Regulation of Adenovirus E1a Gene Expression and the Cytolytic Immune Response

by

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STATEMENT

The flow cytometry experiment described in Chapter 3 was done by Dr. Alan Bellett. Experiments on Tc cell cytolysis were done in collaboration with Dr. Arno Mullbacher. The rest of the work in this thesis is my own.

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Finally, I thank my family for all of their support and sacrifice.
ABSTRACT

Adenoviruses, like some other viruses, are able to evade the host's immune defence system and establish persistent infections. Some mechanisms have been proposed by which adenoviruses may evade cellular immune surveillance. Most of these are concerned with the effects of proteins coded in the E3 region. A 14.7k product of the E3 gene has been shown to inhibit cytolysis of infected cells by tumor necrosis factor. Another E3 gene product, E3 19k, has been reported to bind to some class I MHC antigens of certain haplotypes and reduce their cell surface expression. This results in a decrease in cytotoxic T (Tc) lymphocyte recognition. However, under some conditions, E3 gene products from group C adenoviruses were shown to reduce Tc cell recognition without affecting cell surface expression of class I antigen expression. These observations suggest that there may be an additional mechanism(s) for E3 to interfere with Tc cell recognition.

Consistent with the proposal mentioned above, studies in this thesis demonstrate that both newly synthesized and total E1a protein products, the immunodominant antigens for Tc cell recognition of certain mouse strains during adenovirus infection, are substantially lower in w.t. group C adenovirus infected L cells than in cells infected with E3 deletion mutants. Immunological studies showed that there was a strong relationship between the level of E1a expression and Tc cell recognition. Studies on cell lines from different species showed that down regulation of E1a by E3 gene products occurred in all of the mouse cell lines tested and also in some human cell lines, indicating that it is a rather general phenomenon. Results from experiments on the possible mechanisms by which E3 gene products interfere with E1a expression indicated that E3 gene products had little effect on E1a transcriptional efficiency or posttranscriptional modification of E1a mRNA, suggesting that they interfere with E1a expression by translational regulation.

The adenovirus E3 gene has been shown to be nonessential for virus growth in tissue culture studies. However, E3 gene products have been found to be important immunological regulators during adenovirus infection. The results obtained from the studies in this thesis
demonstrate yet again the diversity of E3 gene immunological regulating functions which will allow adenovirus to evade immune recognition and build up a persistent infection \textit{in vivo}. 
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>Ad2</td>
<td>adenovirus type 2</td>
</tr>
<tr>
<td>Ad5</td>
<td>adenovirus type 5</td>
</tr>
<tr>
<td>Ap</td>
<td>autopow media</td>
</tr>
<tr>
<td>Blotto</td>
<td>10% nonfat dry milk</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy-adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxy-cytidine-5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy-guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>dep</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dH₂O</td>
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</tr>
<tr>
<td>dl</td>
<td>deletion</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>dTTP</td>
<td>Deoxy-thymidine-5'-triphosphate</td>
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<tr>
<td>EDTA</td>
<td>disodium ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IU</td>
<td>infectious units</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton molecular weight</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>m.u.</td>
<td>map unit</td>
</tr>
<tr>
<td>NMS</td>
<td>normal mouse serum</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAse</td>
<td>Ribonuclease, Promega</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
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<td>0.15M NaCl/0.015M Na citrate</td>
</tr>
<tr>
<td>SSPE (1x)</td>
<td>0.18M NaCl/10mM Na₂HPO₄/1mM EDTA</td>
</tr>
<tr>
<td>STE</td>
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</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>Tc</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris pH 8.0/1mM EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>units of enzyme activity</td>
</tr>
<tr>
<td>VA RNA</td>
<td>virus associated RNA</td>
</tr>
<tr>
<td>w.t.</td>
<td>wild type</td>
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Chapter 1

Adenovirus gene expression and the cellular immune response: A Literature Review

1.1 INTRODUCTION

As the field of virology continues to expand, it is becoming increasingly clear that long-term infection of the host is a highly significant phenomenon in the biology of many virus groups including adenoviruses. Adenoviruses are ubiquitous lytic DNA viruses that are found in the majority of human populations. They are also responsible for a variety of infections ranging from mild respiratory illnesses to more severe diseases such as acute hemorrhagic cystitis and gastroenteritis. The role of adenoviruses in human disease is not well understood at the molecular level. However, the recent application of contemporary techniques in molecular biology, virology, and cell biology provide powerful means for analysis and, over the past term, these should allow a meaningful dissection of the overall process.

In this review, adenovirus molecular biology is first discussed, with particular reference to adenovirus early gene expression and regulation. This is followed by a review of basic knowledge of the specific cytopathic effect of adenovirus infection and its role in cellular immune response. The review concludes with a discussion on the cellular immune response to adenovirus infection.

1.2 Adenovirus Early Gene Expression

1.2.1 Introduction

Although the original identification of adenoviruses was the result of clinical investigations (Kilbourne and Wachsmann, 1963; Runge et al., 1963), they have been important as a tool for molecular biological research. Adenoviruses have been extensively used as model systems for the study of eukaryotic gene expression. For example, during the study of the biology of adenovirus mRNA, the first reverse transcriptase activity of RNA viruses was discovered (Berk et al., 1976; Charles et al., 1977). In vitro transcription assay systems were first developed for mammalian
1.1. INTRODUCTION

As the field of virology continues to unfold, it is becoming increasingly clear that long-term infection of the host is a highly significant phenomenon in the natural history of many virus groups including adenoviruses. Adenoviruses are ubiquitous lytic DNA viruses that infect many animal species. Although acute infection with adenoviruses is sometimes severe, it is rarely fatal. In addition to acute disease, adenoviruses also cause persistent infections characterized by prolonged existence of viruses in lymphoid tissues and peripheral blood lymphocytes (Green et al., 1979b; Horvath et al., 1986). The precise mechanism(s) by which viruses evade the host’s immune surveillance and build up persistent infections in vivo is currently not well understood at the molecular level. However, the proper application of contemporary techniques in molecular biology, immunology, and cell biology provide powerful means for analysis and, over the near term, these should allow a meaningful dissection of the overall process.

In this review, adenovirus molecular biology is first discussed, with particular reference to adenovirus early gene expression and regulation. This is followed by a review on basic knowledge of virus-specific cytotoxic T (Tc) lymphocyte recognition and its role in combating viral infection. The review concludes with a discussion on the cellular immune response to adenovirus infection.

1.2. Adenovirus Early Gene Expression

1.2.1. Introduction

Although the original identification of adenoviruses was the result of clinical investigations (Hilleman and Werner 1954; Rowe et al., 1953), they have been important as a tool for fundamental scientific research. Adenoviruses have been extensively used as model systems for the study of eukaryotic gene expression. For instance, during the study on the biogenesis of adenovirus mRNA, the important processing event of RNA splicing was discovered (Berget et al., 1977; Chow et al., 1977). In vitro transcription assay systems were first developed for mammalian
cells using adenovirus (Manley et al., 1980; Weil et al., 1979). Similarly, the finding that the promoter of an adenovirus gene determined the cap site for mRNA, led to the discovery that the start site of transcription of polymerase II became the 5' end of the mRNA (Ziff and Evans 1978). Adenoviruses have continued to fulfil expectations of their role as a model for studying some important biological functions.

47 serotypes of human adenoviruses have been isolated. These have been divided into five groups, based on their oncogenicity and their DNA restriction mapping (Gallimore et al., 1985; Larson et al., 1965 Green et al., 1979a; Pereira et al., 1965). As adenovirus type 2 (Ad2) and type 5 (Ad5), the representatives of the non-oncogenic group C adenoviruses, have been well characterized, more information for these two serotypes are available. For example, the whole genome of Ad2 has been sequenced (for review, see: Roberts et al., 1986). They have been extensively used in experiments in this thesis. Accordingly, this literature review will concentrate on data obtained from studies with Ad2 and Ad5, but will also deal with some data from adenovirus type 12 (Ad12), a representative of the highly oncogenic group A adenoviruses.

1.2.2. The structure of the adenovirus genome

The genomes of adenoviruses are linear, double-stranded DNA molecules with molecular weights in the range of 20-25x10^6 daltons (van der Eb et al., 1969). In the case of Ad2 the genome has a length of 35,937 base pairs (Roberts et al., 1986), and where the data is available it suggests that the genomic organization of other serotypes is similar. For convenience, the genome is arbitrarily divided into 100 map units (m.u.), i.e. one m.u. equals nearly 360 bp. Both ends of the viral genome contain a protein, the terminal protein, which is covalently linked via a serine residue to the 5'-end of each DNA strand (Desideiro and Kelly 1981; Rekosh et al., 1977; Robinson et al., 1973). Evidence has been presented that the terminal protein plays an essential role in the initiation of adenovirus DNA replication (Challberg and Kelly 1979; Pettit et al., 1989; Tamanoi and Stillman 1982). Another interesting structural character of the adenovirus genome is that the viral genome contains inverted
terminal repeats ranging from 100 to 200 bp depending on the serotype of the virus (Kelly 1984; Sussenbach 1984). Using mutational analyses, a number of investigators have identified that some sequences within the inverted terminal sequences are important for initiation of DNA replication (Challberg and Rawlins 1984; Hay and McDougall 1986; Rawlins et al., 1984). Inverted terminal sequences have also been shown to be important for the viability of adenoviruses (Lippe and Graham 1989).

Adenovirus DNA is transcribed in both the rightward (from the r-strand) and leftward (from the l-strand) direction, as illustrated in the transcription map of the Ad2 genome (Fig.1.1). Individual genes have been mapped on the genome using a variety of techniques. During adenovirus infection, the viral genes are expressed in two broadly defined phases—early and late. Early genes are the genes which are expressed before the onset of viral DNA replication and are grouped in six transcription units denoted E1a, E1b, E2, E3, and E4, and L1. Among them, E1a is the first to be expressed during infection (Nevins et al., 1979) and is thus referred to as an "immediate early" gene block (Flint 1986; Galos et al., 1979). E1a gene products are required for the transcriptional activation of the other adenovirus early genes (Berk et al., 1979; Jones and Shenk 1979a). Late genes are transcribed after the onset of viral DNA replication and include those coding for structural proteins. However, there also exists a group of intermediate genes which are expressed at intermediate times in infection. A summary of adenovirus gene transcription and expression is illustrated in Fig.1.1.

