expression of the 19 kd glycoprotein may help to maintain viral persistence in lymphocytes (Persson *et al.*, 1979, 1989; Signas *et al.*, 1982).

Differences in the primary sequence of the NF-$\varkappa$ B motifs may alter the ability of these factors to modulate gene expression. Mutations of the L2 motif which contained a concensus NF-$\varkappa$ B binding motif resulted in severe decreases in E3

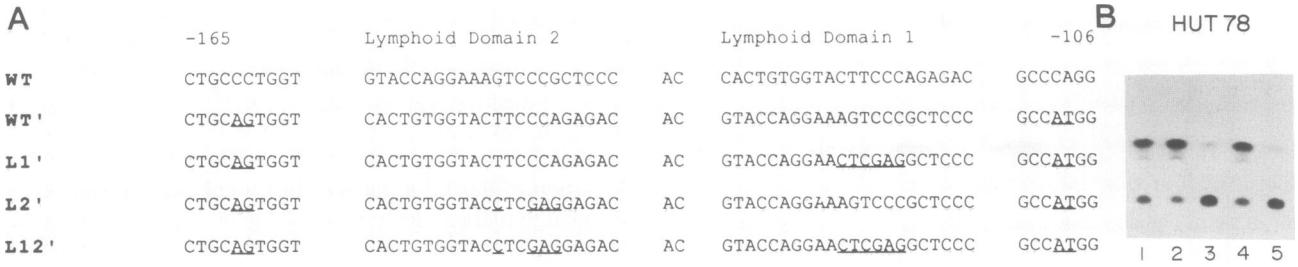



**Fig 6.** Reversal of the L1 and L2 domains in the E3 promoter. (**A**) Sequences from −165 to −108 in the E3 promoter. The wild-type E3 promoter (WT) and reversal of the L1 and L2 domains (WT′) are indicated, as are mutations (L1′, L2′, L12′) of these domains. The underlined nucleotides indicate changes in the sequence relative to the wild-type promoter. The regions corresponding to the lymphoid-specific elements are offset to allow comparison of the transversed sequences. (**B**) The wild-type E3 promoter (lane 1), the inverted L1/L2 domains in the E3 promoter (WT) (lane 2), or this latter promoter containing mutations of the L2 sequences (L1′) (lane 3), L1 sequences (L2′) (lane 4), or L1 and L2 sequences (L12′) were electroporated into HUT 78 cells and CAT assays performed at 30 h post-electroporation.

gene expression in lymphoid cells. This motif had a higher binding affinity than the L1 motif. This latter site contained 7 out of 10 bp homology with the NF-κ B motif. However, mutations of this site in the E3 promoter resulted in a slight, but reproducible, increase in E3 gene expression when coupled to the L2 mutation suggesting a potential negative regulatory role. Reversal of these motifs in the E3 promoter indicated that the effect of the L1 and L2 sequences on regulating E3 gene expression was dependent on their primary sequence rather than their position. It is also important to note an additional constraint on the E3 promoter, in that it is contained within the coding region the adenovirus 100 kd late protein (Lewis *et al.*, 1975). Thus, limitations may exist on the primary nucleotide sequence of the E3 regulatory elements due to restrictions imposed by conservation of the amino acid composition of this late protein.

The ability of NF-κ B motifs to modulate viral gene expression has been reported with other viral promoters including SV40 (Davidson *et al.*, 1986; Nomiyama *et al.*, 1987; Ondek *et al.*, 1987; Schirm *et al.*, 1987; Kanno *et al.*, 1989; Macchi *et al.*, 1989), cytomegalovirus (Boshart *et al.*, 1985), and the human immunodeficiency virus (Nabel and Baltimore, 1987; Wu *et al.*, 1988a,b; Gaynor *et al.*, 1988). A single NF-κ B motif from the SV40 promoter has been shown to have little effect on heterologous promoter activation (Kanno *et al.*, 1989; Macchi *et al.*, 1989). However, multimerizing these motifs resulted in enhancer function in a wide variety of cells (Nomiyama *et al.*, 1987; Ondek *et al.*, 1987; Schirm *et al.*, 1987; Kanno *et al.*, 1989; Macchi *et al.*, 1989). This suggested that proteins present in a wide variety of cells including EBP1 (Clark *et al.*, 1988; Wu *et al.*, 1988b), H2TF1 (Baldwin and Sharp, 1987, 1988), and KBF1 (Yano *et al.*, 1987), can bind to these motifs and activate gene expression. This was confirmed by gel retardation and methylation interference analysis indicating that both H2TF1 and NF-κ B, which bound to the SV40 κ B motif, were present in a wide variety of lymphoid and non-lymphoid cells (Macchi *et al.*, 1989).