1.2.3. Expression of E1a

The transcription unit of E1a is located between 1.3 and 4.6 m.u. and is transcribed from the r-strand. The primary E1a transcript is differentially spliced to produce at least five mRNAs, four of which (13S, 12S, 11S, and 10S) differ from each other by the size of the intron which is removed during mRNA maturation. They consequently encode highly related proteins and share their N-terminal and C-terminal segments (Perricaudet et al., 1979; Stephens and Harlow 1987; Ulfendahl et al.,
Fig. 1.1. Transcriptional organization of early regions of the Ad2 genome. The genome is divided into 100 map units. The r-strand is transcribed into RNA rightward and the l-strand leftward. The direction of transcription is indicated by arrows. The 5' ends of the cytoplasmic RNA indicate the positions of transcriptional promoters, while the arrowheads represent the 3' polyadenylation sites. Gaps in arrows indicate intervening sequences, which have been removed from the cytoplasmic RNA by splicing. Adapted from Nevins (1987).
1987). The fifth message, a 9S transcript, shares the same 5' and 3' ends with the other E1a mRNAs, but is spliced such that its second exon is predicted to be translated in a different reading frame (Virtanen and Pettersson 1983). The 13S and 12S mRNAs are expressed both early and late after infection (Berk and Sharp 1978; Chow et al., 1979), whereas the 9S, 10S, and 11S transcripts are detected only at late times of infection (Ulfendahl et al., 1987). In Ad2, the 13S and 12S transcripts encode 289 amino acid (289R) and 243 amino acid proteins (243R), respectively. The only difference between these two proteins is that the 289R contains an additional 46 amino acids between residues 144 and 189. The 11S and 10S mRNAs encode proteins of 217R and 171R which are alternatively spliced products of the 289R and 243R, but lack amino acids 27 to 98 (Stephens and Harlow 1987; Ulfendahl et al., 1987). The functions of these two polypeptides are still unknown (Tremblay et al., 1989). All of the E1a gene products are post-translationally modified, principally by phosphorylation (Gaynor et al., 1982; Yee et al., 1983), giving rise to many different species of E1a proteins which can be distinguished by their electrophoretic mobility (Harlow et al., 1985; Yee and Branton 1985).

1.2.4. Functions of E1a

1.2.4.1. E1a mediated transactivation.

Using a variety of Ad5 mutants with lesions in the E1a gene it was originally shown that expression of the E1a gene products was required for the accumulation of early viral mRNAs encoded from other adenovirus early genes (Berk et al., 1979; Jones and Shenk 1979a). Viruses whose E1a transcription unit has been deleted, such as Ad5dl312 (Jones and Shenk 1979b), or altered in more subtle ways, for example, Ad5hr1 (Harrison et al., 1977), were found to be defective for growth on HeLa cells, failing to produce cytoplasmic or nuclear RNA species complementary to the early transcription units (Berk et al., 1979; Jones and Shenk 1979a). This observation has been confirmed by studies using appropriate expression plasmids cotransfected into HeLa cells (Leff et al., 1984; Weeks and Jones 1983). Although E1a coordinately activates all of the early genes, the timing and level of activation for each
gene varies (Neuwald et al., 1977; Nevins et al., 1979; Ross et al., 1980; Rowe et al., 1984; Spector et al., 1978). The E4 transcription unit is activated first, followed by E3 and E1b and finally E2. The transcription of E4 peaks very early and is rapidly shut off, coinciding with the activation of E2. E1a and E1b transcripts are present throughout infection, but transcription of E2 and E3 gradually decline.

Studies with Ad5hr1 mutant, in which 289R product of the 13S mRNA is truncated but 243R product of the 12S mRNA is normal, showed that 289R mediates transcriptional activation of viral early gene expression (Harrison et al., 1977). This conclusion has been confirmed by studies of several other mutant viruses that can express only the 13S or 12S E1a mRNA (Montell et al., 1982) or the viruses whose E1a region has been replaced by cDNA copies of E1a 12S or 13S mRNA (Moran et al., 1986). As mentioned previously, the only difference between 289R and 243R is that the former has an extra 46 amino acid residues, which includes most of a region known as "conserved domain" 3 (CD3). The transactivating function of E1a has been therefore exclusively ascribed to CD3. However, there is accumulating evidence against this hypothesis (for review: Braithwaite et al., 1991b). The dispute is based on the following observations: (1) When the purified 243R and 289R from Escherichia coli were seperately microinjected into Xenopus oocytes along with the E3 plasmid, both of the E1a proteins transactivated the E3 promoter with a nearly equal efficiency (Ferguson et al., 1984; Richter et al., 1985); (2) The 243R protein has been observed to stimulate the transcription of the E2 and E3 transcription units when appropriate plasmids are cotransfected into HeLa cells, although less efficiently than E1a 13S mRNA products (Leff et al., 1984); (3) A 13S defective mutant of Ad5, in500, which failed to produce CD3, has been shown to have a substantial ability to transactivate E2 and E3 efficiently (Carlock and Jones 1981). All these evidence suggest that the unique CD3 region of 289R is extremely important in transactivation, but not all transactivation function is located exclusively within this region. Regions of E1a other than CD3 also significantly contribute to the ability of E1a proteins to transactivate viral promoters, but is considerably less
effective in this role. It also appears that transactivation of different promoters may involve different domains of the E1a protein (reviewed in: Braithwaite et al., 1991b).

It is worth mentioning that E1a can autoregulate at the level of transcription (Borrelli et al., 1984; Hearing and Shenk 1985). The 289R protein appears mainly responsible for the positive autoregulation, while the 243R protein is much less effective (Hearing and Shenk 1985; Montell et al., 1984; Tibbetts et al., 1986). Genetic studies have shown both positive and negative E1a autoregulation (Larsen and Tibbetts 1987; Smith et al., 1985). The minimal upstream sequence required for up-regulation is the TATA box (Hearing and Shenk 1985). It is possible that the mechanisms for initial activation and both positive and negative autoregulation involve a number of transcription enhancers as well. Several studies have identified enhancer-like sequences upstream of the E1a promoter which act to stimulate transcription from E1a (Hearing and Shenk 1986; Imperiale et al., 1983). Negative regulation late in infection may possibly serve as part of the early to late switch.

The 289R E1a protein can stimulate transcription not only of adenoviral transcription units but also of cellular genes, under at least some circumstances. Obviously this is not a generalized phenomenon, as the overall rate of transcription of cellular DNA does not increase following adenovirus infection. Also there does not appear to be a common E1a responsive enhancer element to all of the different genes (Jones et al., 1988). The cellular genes which can be up regulated include hsp70 (Kao and Nevins 1983), c-fos (Sassone-Corsi and Borrelli 1987), c-myc (Hiebert et al., 1989), dihydrofolate reductase (Yoder and Berget 1985; Yoder et al., 1983), thymidine kinase (Braithwaite et al., 1983; Cheetham and Bellett 1982), β-globin (Green et al., 1983), β-tubulin (Stein and Ziff 1984), p53 (Braithwaite et al., 1990), class I MHC antigen (Rosenthal et al., 1985), and cell cycle dependent genes (Liu et al., 1985).

Although the activation of early genes by E1a has been shown to occur through an increase in the rate of transcription of the target genes (Nevins 1981; Weeks and Jones 1983), the detailed mechanism is still not clear. The variety of cellular and viral transcription units, normally
expressed in quite different circumstances, whose transcription can be enhanced by E1a proteins, suggest that such stimulation of transcription cannot be mediated directly by E1a itself. It has been shown that DNA sequences present in the 250 nucleotides 5' to the initiation sites for E1b, E2A, E3 and E4 are required for activation (Imperiale and Nevins 1984; Weeks and Jones 1983). However, these regions do not share a unique E1a-inducible regulatory sequence, instead, they contain a composite of an E1a-inducible element and binding site for trans-acting factors. Since E1a has not been shown to bind directly to DNA, E1a may modulate transcription via modification of pre-existing transcription factors, for example, by phosphorylation (Bagchi et al., 1989; Sassone-Corsi 1988). There is also evidence that E1a may transactivate different cellular and viral gene expression by interacting with different classes of transcription factors to indirectly bind to viral DNA (Ko et al., 1986; Mirza 1988).

1.2.4.2. E1a mediated repression.

In addition to transactivating viral and cellular genes, E1a products have been shown to be able to repress some gene expression. Both the 243R and 289R E1a proteins have been independently observed to repress SV40 or polyomavirus enhancer-mediated transcription, following cotransfection of appropriate plasmids into HeLa cells (Borrelli et al., 1984; Velcich and Ziff 1985). Such repression required the synthesis of E1a proteins, but was not dependent upon the production of papovaviral early gene products, and was specific for enhancer-mediated transcription. Plasmids that could express only the 243R or 289R protein were equally effective in repressing transcription from these enhancer-containing plasmids (Borrelli et al., 1984; Velcich and Ziff 1985), suggesting that the domains of the E1a protein responsible for transcriptional activation and repression are separate. During infection of plasmacytoma, the E1a proteins have been shown to repress the enhancer of the immunoglobulin heavy chain (Hen et al., 1985). The E1a gene products also inhibit enhancer-stimulated transcription of the rat insulin II gene (Stein and Ziff 1987) and the promoter regions of the
muscle-specific α-skeletal and α-cardiac actin genes (Webster et al., 1988). E1a represses the transcription of histone (Flint et al., 1984). E1a gene products can also achieve repression by destabilizing mRNAs encoded by some genes like JE and stromelysin (Offringa et al., 1988; Timmers et al., 1989). Recently it was reported that E1a can repress the expression of the secreted proteases, type IV collagenase, interstitial collagenase and urokinase. These proteins are important contributors to the metastasis of tumor cells. E1a may suppress the metastatic potential of human tumor cells (Frish et al., 1990).

As mentioned earlier, E1a can also repress its own expression (Smith et al., 1985). This may have another functional significance in the context of viral gene expression during infection. As we have seen, transcription of the E1a unit can also be activated by the 289R E1a protein. It seems likely that transcription of the E1a region must be tightly controlled, presumably responding to the total concentration of the two E1a proteins as well as to the ratio of the 289R to the 243R protein. When E1a products reach certain high levels, they then repress their own expression, whereas when the E1a level is too low, they stimulate the expression.

1.2.4.3. E1a mediated transformation.

Early studies have shown that oncogenic transformation by adenoviruses is a function of both E1a and E1b regions. This conclusion is based on three main lines of evidence. First, cell lines transformed by adenoviruses always contain and express the E1a and E1b regions and some possess only those sequences (Flint et al., 1976; Gallimore et al., 1974). Second, transfection of rodent cells in vitro with DNA fragments has shown that left-end fragments alone are sufficient to bring about transformation (Graham et al., 1974). Third, it has been reported that host range (hr) mutants with mutations mapping to either E1a or E1b are transformation defective (Chinnadurai 1983; Harrison et al., 1977; Jones and Shenk 1979b). Cells transformed by DNA encoding E1a only exhibit a partial transformed phenotype. They become immortalized and morphologically resemble fully transformed cells but show intermediate
levels of anchorage independent growth, do not reach high saturation densities and are not tumourigenic in vivo (Houweling et al., 1980; Senear and Lewis 1986). In addition to the immortalization function, E1a also appears to have a transforming function, since immortalized rat embryo cells cannot be transformed by DNA constructs capable of expressing E1b without E1a (van den Elsen et al., 1983).