Our results with the E3 promoter and previous results with the immunoglobulin κ enhancer suggest that NF-κ B binding is restricted to lymphoid cells in the absence of substances such as phorbol esters (Sen and Baltimore, 1986a,b; Atchison and Perry, 1987; Lenardo *et al.*, 1987; Nelson *et al.*, 1988; Pierce *et al.*, 1988; Wirth and Baltimore, 1988). However, using oligonucleotides from the HIV enhancer which contains two NF-κ B motifs, we have detected binding to these motifs with HeLa extract by gel retardation and DNase I footprinting (Wu *et al.*, 1988a; Gaynor *et al.*, 1988). These results suggest that cellular factors from HeLa cells are capable of binding to κ B motifs, but this binding may also depend on the nucleotide sequence of regions flanking the NF-κ B motif. Our results indicate the mutation of the L1 and L2 domains did result in small but reproducible decreases in E3 gene expression in HeLa cells, indicating that factors present in HeLa cells can likely interact with these binding domains. However, we have been unable to detect binding to these motifs with HeLa extract even in concentrations 5- to 10-fold higher than with lymphoid extracts.

Domains other than NF-κ B have also been shown to be important in E3 gene expression (Garcia *et al.*, 1987; Kornuc *et al.*, 1990). Both CREB and AP1 binding sites were found to be important for gene expression on HeLa cells (Garcia *et al.*, 1987; Kornuc *et al.*, 1990). These sites were also critical for gene expression in HUT78 cells. E1A was critical for activation of the E3 promoter in HeLa cells but not in lymphoid cells. These results suggest that lymphoid specific regions may lessen the requirement for E1A to obtain high levels of E3 gene expression. Our results are consistent with a role for these lymphoid specific domains in the maintenance of high levels of E3 gene expression. Such expression may be one determinant of the maintenance of adenoviral persistence in lymphoid cells.

## Materials and methods

### *Cells and virus stocks*

Wild-type Ad5 was grown in HeLa suspension cultures maintained in MEM + 5% newborn calf serum, and titers were determined by plaque formation on 293 cells (Graham *et al.*, 1977). Ad5 dl 434 (Grodzicker and Klessig, 1980) was grown on monolayers of the complementing 293 cell line and titers were determined by plaque assay on this cell line. HeLa monolayers were maintained in Iscovers media with 5% newborn calf serum containing penicillin and streptomycin. HUT 78 cells (Gootenberg *et al.*, 1981), H9 (Popovic *et al.*, 1984), Jurkat (Gillis and Watson, 1981), and Raji cells were maintained in Iscoves media with 10% fetal calf serum at a density of $10^5-10^6$ cells/ml.

### *Transfections and CAT assays*

HeLa plates were split on the day prior to transfection so that each 100 mm plate was 50−70% confluent at the time of transfection. Cells were infected with either wild-type adenovirus or dl 434 at an m.o.i. of 20. At 1 h post-infection, fresh media with cytosine arabinoside (ara-C) (20 μg/ml) was added. At 4 h post-infection, media was removed and 5 μg of each E3 CAT construct was transfected onto identically prepared plates. Fresh media containing ara-C was added, the media changed 12 h later and the plates harvested at 32 h post-transfections for CAT analysis as described (Gorman *et al.*, 1982).

### Plasmids

An *Eco*RI−*Sac*I (−236 to +31) fragment containing the E3 promoter was cloned into *Sma*I/*Sac*I mp18 (Garcia *et al.*, 1987). Oligonucleotides complementary to regions L1 and L2 containing specified point mutations were synthesized (courtesy of T.Sutherland, Molecular Biology Institute and Jonsson Cancer Centre) and used for oligonucleotide-directed mutagenesis according to the manufacturer's protocol (Amersham). Each mutant was verified by dideoxy sequencing. The RF DNA from each isolate was prepared, the E3 upstream region was isolated and cloned into a Rous sarcoma virus (RSV) CAT derivative. The fragments were also cloned into pUC19 for use in DNase I footprinting (Galas and Schmitz, 1978).

### Plasmid constructions

An *Eco*RI−*Sac*I (−236 to +31) fragment from the E3 promoter was cloned into mp 18 (Garcia *et al.*, 1987). Oligonucleotides complementary to regions L1 or L2 containing the indicated point mutations were synthesized (courtesy of T.Sutherland, MBI and Jonsson Cancer Center) and used for oligonucleotide-directed mutagenesis according to the manufacturer's recommended protocol (Amersham). All mutations were verified by dideoxy-sequencing. A *Bam*HI−*Sac*I fragment was prepared from the RF DNA of each mutant and ligated into *Bam*HI−*Sac*I pJGFCAT19, a derivative of RSV CAT, for transfection analysis and into pUC19 for DNase I protection studies.