Analysis of transformation studies has allowed a number of conclusions to be drawn about E1a mediated transformation. The E1a mediated transactivation and transformation activities are separate events. This was first indicated by studies showing that the 12S cDNA sequences of E1a, which have little transactivation activity (Esche et al., 1980; Montell et al., 1982), were fully capable of establishing primary rat cells and cooperating with the activated ras gene to effect full morphological transformation (Haley et al., 1984; Zerler et al., 1986). It has been shown that mutations in two different domains of the E1a coding region result in reduced transformation without affecting transactivation and therefore further strengthens the conclusion that these two activities are not related (Schneider et al., 1987). It appears that both major E1a proteins are required for complete transformation (Montell et al., 1984). When cells are infected with a deletion mutant which produces normal amounts of 13S but much reduced amounts of 12S, the number of transformed foci is only one-fifth to one-half of those induced by wild type virus, and the transformed cells are defective in their ability to form anchorage-independent colonies in semi-solid medium (Hurwitz and Chinnadurai 1985). E1a gene products also determine to a large extent, morphological transformation (van der Elsen et al., 1983). Individual E1a gene products can elicit distinct alterations of cellular morphology and gene expression (Roberts et al., 1985). It has been suggested that it is transcriptional repression rather than transcriptional activation of cellular gene expression that is important for transformation (Schneider et al., 1987).
1.2.5. The E1b region.

Region E1b is located immediately adjacent to E1a (4.6-11.2 m.u.) and is also transcribed in the rightward direction (see Fig.1.1). The E1b transcription unit expresses at least five differentially spliced mRNA species (Chow et al., 1979; Virtanen and Pettersson 1985). Two major mRNAs (22S and 13S in group C adenoviruses) and two minor mRNAs (14.5S and 14S ) are generated by splicing of a common precursor RNA. In addition, an unspliced 9S mRNA encoding viron polypeptide IX, is transcribed from a separate promoter at intermediate and late times after infection (Alestrom et al., 1980). The E1b mRNAs differ from each other by the size of the introns that are removed. The 22S and 13S mRNAs are the predominant species. They encode the 495R (55kD) and the 175R (19kD) tumor antigens, respectively (Bos et al., 1981; Esche et al., 1980; Ross et al., 1980). It should be noted that these two polypeptides are translated in different reading frames and therefore have no amino acid sequence in common. A minor E1b protein, which is predicted to be synthesized from the 14S mRNA has been observed. It shares its N- and C-terminal amino acid residues with the large E1a 55kD polypeptide (Anderson et al., 1984; Virtanen and Pettersson 1985).

Although the function of E1b 19kD is essentially unknown, the phenotypic description of adenovirus mutants which have deletions in the E1b 19kD protein suggests that E1b 19kD may have some potential functions (Stillman 1986). The first, and most common, of the phenotypes that these mutants induced on infection is that E1b 19kD mutants form large plaques (lp phenotype) (Chinnadurai 1983; Subramanian et al., 1984; White et al., 1984). Second, infection with an E1b 19kD protein gene mutant virus induces an enhanced and abnormal cytopathic effect (cyt , or cytocidal phenotype) (Pilder et al., 1984; Subramanian et al., 1984; White et al., 1984). Third, E1b 19kD mutant can induce degradation of host cell chromosomal and viral DNA (deg phenotype) (D’Halluin et al., 1979; Enzo et al., 1981; Lai Fatt and Mak 1982; Pilder et al., 1984; White et al., 1984). Fourth, E1b 19kD mutant viruses possess an unusual host range (hr ) phenotype, in which mutant viruses have a substantial growth advantage over the wild-type virus in human W138 cells (White
et al., 1986; White and Stillman 1987). All the phenotypes attributed to the E1b 19kD gene, including the hr phenotype, are E1a dependent, beyond the requirement for E1a to activate transcription, since infection with an E1a 9S virus carrying a 19kD gene point mutation did not induce cyt, deg, lp, and hr phenotypes even at high multiplicities of infection (White et al., 1986; White and Stillman 1987). This suggested a functional interaction between E1a and E1b 19kD gene products.

The analysis of a number of mutants with specific lesions in the E1b 55kD has also defined some of the unique functions of this E1b protein. Mutation in the 55kD gene results in approximately 100-fold reduction of virus yield. This is because the 55kD protein is required for efficient shut-off of host protein synthesis and efficient transition from the early to the late mode of viral gene expression (Babiss and Ginsberg 1984; Pilder et al., 1986). E1b 55kD has also been shown to have gene regulatory function. Mutants with defects in the Ad 5 E1b protein of 55kD showed reduced levels of late messages in the cytoplasm, caused either by a defect in the transport of late messages to the cytoplasm or by a reduction in the stability of cytoplasmic mRNA (Babiss et al., 1985). More detailed studies have ascribed additional functions to E1b 55kD. Many separate functional regions in 55kD have been defined. The structure of the E1b 55kD may somehow contribute to its own stability. Deletions of the first 24 amino acids and of amino acid residues 114-155 results in an instability of the E1b 55kD itself. The N-terminal 24 amino acid residues are not required for virus replication, whereas amino acid residues between 114 and 155 are absolutely necessary for viral DNA synthesis (Mak and Mak 1990).

The regulatory activities displayed by E1b 19kD protein are more intriguing. Like E1a, E1b 19kD protein also has transactivation activities. By use of a chloramphenicol acetyltransferase (CAT) gene transient assay system, it was shown that the E1b 19kD protein from Ad5 can activate all the adenovirus early promoters (E1a, E1b, E2, E3 and E4) and a cellular heat shock promoter, but not the adenovirus late promoters. The effect is greatest under conditions where cell growth is inhibited (Herrmann et al., 1987). This activation appears to operate at the
transcription level. This transactivation by E1b of other early genes does not require the presence of E1a gene products, but a synergistic effect is observed in the presence of E1a 13S product. The transactivating activity of the E1b gene is also observed during virus infection (Herrmann et al., 1987). This observation implies that the regulatory function of E1b 19kD may have important consequences in lytically infected and in transformed cells. The E1b 19kD protein has also been shown to transactivate reporter genes linked to the SV40, polyoma, and immunoglobulin heavy-chain enhancers during co-transfection in fibroblasts, but not in myeloma cells (Natarajan 1986; Yoshida et al., 1987). Furthermore, E1b 19kD activates the polyoma enhancer in F9 cells (Yoshida et al., 1987), where the enhancer is normally inactive (Hen et al., 1986).

As mentioned earlier, E1a can not completely transform cells, it can only immortalize them. Induction of a fully transformed phenotype and correct gene regulation requires both E1a and E1b genes (Chinnadurai 1983). It seems that both major E1b proteins are required for full transformation (Barker and Berk 1987). A clear requirement for the E1b 19kD protein in transformation is well documented by studies with viral mutants (Babiss and Ginsberg 1984; Barker and Berk 1987; Chinnadurai 1983; Pilder et al., 1984; Subramanian et al., 1984). A similar requirement is also seen in transfection studies (Babiss and Ginsberg 1984; Chinnadurai 1983). Mutant viruses that fail to express the E1b 19kD protein or encode potentially truncated forms of this protein are unable to transform a number of different rodent cells, even at high multiplicities of infection (Babiss et al., 1984; Barker and Berk 1987; Chinnadurai 1983; Pilder et al., 1984). Less clearly defined is the role of the E1b 55kD protein in transformation. In cooperation with E1a proteins and the E1b 19kD protein, this protein appears to play a role both in initiating and in maintaining the transformed cell phenotype (Babiss et al., 1984; Barker and Berk 1987; Pilder et al., 1984). When introduced into nonpermissive rodent cells, viruses that express truncated forms of this protein induce foci at a reduced frequency and with fewer transformed-cell characteristics as the truncation approaches the amino terminus of
the protein (Babiss et al., 1984). However, the complete absence of E1b 55kD protein expression still results in focus formation, suggesting that this protein is involved in a secondary pathway leading to the transformed state. The exact mechanisms of how the two major E1b proteins are involved in transformation has not been elucidated, but there are reports that the E1b 55kD protein can form complexes with the cellular proto-oncogene p53 (Sarnow et al., 1982; Zantema et al., 1985; Braithwaite et al., 1989; Braithwaite et al., 1991a). Based on a series of recent important findings, wild type p53 is considered to have dominant anti-oncogene properties (Baker et al., 1990; Eliyahu et al., 1989; Finlay et al., 1989). It is speculated that E1b might inactivate p53 or interfere with its anti-oncogene function by binding to it, and then cause transformation. However, there is no direct evidence for this. A recent report showed that mutations which prevented E1b 55kD binding with p53 were not more defective for transformation than other mutations which did not affect binding (Kao et al., 1990). This indicates that there may not be a direct relation between E1b 55kD binding with p53 and its transforming activity. It has been reported that activated Ha-ras can replace E1b and cause transformation when it is introduced together with Ad2 E1a into primary BRK cells whereas Ha-ras itself cannot induce transformation (Ruley 1983). This suggests that there may be similarities in mechanisms between E1b and ras induced transformation.

1.2.6. The E2 region.

The E2 region is located at 11.3-75.4 m.u.. Unlike E1 genes, region E2 is transcribed from the viral I-strand. The transcription pattern of this region is drastically changed between early and intermediate times of infection. At early times of infection, a promoter at 75 m.u. is active (Berk and Sharp 1977). Following the switch from the early to the late phase, a promoter shift occurs, and the promoter located at 72 m.u. becomes the most active one (Chow et al., 1979). Two major classes of transcripts are generated from the E2 region. The first class of mRNA, designated E2A, is the one which extends from the transcriptional
initiation site to a polyadenylation site at 62.4 m.u. (Fig.1.1) The E2a region encodes a single stranded DNA binding protein, which is synthesized both early and late during infection (Linne et al., 1977). The second set of transcripts bypass the polyadenylation signal used by E2A mRNA and extends to a second polyadenylation site at 11 m.u.. The mRNA from E2B codes three polypeptides with estimated molecular weights of 87, 105, and 75kD (Stillman et al., 1981). Subsequent studies have shown that the 87kD polypeptide is the precursor for the terminal protein that is covalently bound to the termini of adenovirus DNA (Gingeras et al., 1982). The 87kD protein is cleaved to the 55kD terminal protein during assembly into virus particles (Stillman et al., 1981). Accumulating evidence suggest that the 105kD polypeptide is a viral DNA polymerase that is responsible for the replication of adenovirus DNA (Stillman et al., 1982).

Proteins encoded by E2 play a substantial role in viral DNA replication. Three viral proteins required for adenovirus DNA replication are all encoded in the E2 region. Functionally, the 72kD DNA binding protein is the best characterized of these proteins and much of what we know about it has come from studies of temperature sensitive (ts) and other mutants whose mutations map in the DNA binding protein gene. The protein binds efficiently, but not covalently, to single stranded DNA (van der Vliet et al., 1978), and, to a lesser extent, to double-stranded DNA (Fowlkes et al., 1979). The DNA binding protein of E2a prototype mutant H5ts125, a mutant with a proline-serine position substitution at the C-terminal region of the protein (Kruijer et al., 1983), produces a thermolabile protein and is defective for viral DNA replication at the non-permissive temperature (Ensinger and Ginsberg 1972; Horwitz 1978; van der Vliet et al., 1975). There is evidence that the DNA binding protein is required for both initiation of DNA synthesis and subsequent elongation (Horwitz 1978; van der Vliet et al., 1977; van der Vliet and Sussenbach 1975). The another E2 encoded protein, the 87kD protein which is the precursor for terminal protein, has been shown to be involved in initiation of viral DNA replication, and the 105kD protein,
which functions as a polymerase, is also responsible for the replication of adenovirus DNA (reviewed in: Kelly 1984).

The DNA binding protein from E2 is a multifunctional protein, with regulatory effects on viral gene expression. Cells infected with H5ts125 at the non-permissive temperature, contain elevated levels of early DNA binding protein mRNA and other early mRNAs (Carter and Blanton 1978). This finding suggests that DNA binding protein can repress its own synthesis during a normal wild-type infection and also exerts a similar influence on the expression of other early proteins. This increase in early protein synthesis in H5ts125 infected cells seems to occur by a prolonged half-life of the message (Babich and Nevins 1981). Experiments have shown that in the absence of E2 DNA binding protein, region 4 transcription reaches a maximal rate which is maintained throughout the course of the experiment, whereas in the presence of functional DNA binding protein (wild type infection), the rate of E4 transcription is reduced late in infection. This suggests that DNA binding protein can down regulate E4 expression at a certain stage during w.t. virus infection, and it seems that the down regulation is the result of a block at the initiation of transcription (Nevins and Winkler 1980). There is recent evidence of down regulation of E4 by the E2 DNA binding protein in an in vitro system. It has been demonstrated that purified E2a protein can specifically down regulate transcription from the E4 promoter (Handa et al., 1983).

1.2.7. The E3 region.

1.2.7.1. The transcription unit of E3.

Region E3 is located at 76-86 m.u. and is transcribed from the viral r-strand. Nucleotide sequence analysis of this region has revealed that a TATA box of the structure TATAA is located at nucleotide -30 (relative to the transcription start site). The first ATG in the E3 region is found at position 291 (Cladaras and Wold 1985). Analysis of deletion studies suggests that a critical element is located between positions -105 and -82. Deletion of these sequences significantly reduced transcription efficiency both in the presence and absence of E1a (Weeks and Jones 1985). This
region is considered to be enhancer region. In group C adenovirus, all the E3 mRNAs initiate from a common promoter and use a single cap site, but differ from each other by the different positions of their 3'-termini, termed E3A and E3B. One of the E3 polyadenylation sites is located at coordinate 85.9 m.u. (E3B) and is preceded by the hexanucleotide sequence AAUAAA. The second poly(A) site is located at coordinate 82.9 (E3A) (Cladaras et al., 1985). It seems that this site is less frequently used (Fig1.2).

1.2.7.2. E3 proteins and their functions.

Region E3 encodes at least nine overlapping mRNAs (a-i) by differential splicing. There are two 5' splicing sites and four 3' splicing sites. The precise positions of these splicing sites have been determined by a variety of methods (Chow et al., 1979; Cladaras et al., 1985; Kitchingman and Westphal 1980). The major mRNAs are a (about 40% of the total) and c (about 15%), which are spliced once, and f (about 15%) and h (about 25%), which are spliced twice. mRNA a uses the upstream E3A polyadenylation site, and the other major mRNAs use the downstream E3B polyadenylation site. Theoretically, there should be approximately nine proteins synthesized from the E3 mRNAs. Up to date, 6 of them have been found in vivo (reviewed in: Gooding and Wold 1990; Wold and Gooding 1989). A schematic transcriptional and translational map of E3 unit is shown in Fig. 1.2.

Among the identified E3 proteins, a protein with a molecular weight of 19kD in group C adenoviruses, designated E3 19k, was the earliest discovered and has been most studied (Jeng et al., 1978; Persson et al., 1979; Wold et al., 1985). It is 142 amino acid residues long and is an abundant early glycoprotein. Immunofluorescence and other studies have shown that it is localized exclusively in the membrane portions of fractionated cells, mainly in the endoplasmic reticulum (Paabo et al., 1987). At the N-terminus of this protein there is a sequence of 17 residues which may function as a signal for E3 19k insertion into the membrane of the endoplasmic reticulum (Kampe et al., 1983; Persson et al., 1980). Near the C-terminus there is a hydrophobic domain about 20-23 residues
Fig. 1.2. Schematic representation of Ad2 E3 region. The spliced arrows represent the 7 major mRNAs (a through i) out of 9 putative E3 mRNAs. The broken portions of the arrows depict the spliced introns from the mRNA. The thickness of the arrow implies the relative abundance of the mRNAs. The mRNAs are co-terminal at their 5’ ends (at the left) and they form 3’ ends at one of two polyadenylation sites termed E3A and E3B. The bars above the arrows indicate coding positions for proteins. Hatched bars are proteins that have been identified in infected cells, as follows: 14.7kD, 14.5kD, 10.4kD, 11.6kD, 6.7kD, and 19kD. Stippled bars indicate open reading frames that are proposed to exist. Adapted from Gooding and Wold (1990).
long followed by a polar domain of 15 residues (Persson et al., 1980). It seems that the hydrophobic domain is the trans-membrane domain and the C-terminal 15 residues extend into the cytoplasm. There are two N-linked glycosylation sites, both of which are glycosylated (Wold et al., 1985). The main observed property of E3 19k is to form a complex with class I major histocompatibility complex (MHC) antigens, preventing their glycosylation and subsequent expression on the cell surface. This will be described later in more detail (section 1.4.2.2). There is one report which shows that E3 19k has sequence homology with the α-chain of the human class II MHC antigen HLA-DR and the κ-chain of human Ig-M. The authors suggest that this sequence homology may have evolutionary significance (Chatterjee and Maizel 1984).

Recently a 10.4kD protein of E3, which is translated primarily from E3 mRNA, has been identified (Tollefson et al., 1990). This protein migrates as two bands with apparent molecular weights of 16kD and 11kD respectively. It seems that the 16kD protein is the precursor of the 11kD polypeptide since only the 16kD band is obtained by cell-free translation. The 10.4kD protein is also a membrane protein. This protein has been implicated in down-regulating the epidermal growth factor receptor (EGF-R) by stimulating endosome mediated internalization and degradation of EGF-R (Carlin et al., 1989). The 10.4kD protein appears to mimic EGF in inducing internalization and degradation of EGF-R, instead of affecting its synthesis. As EGF-R oligomerization is thought to be obligatory for ligand-induced EGF-R activation, it is speculated that 10.4kD elicits the same biological response by promoting the formation of heterologous oligomers composed of 10.4kD and EGF-R molecules.

The presence of another E3 protein, the 14.7kD polypeptide which is designated E3 14.7k, has also been described (Tollefson and Wold 1988). This protein is coded by mRNA. It does not have an hydrophobic domain, rather, it is highly charged. It exists in three forms with different mobilities on SDS-PAGE (Persson et al., 1979; Tollefson and Wold 1988). This may be due to posttranslational modification, or perhaps due to synthesis from alternatively spliced mRNAs. It seems that the 14.7k protein has both nuclear and cytoplasmic locations.
chase experiments indicate that the protein is very stable. It is an abundant early protein that accumulates throughout the entire lytic cycle of the virus infection. The only function ascribed to the 14.7k protein is that it can inhibit cytolysis by tumor necrosis factor (TNF) which is induced by adenovirus E1a conserved domain 1 (Duerksen-Hughes et al., 1991; Gooding et al., 1988). The original finding comes from the observation that cells infected with E3 deletion mutants of adenovirus are killed when exposed to supernatants from activated macrophages. Uninfected cells and cells infected with wild-type adenovirus are not killed. The active agent in this supernatant was identified as TNF. Further studies using a series of E3 deletion mutants identified the gene product responsible for protection from TNF killing as the 14.7k protein (Gooding et al., 1988). This 14.7k mediated protection may have important significance in natural infection. During the early stages of infection, among the first cells to migrate to a virus infection site and provide an immune response to infection include monocytes and natural killer cells. The action of both of these cells involves TNF (Old 1985). The insensitivity of infected cells to TNF, caused by E3 14.7k, may provide a viral strategy for evasion of the host’s first defense mechanism and creates an opportunity for the virus to set up a widespread infection.

Another three proteins out of the nine possible products of E3 region have also been identified. They are 11.6kD (Wold et al., 1984), 14.5kD (Tollefson et al., 1990) and 6.7kD (Wilson-Rawls et al., 1990). Based on the DNA sequence, it is predicted that these proteins will also have hydrophobic domains typical of membrane proteins. The functions of these three proteins are still unknown.

1.2.8. The E4 region.

The adenovirus early region E4 transcription unit is located at the extreme right end of the genome between 91 and 100 m.u.. It is transcribed in the leftward direction. The promoter of this region has been mapped at 99.2 m.u.. Transcription from the E4 promoter is subjected to both positive and negative regulation during the infectious cycle (Nevins 1987; Nevins and Winkler 1980). All E4 mRNAs share their
5' and 3' terminal nucleotide sequences, but vary in the locations of splice points (Chow et al., 1979). The E4 nucleotide sequence of adenoviruses reveals seven open reading frames with coding capacities of greater than 25 amino acids, among which five contain AUG start codons (Herisse et al., 1981). A family of differentially spliced mRNAs is synthesized from this region. It is suggested that E4 encodes at least seven distinct protein products. Three protein products encoded by E4 have been identified serologically, including a 34kD protein from ORF 6 (Challberg and Ketner 1981), an 11kD protein from ORF3 (Sarnow et al., 1982), and a 19kD protein from ORF6/7 (Cutt et al., 1987).

By using of E4 deletion mutants, some important functions of E4 have been found. Cells infected with E4 deletion mutants show a reduction in late protein synthesis (Halbert et al., 1985); failure to accumulate normal nuclear and cytoplasmic levels of late messages (Sandler and Ketner 1989); a reduction in viral DNA accumulation; a failure to inhibit host protein synthesis (Weinberg and Ketner 1986); and a defect in viral particle assembly (Falgout and Ketner 1987). It seems that the 34kD protein from ORF6 plays a key role in these functions. Since the 34kD protein from E4 forms a complex with E1b 55kD (Sarnow et al., 1984), and mutants in the E1b 55kD polypeptide are also defective in late mRNA synthesis and in the control of cellular gene expression (Babiss et al., 1985; Pilder et al., 1986), it seems likely that the physical complex formed between the E1b 55kD and the E4 34kD polypeptide is a functional unit. A recent report has confirmed this hypothesis (Bridge and Ketner 1990).