For construction of the lymphoid domain transversion plasmids WT′, L1′, L2′ and L12′, site-directed mutagenesis was used to introduce an *Nco*I site at position −108 of the E3 −236/+31 fragment from a mutation, MIV, used in an earlier study (Kornuc *et al.*, 1988) which contains a *Pst*I site in the MIV mutation at position −165. A *Pst*I−*Sac*I fragment from the RF DNA of the MIV/*Nco*I$^+$ mutant construct was isolated and cloned into pUC19. This plasmid served as a shuttle vector for construction of the transversion plasmids. To construct the transversion plasmids, complementary oligonucleotides for each of the constructs were synthesized with *Pst*I and *Nco*I overhangs, annealed and ligated into a *Pst*I/*Nco*I shuttle vector. Verification of the plasmids was performed by dideoxy sequencing. *Pst*I−*Sac*I fragments from each of the transversion constructs, the shuttle vector and the parent MIV plasmid were ligated into *Pst*I/*Sac*I pJGFCAT19 for use in transfection analysis.

### RNase protection

Cytoplasmic RNA was prepared from either HeLa or HUT 78 cells ($10^8$ cells) at 36 h post-infection with either dl 434 or Ad5 at an m.o.i. of 20 as described (Garcia *et al.*, 1987). A *Sma*I−*Xba*I from an Ad5 E1A 13S cDNA was cloned into pGEM 3, linearized with *Eco*RI and SP6 polymerase and α-UTP was used to generate a 265 bp internally labeled RNA probe for use in an RNase protection assay. Protected bands of 220 bp indicated the 13S form of E1A produced in wild-type adenovirus infected cells.

### Electroporation

HUT 78 cells ($3 \times 10^8$ cells) were infected with either wild-type Ad5 or dl 434 at an m.o.i. of 20 for 1 h in 3 ml media and then diluted to 30 ml. After 4 h of the infection, cells were spun at 1K for 5 min and washed with 5 ml of serum-free Iscoves media. Cells were resuspended at a concentration of $8 \times 10^7$ cells/ml in electroporation media (Iscoves media and 20% FCS). $2 \times 10^7$ cells were incubated with 10 μg of DNA on ice for 10 min. The cell/DNA mixture was then electroporated at 1180 μ Farads and 250 volts using a BRL electroporator. Cells were suspended at $3 \times 10^6$ cells/ml and harvested at 30 h post-electroporation. The presence or absence of *ara*-C to block viral replication did not alter E3 gene expression on HUT 78 cells.

### DNase I footprinting

HeLa, HUT 78, Jurkat, H9 or Raji nuclear extract (Dignam *et al.*, 1983) was loaded onto a heparin−agarose column, and eluted with buffer containing 0.4 M KCl. The extract was dialyzed into 20 mM Tris pH 7.9−100 mM KCl−0.2 mM DTT−0.2 mM PMSF and used in DNase I footprinting assays. DNase I footprinting studies were done with a clone containing the E3 upstream region (−236/+31) either wild-type or containing L1, L2 or L1/L2 mutations cloned into *Sma*I/*Sac*I pUC19. The coding strand was labeled with T4 kinase and [$\gamma$-$^{36}$P]ATP at the *Hin*dIII site in the polylinker and a 400 bp fragment was isolated by electroelution after cutting with *Pvu*II. Fragments for footprinting each of the point mutants were labeled and isolated in the same manner. From 1 to 5 ng of end-labeled probe was added to each 50 μl reaction mix with 100 μg of HeLa extract, poly(dI-dC) (3 μg), and final concentrations of 10 mM Tris pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT and 58 (vol/vol) glycerol. The DNA and extract were allowed to bind for 30 min at room temperature, the reaction volume was increased to 100 μl, and final concentrations of DNase I (0.4−3.0 μg/ml), 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ were added. The reaction was stopped after 30 s with phenol−chloroform, ethanol precipitated, and loaded onto a 10% polyacrylamide−8 M urea sequencing gel. All gels were then subjected to autoradiography.

### Gel retardations

HeLa or HUT 78 nuclear extracts were used in gel retardations with oligonucleotides complementary to the NF1, L1 and L2 sites. Double stranded oligonucleotides corresponding to the NF1 (5′-TAGTTGG CCCGCTGCCCTGGTGTA-3′), L1 (5′-CGCACCACTGTGGTACTT CCCAGAGAGACG-3′) and L2 (5′-GGGTACCAGGAAAGTCCCG CTCCCGCTCCC-3′) binding sites of the E3 promoter were purified and labeled with T4 polynucleotide kinase and 20 000 c.p.m. (~1 ng) used in gel retardations with 3 μg of either HUT 78 or HeLa nuclear extract as described (Kornuc *et al.*, 1990). Oligonucleotide competitions were performed with either a 2, 6, 15 or 30-fold molar excess of either L1, L2 or NF1 oligonucleotides.

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