Recent experimental data have shown that E4 gene products have a profound effect on E2 gene expression. E4 can up-regulate E2 expression in different ways. The E4 19kD protein from ORF 6/7 has been shown to form a complex with the cellular transcription factor E2F and trans-activates transcription dependent on E2F. This stimulation of E2F is E1a independent. The formation of a complex between E4 19kD and E2F can also make E2F bind cooperatively to pairs of properly spaced and oriented E2F recognition sites (Babiss 1989; Marton et al., 1990; Neill et al., 1990). This makes the E2F efficiently stimulate the accumulation of
E2 mRNA. In transient expression systems, E4 can directly stimulate specific transcription from the major E2A start site by 5-15 fold (Goding et al., 1985). It has been shown that the extent of the E4-induced stimulation is similar to that achieved by E1a under identical conditions. However, unlike E1a-mediated stimulation, where no unique E2A promoter elements are implicated, maximal induction by E4 requires sequences between positions -48 and -19 (Goding et al., 1985).

1.2.9. VA RNA and its role in translational regulation

The adenovirus genome contains two genes that encode the virus-associated (VA) RNAs, VA RNAI and VA RNAII (Akusjarvi et al., 1980; Mathews 1975; Pettersson and Philipson 1975). The genes are arranged in tandem and are located between 29 and 31 m.u on the viral chromosome (Akusjarvi et al., 1980; Mathews 1975). Unlike the other viral transcription units, which are transcribed by RNA polymerase II, VA RNAI and RNAII are synthesized by RNA polymerase III (Price and Penman 1972; Weinmann et al., 1974). The VA RNAI and RNAII are approximately 160 nucleotides in length and are both present from early times of infection, but the synthesis of VA RNAI increases dramatically with the onset of the late phase and this species becomes the predominant cytoplasmic RNA of infected cells (Soderlund et al., 1976). The VA RNAs are capable of extensive intramolecular base pairing and have been shown to anneal with viral mRNAs (Akusjarvi et al., 1980; Mathews 1980).

Analysis of mutant viruses that are defective in the production of VA RNAs revealed that the absence of the VA RNAI resulted in poor virus growth (Thimmapaya et al., 1982). In cells infected with the VAl mutant, protein synthesis is reduced by over 90% at late times of infection. It was concluded that VA RNAI was required for the efficient translation of both viral and cellular mRNAs late in infection. The mutant virus proceeds through the early phase of infection but fails to make late viral proteins, even though undiminished levels of late viral mRNAs are present. These mRNAs are normal in structure and are capable of efficient translation in a heterologous cell-free system,
suggesting that the defect lies in the translational apparatus (Thimmapaya et al., 1982). Subsequent work (Schneider et al., 1984) excluded defects in polypeptide chain elongation or termination and led to the conclusion that the defect occurs at the level of chain initiation. The same conclusion was also reached by the studies conducted in vitro (Reichel et al., 1985). It was thought that VA RNAI was necessary to facilitate interaction between the 43S pre-initiation complex and mRNA to form the 48S species that binds to the 60S ribosomal subunit to yield an 80S initiation complex (Schneider et al., 1984). The initiation of protein synthesis requires the covalent modification of initiation factor eIF2 to bind to a molecules of GTP to form eIF2-GTP, which then forms the ternary complex, eIF-GTP- Met-tRNAf. This complex binds to mRNA and the small ribosomal subunit (plus other initiation factors) to make an initiation complex (the 40S complex in eukaryotes). After the ternary complex is positioned correctly at the AUG initiation codon, a large ribosomal subunit then joins the complex; the bound GTP is hydrolyzed and the initiation factors detach. In the presence of guanosine nucleotide exchange factor (GEF) the eIF2 is catalytically recycled. As an anti-viral response the cell produces interferon, which in turn induces synthesis of a protein kinase, the double-stranded RNA activated inhibitor (DAI). The DAI phosphorylates eIF2, preventing recycling and inhibiting chain elongation (Ochoa 1983; Schneider and Shenk 1987). VA RNA binds to DAI, thus preventing phosphorylation of eIF2 (Katze et al., 1987; Mellitis and Mathews 1988; O'Malley et al., 1989). It was noted that eIF2 phosphorylation increased during Ad2 infection, correlating with the shut off of host protein synthesis.

A model was proposed by O'Malley et al (1989) that VA RNAI is involved in both the selective translation of adenovirus and the shut down of host cell protein synthesis. The model relies on the compartmentalization of viral and cellular mRNAs. The viral compartment would contain viral mRNAs and VA RNAI, which would prevent DAI activation and thus permit active protein synthesis. The host mRNA compartment would not contain VA RNAI, thus DAI would be active in phosphorylating eIF2 and inhibiting host protein
synthesis. This model is supported by some experimental results which show that VA RNAI has a specific affinity for late viral mRNA (Mathews 1980).

1.3. CLASS I MHC ANTIGEN-RESTRICTED RECOGNITION AND ITS ROLE IN COMBATING VIRAL INFECTION.

1.3.1. Introduction

The most basic feature of the immune response to a foreign antigen is its specificity for that particular antigen. Lymphocytes are the agents of antigenic specificity in immune response. They are divided into two groups: B cells that make immunoglobulins (antibodies), and T cells that perform a heterogeneous set of regulatory functions including help for B cells, production of delayed-type hypersensitivity reactions, release of lymphokines, and the specific killing of virus-infected cells. Immunoglobulins are the sole source of B cell specificity, whereas molecules from two diverse families of cell surface glycoproteins, the T cell receptors and the major histocompatibility complex glycoproteins, are the key elements of specificity in the T cell response to foreign antigens.

Although immune activities involving these two sets of lymphocytes are equally important, functionally they are different. For instance, antibody to a virus is primarily important in resistance to reinfection by a given virus, and a susceptible host can be passively protected by injecting antibodies; but antibody plays a small part in recovering from the first infection with a given virus, whereas T cell-mediated immune mechanisms are crucial.

Cytolytic lymphocytes, defined by their capacity to kill target cells in vitro, are major effector cells in cell-mediated immunity. At least 3 types of cytolytic cells have been described, namely, cytotoxic T (Tc) cells, nature killer (NK) cells and K cells. Among them, Tc cells recognize viral antigen peptides only in association with class I MHC antigens, a phenomenon termed MHC-restriction. The immune recognition mediated by Tc cells is important as it focuses on the infected cell, which is the source of progeny virus, rather than on free viral particles.
Therefore, it is a very efficient way of combating viral infection and eradicating infected cells in the body. In the following review, the mechanism of Tc cell recognition and its role in limiting viral infections is discussed.

1.3.2. Structure of the class I MHC antigen and the T cell receptor

There are two structurally distinct, but related families of MHC molecules that present antigens to T cells: class I MHC molecules (H-2K, D and L in mouse; HLA-A, B and C in human), present antigens to T cells that express the CD8 cell-surface glycoproteins, and class II MHC molecules present antigens to T cells that express the CD4 cell-surface glycoprotein (Parnes 1989; Teh et al., 1988). Class I antigens commonly present peptides derived from endogenously synthesized proteins such as viral components produced upon virus infection and result in the stimulation of CD8-bearing Tc cells (for review: Townsend and Bodmer 1989). On the other hand, class II molecules generally present peptides derived from exogenously synthesized proteins and stimulate CD4-bearing helper T cells (Unanue and Allen 1987).

In both mouse and man, the antigenic structure of class I molecules consists of two non-covalently associated polypeptides (Silver and Hood 1974; Springer et al., 1974). A large MHC encoded subunit of 44kD (heavy chain) is complexed with a smaller subunit of 12kD which is not MHC encoded. This small subunit is β2-microglobulin (Grey et al., 1973). The heavy chain spans the membrane bilayer, oriented with its N-terminal major portion on the outside of the cell. The extracellular portion of the heavy chain has been divided into three domains called α1, α2, and α3 (Orr et al., 1979), composed of 90, 92 and 92 amino acids, respectively. Most amino acid substitutions between the products of different class I alleles are localized to the α1 and α2 domains with the α3 domain and β2-microglobulin being relatively conserved (Parham et al., 1988).

The α1 and α2 domains have been the focus of most interest, because this part of the class I antigen is believed to be the binding site for peptides which will be recognized by Tc cells. X-ray crystallography
of a purified class I antigen (HLA-A2) revealed that domains $\alpha_1$ and $\alpha_2$ form a platform composed of a single $\beta$-pleated sheet topped by $\alpha$-helices with a long groove between the helices. Within the groove, it appeared to contain a bound peptide (Bjorkman et al., 1987a). This groove is linked by amino acid residues that are hypervariable and are the reason for the class I gene polymorphism (Bjorkman et al., 1987b). The hypervariability is believed to reflect the ability of different class I antigens to bind different antigen peptides and to interact with different T cell receptors (Bjorkman et al., 1987b).

T cell receptor (TCR), in common with the immunoglobulin receptors of B cells, are products of somatically rearranging genes and are clonally expressed (for review: Hedrick 1988). The mature TCR chains are glycoproteins of 260-300 residues in length, comprising a short cytoplasmic tail, a transmembrane region, and two extracellular domains—one constant and one variable. The V region is the main component of the antigen recognition site, which recognizes the complex of antigen peptide and MHC (reviewed in: Danska 1989). The overall structure of the TCR molecule is similar to the distal portion of an antibody molecule. There are, however, differences in antigen recognition by these related groups of lymphocyte receptors. For instance, the type of antigenic determinant (epitope) recognized by TCR differs from that of B cell receptors. TCRs recognize short, liner peptide determinants of 10-20 amino acids which are products of degradated or processed antigenic proteins (Maryanski et al., 1986; Townsend et al., 1986), whereas immunoglobulins usually interact with epitopes formed by the three-dimensional structure of native proteins, although they can also be raised against peptides.

1.3.3. Tc cell recognition is class I MHC antigen restricted

Tc cell mediated cytolysis is an important mechanism in allograft rejection, tumor destruction and lysis of syngeneic or autologous cells infected by viruses. The first step of Tc cell mediated cytolysis is recognition. The mechanism of Tc cell recognition has been most extensively characterized in the mouse, but current studies are
revealing equivalent findings in humans. Evidence that the recognition was MHC antigen restricted was first obtained in mice when cytolytic activity against cells infected with lymphocytic choriomeningitis virus was shown to be H-2 restricted (Zinkernagel and Doherty 1974). The observation has been widely confirmed in other vertebrates, including man (Zinkernagel 1979). This restriction in both man and mouse is mediated via class I MHC antigens. When cells of known class I type are infected by a given virus (A), Tc cells are induced which will only kill infected cells of the same class I type, but not cells with a different class I type, even though they are infected with the same virus (A). The same cells infected with virus B or uninfected cells of the same or different class I type are not killed, either. Sophisticated studies have since confirmed that the Tc cell recognizes neither the viral antigen nor MHC antigen alone, but both in association, i.e. the viral antigens must be correctly presented on the cell surface in conjunction with the class I antigens (Schwartz 1985). The specificity of the recognition mechanism is against both the MHC haplotype and the viral specific antigen.

A central question still to be answered about Tc cell recognition is the conformation of the TCR and its orientation when engaged with MHC/antigenic peptide complexes. There have been two theories proposed to account for Tc cell recognition. The model that Tc cells carry two distinct receptors (dual recognition) for recognition of both foreign antigen and a self-MHC molecule was first suggested by cellular immunologists in the early 1970s (Blanden and Ada 1978; Janeway et al., 1976). The other theory is based on single recognition model (Matzinger 1981) which states that TCR has a single recognition unit recognizing self MHC plus foreign epitopes. This means that the epitopes recognized by TCR are formed by interaction of foreign antigens with self MHC. Recent reports have supported the latter theory, as transfection experiments have demonstrated that a single TCR recognizes both foreign antigen and MHC molecule (Dembic et al., 1986).
1.3.4. Antigens recognized by Tc cells

Although Tc cells generated as a result of a variety of virus infections could be shown to be specific for the viruses as far as groups are concerned, what remained unclear for several years was the nature of the antigen(s) recognized by class I-restricted Tc cells and how they might interact with the class I MHC molecules. The traditional concept was that Tc cell recognizes foreign glycoproteins inserted alongside MHC molecules in the membrane of the target cell (Gething et al., 1978; Schrader and Edelman 1977). However, recent work has shown that the epitopes recognized by class I MHC-restricted Tc cell can be defined in the lysis assay with short synthetic peptides (Maryanski et al., 1986; Townsend et al., 1986). In addition, target cells that produce rapidly degraded fragments of viral proteins, which are not detectable serologically at the cell surface, are recognized efficiently by Tc cells (Townsend et al., 1985). Additional evidence that expression of an intact viral protein at the surface of a target cell is not by itself sufficient for recognition by Tc cell has arisen from work with vaccinia virus as a vector for the expression of influenza virus hemagglutinin (Coupar et al., 1986). It was found that despite the presence of serologically detectable hemagglutinin molecules at the cell surface, presentation of hemagglutinin peptides to Tc cells was inhibited during the late phase of vaccinia virus infection. These results are consistent with the concept that class I-restricted Tc cells recognize protein antigens only after they have been degraded in the cytoplasm of the cell in which they are synthesized (Townsend et al., 1985), or into which they have entered by fusion of virus with an endosome membrane (Yewdell et al., 1988).

The new view of antigen processing and presentation in the Tc cell response to a virus includes the following steps (for reviews refer to: Townsend and Bodmer 1989; Yewdell and Bennink 1990). The antigenic proteins are first degraded to short peptides. The site of degradation is supposed to be the cytosol. The mechanism of antigen processing is a poorly understood event, but it appears to involve limited proteolytic degradation (Townsend et al., 1986). The peptides produced are transported into the endoplasmic reticulum, where they interact with
class I heavy chains. This in turn triggers proper folding and association with β2-microglobulin (Townsend et al., 1989). The interaction between peptides and class I MHC antigen may be also important for the stability, conformation and cell-surface expression of the class I MHC antigen (reviewed in: Parham 1990). The class I antigen-peptide complex is then transported to the cell surface, where it can be recognized by Tc cells (Cox et al., 1990).

1.3.5. The role of Tc cells in combating viral infection

Considerable progress has been made in documenting the biological significance of virus-specific Tc cells in elimination and control of viral infections. The generation of virus specific Tc cells, which recognize and lyse virus infected cells in a class I MHC antigen restricted manner, has been demonstrated in many viral infections (Ahmed and Stevens 1990). It is well established that Tc cell responses decisively influence the outcome of some virus infections. For instance, in murine lymphocytic choriomeningitis virus (LCMV) acute infection, clearance of the virus is effected by specific Tc cells (Lehmann-Grube et al., 1988). In contrast to the acute infection, mice infected with LCMV at birth or in vitro become persistently infected with lifelong viremia, and most of their organs contain high levels of infectious virus. The persistence of LCMV in these carrier mice is accompanied by T-cell unresponsiveness to the virus. This is a highly specific defect. Such persistently infected mice exhibit no generalized immune suppression, but they show limited or no detectable Tc cell response against LCMV (Ahmed et al., 1984; Pircher et al., 1990). This T-cell defect is the primary reason why these carrier mice are unable to eliminate the virus. The best evidence for this comes from experiments which show that adoptive transfer of CD8+ T cells from LCMV-challenged mice results in the clearance of virus from carrier mice (Ahmed et al., 1987). In influenza virus infection, virus spread is limited either by γ-interferon released by Tc cells at the site of virus infection, or solely by lysis of infected cells mediated by Tc cells. Cloned influenza virus specific Tc cells have been shown to protect mice against 10 lethal doses of the specific virus but not
against one lethal dose of a non-cross-reacting virus. (Lukacher et al., 1984). Although other viral systems have not been studied as extensively, available evidence suggests that Tc cells are important for the control of infection. For example, in human cytomaglovirus (CMV) infection, immuno- suppressed humans die of progressive CMV infection in the presence of normal or high antibody levels (Rasmussen et al., 1982). Furthermore, T cell deficient mice are more susceptible to mouse CMV infection than normal controls (Grundy and Melief 1982), and transferred T cells confer protection against CMV infection (Grundy and Melief 1982; Reddehase et al., 1985).

As discussed previously, Tc cell recognition requires viral antigen as a short peptide fragment in association with a class I MHC glycoprotein. Hence, any event that alters the expression or appropriate processing of viral proteins to peptides or influences the expression of class I MHC antigens would prevent or minimize Tc cell response.

Reduction of class I MHC antigen expression on host cells as a consequence of viral infection has been reported for several viruses. These include Herpes virus type 2 (Jennings et al., 1985), some types of adenovirus and ectromelia virus (Gardner et al., 1975). Among them, the best documented example of virus mediated reduction of cell surface expression of class I MHC antigen is by adenovirus (discussed in detail in the next section). As expected, the reduction in the surface levels of class I MHC antigen required for antigen presentation and Tc cell recognition is accompanied by reduced Tc cell activation and Tc cell mediated lysis (Yewdell et al., 1988).

It has also been observed that some viruses escape Tc cell recognition by their failure to express or present viral antigens which are required for Tc cell recognition. For instance, recognition by Tc cell of the phosphoprotein pp89, the immunodominant viral antigen expressed in the immediate-early phase of CMV infection, was selectively prevented during the subsequent expression of viral early genes (Del Val et al., 1989). This resulted in reduced or lack of lysis of infected target cells by Tc cell, although the surface expression of class I
MHC glycoproteins and their capacity to present externally added pp89-derived antigenic peptides were not affected. Studies carried out with LCMV infected cells have shown that in contrast to acutely infected mice and cells, mice persistently infected in vivo, as well as cultured mouse cells persistently infected in vitro with LCMV, selectively lose some viral antigen expression (Oldstone and Buchmeier 1982), which includes the immunodominant Tc cell antigen. This failure of expression of some genes has been considered to be the mechanism used by the virus to set up persistent infection. It has also been documented that some tumor cell lines induced by viruses can escape Tc cell recognition by failure to express viral antigens (Green 1983; Vasmel et al., 1989).

Interactions between Tc cell and their targets involve an initial phase of effector/target adhesion in which some of the cell-surface adhesion molecules are involved (Shaw and Luce 1987). Studies have shown that down-regulation of these cell-surface adhesion molecules is involved in the escape of EBV positive Burkitt's lymphoma cell lines from EBV-specific Tc cell recognition. Certain EBV-positive lymphoma cell lines were not killed by EBV immune Tc cells when tested. The resistance of these lymphoma cells to Tc cell killing was not due to altered expression of MHC class I genes nor due to a lack of viral gene expression, but correlated with a reduced level of adhesion molecules. The mechanism involved in the selective suppression of these molecules is not known, but such studies nicely illustrate yet another viral strategy of evading Tc cell recognition.

1.4. THE CELLULAR IMMUNE RESPONSE TO ADENOVIRUS INFECTION.

1.4.1. NK cells

NK cells are one of the major components in the host's non-specific natural defence system. It has been found that mammalian cells transformed by nononcogenic human adenoviruses exhibit high susceptibility to destruction by host NK cells and macrophages. Comparison of target cell lines transformed by overlapping segments of the adenovirus E1-transforming gene region revealed that isolated
expression of a single oncogene, E1a, was sufficient to cause increased susceptibility to NK cells (Cook et al., 1986). In rodent cells, E1a induces susceptibility to lysis by NK cells and activated macrophages in both transformed and infected cells. E1a mutant viruses that express only one of the 289R and 243R products in infected cells are both competent to induce cytolysis of NK cells, suggesting either of these can sensitize infected cells to NK cells. Furthermore, there is a correlation between the accumulation of E1a gene products in Ad5-infected cells and the level of susceptibility of such target cells to lysis by NK cells (Cook et al., 1987). However, E1a of Ad12 fails to induce susceptibility to NK lysis in transformed cells (Cook et al., 1987; Kenyon and Raska 1986).

1.4.2. Cytotoxic T lymphocytes

1.4.2.1. E1a gene products from adenovirus play a central role as immunogens in Tc cell recognition.

Early studies have shown that mice infected by adenovirus can generate a specific cellular immune response, as assayed by high 51Cr release when labelled mouse adenovirus-infected target cells and immune spleen cells were mixed together (Inada and Uetake 1978). The first clue that E1a may serve as a target for Tc cell recognition came from studies primarily using cells transformed by group C adenoviruses and tumors induced in rat. These tissues displayed a tumor-specific transplantation antigen (TSTA). The viral antigens responsible for the TSTA and recognition by antitumor Tc cell were initially identified as residing in region E1 (Gallimore and Williams 1982; Raska et al., 1982). Direct evidence came from a rat cell study. Fisher baby rat kidney (BRK) cell lines immortalized by transfection with plasmids containing only the E1a gene of group C adenovirus were shown to be effective in stimulating the generation of cytotoxic T lymphocytes in vitro (Bellgrau et al., 1988). In mouse cells a study using deletion mutant viruses also defined E1a from Ad2 and Ad5 as the region whose products can be recognized by group C adenovirus-specific Tc cells (Mullbacher et al., 1989). Further studies have defined the immune domain more precisely in rat cells. Experiments have shown that the products encoded by either
13S and 12S mRNA alone were sufficient for the induction of adenovirus Tc cells. A series of viruses with mutations in the first exon of the E1a mRNA also induced strong Tc cell responses, while Ad5 mutants with lesions within the second exon failed to induce Tc cells. Based on these results, it was concluded that the C-terminal of conserved domain 2 of group C adenovirus E1a products was required for induction of Ad-specific Tc cell in rat cells (Urbanelli et al., 1989). A more recent study in rat cells has exclusively defined the immunodominant epitopes for anti-adenovirus Tc cell recognition to E1a gene products and has excluded the possibility that other adenovirus gene products may also serve as immunodominant antigens (Routes et al., 1991). The most intriguing demonstration is a functional study in which Tc cells generated from Ad5 E1 transformed cells, which were shown to specifically against products of Ad5 E1a (H-2D\^b restricted), were intravenously injected into B6 nude mice bearing Ad5 E1-induced tumors. These Tc cells, if combined with recombinant IL-2, destroyed subcutaneous tumor masses of up to 10 cm\(^3\). The *in vivo* action of these Tc cells was highly specific and long-term "memory" of these cells persisted in treated nude mice months after tumor regression (Kast et al., 1989). These results strongly argue that E1a is also an immunodominant antigen for Tc cell recognition *in vivo*.

1.4.2.2. Adenovirus proteins and class I MHC antigen cell surface expression

As discussed in section 1.3.2, the class I genes of the major histocompatibility complex encode glycoproteins that noncovalently associate with \(\beta_2\)-microglobulin. Virtually all cells of higher eukaryotes express class I MHC on their surface. These proteins play a crucial role in MHC-restricted cytolysis, a process in which cells expressing foreign antigens, for example as a result of viral infection, are recognized and destroyed by cytotoxic T-lymphocytes. Certain gene products from adenoviruses, namely E1a gene products of Ad12 and E3 19k of group C adenovirus, have been shown to interfere with class I MHC antigen expression on the cell surface. This has been considered to be related to
the events leading to adenovirus-mediated transformation and persistent infection.

The E1a gene of serotype 12 of group A adenovirus has been shown to specifically down regulate the class I MHC antigen gene transcription and thereafter reduce the cell surface expression of class I MHC antigen in transformed rat cells (Ackerill and Blair 1988; Schrier et al., 1983). This action is mediated by the 266R in Ad12 (Bernards et al., 1983). The down regulation of class I MHC antigens by Ad12 has been observed in a variety of cell lines including rat (Schrier et al., 1983), mouse (Eager et al., 1985), hamster (Ackerill and Blair 1988) and human cells (Vaessen et al., 1986; Vasavada et al., 1986). Although there are reports that Ad12 E1a can reduce the steady-state levels of class I MHC mRNA (Friedman and Ricciardi 1988; Schrier et al., 1983), to a large extent the molecular mechanism by which the shutdown of MHC class I expression occurs in Ad12 transformed cells is not understood.

The tumor-inducing capacity of adenoviruses or Ad-transformed cells in rodent hosts is dependent on several factors. These include the adenovirus serotype and the degree of immune competence of the host. Since group A adenoviruses are oncogenic in vivo (for review: Graham 1984) and they are also the subgenus whose E1a gene products have been shown to down regulate class I gene expression in transformed cells, it has been suggested that "turn-off" of class I MHC antigen expression by Ad12 E1a may be one reason why group A adenoviruses are more oncogenic than other adenoviruses (Bernards et al., 1983; Hayashi et al., 1985; Tanaka et al., 1985). On the other hand, this hypothesis has been challenged by studies which showed no correlation between tumorigenicity in immunocompetent syngeneic rodents of Ad12- and Ad5- transformed cells and the level of class I MHC molecules expressed on the surface of these cells (Haddada et al., 1986; Haddada et al., 1988). It seems therefore that other mechanisms also play a role in determining whether or not transformed cells will be capable of initiating a tumor in vivo. It has been suggested that NK cells may act as the first line of defence against tumor cells (Raska and Gallimore 1982).
As described earlier, the E3 19k protein is expressed in large amounts during the early phase of an adenovirus 2 infection. It is a fairly typical transmembrane glycoprotein. Although its polypeptide chain has a molecular weight of 19kD, the mature protein is a 25kD molecule due to the fact that it contains two carbohydrate units. By immunoprecipitation of the E3 19k protein from transformed rat cells (Kvist et al., 1978), as well as from productively infected cells (Kampe et al., 1983; Signas et al., 1982), it has been shown that this viral protein can form complexes with class I MHC antigens. The complex is noncovalent (Burgert and Kvist 1987). E3 19k protein can form complexes with class I MHC antigens in HeLa cells which are transfected with an E3 expression vector, where only the E3 19k protein is present. Therefore, this complex formation does not require other adenovirus proteins (Andersson et al., 1985; Paabo et al., 1983). Other studies show that E3 19k from Ad2 binds to class I MHC antigens of different species, like mouse, human and rat. Furthermore, all of the E3 19k proteins from representative serotypes of adenovirus groups B (Ad3, Ad11 and Ad34), C (Ad2 and Ad5), D (Ad9 and Ad19) and E (Ad4), but not of group A, have been shown to bind to class I MHC antigen (Flomenberg et al., 1987; Paabo et al., 1986a). Thus, the binding of E3 19k to class I MHC antigens is a quite common phenomenon in adenovirus infected cells.

Studies have shown that the domains α1 and α2 of the class I heavy chain are essential for E3 19k binding (Burgert and Kvist 1987). A property of E3 19k which is crucial for its function is that it is retained in the membrane of the endoplasmic reticulum (Paabo et al., 1987). It is in the endoplasmic reticulum that complex formation between E3 19k and class I MHC antigen takes place. Pulse-chase experiments have demonstrated that the complexes form immediately upon synthesis of the reactive proteins in the endoplasmic reticulum and that this interaction abrogates the normal glycosylation and then transport of the class I molecules to the plasma membrane via the Golgi apparatus (Burgert and Kvist 1987; Severinsson and Peterson 1985). Consistent with this, a reduction of mouse class I antigen H-2Kd on the cell surface has been demonstrated by fluorescent activated cell sorter analysis when the
cells were transfected with a plasmid containing the Ad2 E3 gene (Burgert and Kvist 1987), and reduction of human class I antigen HLA-B7 on the surface of HeLa cells has been shown by an anti-alloreactive cytotoxic T-lymphocyte assay when cells were infected with Ad2 (Andersson et al., 1987).

Although E3 19k from most groups of adenoviruses has been shown to bind to class I MHC antigens in a variety of cell lines, other cell lines, however, show resistance to the adenovirus E3 effect on class I MHC antigen glycosylation and transportation. In one study, the generality of this phenomenon was tested in a variety of human cell lines. With the exception of the Ad5 E1 transformed cell line, 293, Ad2 and Ad5 infection of fibroblastic, epithelial and lymphoid cells did not cause major decreases in surface class I MHC antigen concentration until the terminal stages of infection when cell death was imminent. Furthermore, newly synthesized class I antigen continued to be expressed on the surface of most cell types at times when infected cells contained large amounts of group C adenovirus E3 19k glycoprotein (Routes and Cook 1990). These data indicate that most types of human cells are resistant to the E3 19k effect, suggesting that virus-specific Tc cell recognition and lysis of most Ad2 and Ad5 infected human cells should not be limited by E3 19k-mediated reduction in class I MHC antigen expression. Evidence has also accumulated that with class I MHC antigen of some haplotypes, E3 19k shows reduced binding ability or no binding at all. For example, in human HLA, HLA-A2 antigen was shown to bind E3 19k protein more than twice as efficiently as HLA-B7 molecules (Severinsson et al., 1986). In the mouse H-2 system, E3 19k protein can bind H-2Kd and Db efficiently, but shows no binding to H-2Kk and Kb (Burgert and Kvist 1987; Tanaka and Tevethia 1988). A more intriguing observation is that although E3 19k does not bind to mouse class I antigen H-2Kk, the H-2Kk restricted, Ad5 specific Tc cell response to cells expressing E3 19k is still decreased, compared with E3 deletion mutant infected cells (Mullbacher et al., 1989). A recent report shows that the E3 19k protein can inhibit the presentation of protein antigens processed from the cytosol without decreasing the levels of
class I expression at the cell surface, thus it is concluded that the relevant parameter for antigen presentation is the rate of MHC class I molecule exocytosis, not the level of class I cell surface expression (Cox et al., 1990).

In summary, recognition of virus infected targets by Tc cells requires that both class I MHC antigen and viral antigenic peptides be presented on the target cell surface. Quantitative and/or qualitative changes of either of these components will affect the Tc cell recognition. As mentioned early in this chapter, an E3 gene product, E3 19k has been shown to be able to bind with class I MHC antigen of certain haplotypes and reduce their expression on the cell surface. This indeed reduces adenovirus specific Tc cell recognition. However, Mullbacher et al (1989) reported that in mouse L929 cell, in which the restricting haplotype of class I antigen is H-2K^k, E3 deletion mutant infection could induce much higher Tc cell response than from wild type (w.t.) Ad5 infection. As E3 19k from group C adenovirus is unable to bind to H-2K^k, it is unlikely that this reduced Tc cell recognition comes from reduced H-2K^k expression on the target cell surface. This observation led me to postulate and test the possibility that E3 gene products from group C adenoviruses may reduce Tc cell recognition by down regulating immunodominant E1a antigen expression.
1.5. SCOPE OF THIS THESIS

The cellular immune response is one of the most important mechanisms of the host's defence system in combating viral infection. Naturally many virus groups have developed some strategies to evade the host's cellular immune surveillance and build up persistent infection and/or transformation. Among these viruses, adenovirus is one of those which have been well studied. One of the mechanisms proposed for adenoviruses to evade cellular immune surveillance is the effect of the E3 19k protein from some groups of adenoviruses on host class I MHC antigen cell surface expression. E3 19k is maximally produced at an early time of infection and binds to newly synthesized class I MHC molecules in the endoplasmic reticulum. This binding blocks their transport to the cell surface and results in reducing Tc cell recognition. However, in some cell lines, like L929, E3 gene products are shown to reduce Tc cell recognition without affecting class I MHC antigen expression on the cell surface. The aim of this thesis is to investigate further a role of E3 gene products in regulating Tc cell recognition and test the possibility that E3 gene products from group C adenoviruses may down regulate expression of the immunodominant E1a antigen.

In Chapter 3 of this thesis, I have described some experiments which showed that E3 gene products did not affect the function and cellular surface expression of H-2K\textsuperscript{k}, but did affect E1a gene protein production. This is followed in Chapter 4 by some experiments designed to investigate possible mechanisms for the E3 gene products to interfere with E1a expression. Results presented in this chapter showed that E3 gene products did not down regulate E1a expression at transcription, but rather at the level of translation. In Chapter 5, more experiments are designed to test the down regulation of E1a by E3 in different cell lines, and cells infected with a different serotype of group C adenoviruses. The data showed that down regulation of E1a by E3 gene products was a rather general phenomenon. Finally, in Chapter 6, preliminary studies of E1a expression in an E3 gene transformed cell line are described.
Chapter 7 provides a general discussion on E3 interference with E1a expression and some possible experiments for the future studies.
Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Cells and viruses

HeLa cells were obtained from the American Type Tissue Culture Collection and were free from adenovirus-associated virus (AAV). The 293 cell line is a human embryonic kidney cell line transfected by adenovirus that expresses X1A and X2A, and was originally obtained from Dr. Bruce Stillman (Cold Spring Harbor, New York). COS cells are a monkey (African green monkey kidney) cell line transfected by SV40 that expresses SV40 large T and small viral antigens of SV40 (Glewman, 1981) and was originally obtained from Dr. John Jenkins, Maria Caris Research Institute, Surrey, U.K. Normal rat kidney cells (NRK 498) were obtained from Dr. J. DeLambe. All the mouse cell lines are standard lines in the Division. All cell lines were routinely screened to ensure they were free of mycoplasma. Wild-type (wtA) adenovirus serotypes 2 (Ad2) and 5 (Ad5) were obtained from the American Type Tissue Culture Collection and were free from AAV. The mutant adenovirus d1312 which is deleted in the E1a region from 447 to 1250 nucleotides relative to the left end of the adenovirus chromosome (Shear et al., 1973) was a generous gift from Dr. Tomas Shear (Princeton University, New Jersey). Mutant d1355 which is a derivative of Ad5 and has a deletion of most of the coding sequences of E1a, plus a 14 bp deletion in exon coding frame 6 in E4 (Ballest et al., 1989; Harkert et al., 1985) was also obtained from Dr. Tom Shear. Mutant d1277 which is also a derivative of Ad5 and has a deletion for most of the E1a coding region, was originally obtained from Dr. J.R. Gutt, Indiana University, Wabashaw, New Jersey. Mutants d1604 and d1808 which have E3 and E4 region deleted, respectively (Childs and Kalber 1971) were generous gifts from Dr. G. Raskin, the John Hopkins University, Baltimore. The Ad5-Ad2-Ad5 recombinant virus rAd700 and a series E3 deleted mutants derived from this virus, d715, d722, d723, d735, d736, d755, and d764 were obtained from Dr. W. Wold, St. Louis University Medical Center.
2.1. MATERIALS

2.1.1. Cells and viruses

HeLa cells were obtained from the American Type Tissue Culture Collection and were free from adeno associated virus (AAV). The 293 cell line is a human embryo kidney line transformed by adenovirus, that expresses E1A and E1B (Graham et al., 1977) and was originally obtained from Dr. Bruce Stillman (Cold Spring Harbor, New York). COS cells are a monkey (African green monkey kidney) cell line transformed by SV40 that expresses SV40 large T and small t antigen of SV40 (Gluzman, 1981) and was originally obtained from Dr John Jenkins, Marie Curie Research Institute, Survey, U.K. Normal rat kidney cells (NRK 49F) were obtained from Dr J. DeLarco. All the mouse cell lines are standard lines at the Division. All cell lines were routinely screened to ensure they were free of mycoplasma. Wild type (w.t.) adenovirus serotypes 2 (Ad2) and 5 (Ad5) were obtained from the American Type Culture Collection and were free from AAV. The mutant adenovirus dl312 which is deleted in the E1a region from 447 to 1350 nucleotides relative to the left end of the adenovirus chromosome (Shenk et al., 1979) was a generous gift from Dr. Tom Shenk (Princeton University, New Jersey). Mutant dl355 which is a derivative of Ad5 and has a deletion of most of the coding sequences of E3, plus a 14 bp deletion in open reading frame 6 in E4 (Bellett et al., 1985; Halbert et al., 1985), was also obtained from Dr. Tom Shenk. Mutant dl327 which is also a derivative of Ad5 and has a deletion for most of the E3 coding region, was originally obtained from Dr. J.R. Cutt, Rutgers University, Piscataway, New Jersey. Mutants dl801 and dl808 which have E3 and E4 region deleted, respectively (Challberg and Ketner 1981), were generous gifts from Dr. G. Ketner, the John Hopkins University, Baltimore. The Ad5-Ad2-Ad5 recombinant virus rec700 and a series E3 deletion mutants derived from this virus, dl712, dl713, dl722, dl739, dl753, dl762 and dl764 were obtained from Dr. W. Wold, St. Louis University Medical Centre.
2.1.2. Antibodies

The antibodies used in this thesis include the following: M73, a monoclonal anti-E1a antibody generously provided by Dr. Ed Harlow, Cold Spring Harbor (Whyte et al., 1988); H14b, a polyclonal serum from tumour-bearing hamsters designated anti-Ad5 E1b 58kD from E. Blair, Leeds University, England (Braithwaite and Jenkins 1989); a polyclonal anti-hexon antiserum from E. Blair, (Braithwaite and Jenkins 1989); a monoclonal antiserum, pAb 421, which detects mouse p53, provided by Dr. H. Sturzbecher (Harlow et al., 1981). A series of antisera against individual E3 gene products, anti-E3 19k, anti-E3 14.7k, anti-E3 14.5k, anti-E3 11.6k, were obtained from Dr W. Wold (Tollefson et al., 1990; Tollefson and Wold 1988; Wold et al., 1985; Wold et al., 1984).

2.1.3. Chemicals, enzymes, and miscellaneous

Restriction endonucleases were purchased from New England Biolabs, Amersham, or International Biolabs Inc. DNAse, RNAse, and proteinase K were molecular biology grade from Sigma Chemical Company. Protein A Sepharose CL-4B was purchased from Pharmacia. All other reagents were analytical grade and obtained from Sigma Chemical Company or British Drug Houses. Autoradiograph film was either MP from Amersham, Cronex from Dupont, or X-AR5 from Kodak. Radioisotopes were $^{32}$P-αdCTP (3000Ci/mmol), $^{125}$I-labelled protein A (30μCi/mg total protein A) and L-[$^{35}$S] methionine (1200 Ci/mmol) from Amersham. Cell culture media DMEM (Flow Laboratories Inc) and phosphate buffered saline (PBS) (0.137M NaCl, 6.75mM Na₂HPO₄, and 2.5mM NaH₂PO₄) were provided by the JCSMR tissue culture support facility. Fetal calf serum (FCS) was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, and was heat inactivated at 56°C for 30 minutes prior to use.
2.2. METHODS

2.2.1. Cells and viruses

2.2.1.1. Cell culture

Continuous human cell lines 293, HeLa, Daudi, K562, monkey cell line COS, rat cell line BRK, and mouse cell lines (L929, C3HOH, MC57, 5R and L929-Ld) were routinely grown in 175 cm² tissue culture flasks (Falcon) with DMEM supplemented with 10% FCS in a 37°C humidified incubator with 5.0% CO₂. Cells were subcultured by washing monolayers twice with PBS, and trypsinizing with 0.025% trypsin, usually for 1-3 minutes. Cells were then seeded to 175 cm² flasks for maintenance or to 9 cm petri dishes for experiments.

2.2.1.2. Adenovirus growth

Adenoviruses were grown by infecting HeLa cells (or 293 cells for the E1a defective virus, dl312) with 1 IU/cell in 10ml/175 cm² flask of DMEM containing 1% FCS at 37°C for 1.5 hours. 50 ml of the same medium was then added to the cells and the infection was allowed to proceed for 4-8 days, until cells were like grape-like clusters and some of the cells detached from the flasks. Cells remaining adherent were dislodged by gentle agitation. The cellular suspension was centrifuged at 500g and the cell pellet was resuspended in 1ml of 20mM Tris pH 7.4/175 cm² flask. Cells were lysed by three 5 minute cycles of freezing (-70°C) and thawing (37°C). The crude lysate was centrifuged at 500g to pellet the cellular debris. The supernatant containing virus was aliquoted and stored at -70°C until use.

2.2.1.3. Viral titration

Two methods were used to titrate every virus used in the experiments of this thesis.

(1) Indirect fluorescent antibody staining. In this technique, titration of viruses were calculated by the fluorescent cell counting method (Philipson 1961). Coverslips within 5 cm petri dishes were seeded with either HeLa or 293 cells. When cells were just confluent, the media were removed and the monolayers were infected for 1.5h with a
series of tenfold dilutions of virus in 10μl DMEM+1%FCS. Then another
5ml of medium was added to each dish. Wild type virus infections were
allowed to proceed for 24 hours whereas all viral mutants grew for 30
hours. The infected monolayers were carefully washed three times with
PBS, and fixed in acetone overnight at -20℃. The coverslips were
extensively washed with PBS before reacting with mouse anti-Ad5
antibody for 30 minutes at 37℃. Excess antiserum was removed by three
5 minute washes in PBS. Cells were then reacted with goat anti-mouse
IgG fluorescein conjugated antibody for 30 minutes at 37℃. Cells were
washed three times for 5 minutes each in PBS and mounted using
PBS:glycerol (50:50). To calculate the titre of virus per ml, approximately
10 fields of cells were viewed under UV and the positive cells were scored
using an Olympus Fluorescent Microscope. The average number of cells
was multiplied by the calibration factor of the microscope at the
magnification used, divided by the dilution of the fraction of a ml used on
the coverslip.

(2) Viral DNA copy number detection. This method is set up to
check whether equal virus input can be obtained after cells are infected
with equal multiplicities of w.t. virus and mutants. L929 cells (2-5x10^5)
in 5 cm petri dishes were infected with a series of 5 fold dilutions of the
same multiplicities of w.t. virus and mutants based on the titres obtained
from indirect fluorescent antibody staining. The duration of infection
was 3h, a time before viruses start to replicate after penetration. Cells
were extensively washed with warm PBS and trypsinized. Viral DNA
copy numbers in these infected cells were measured by a dot blot
hybridization technique (details of this method are described in section
2.2.3.1). Radioactive signals from this hybridization were quantitated
using densitometric scanning. The comparative levels of viruses were
estimated by taking into account the virus dilution and the value of OD
from the densitometric scanning.
2.2.2 DNA Techniques

2.2.2.1. Plasmid growth

Plasmid DNA was isolated by a modified alkaline lysis procedure and purified by CsCl gradient centrifugation (Birnboim and Doly 1979; Ish-Horowicz and Burke 1981). Briefly, a single colony of bacteria was initially inoculated in 10 ml of LB plus selective antibiotic, and grown at 37°C for 8h. Then, 500ml cultures containing the appropriate selective antibiotic with 5ml of the 10 ml of LB culture of the bacteria carrying the indicated plasmid were grown at 37°C. After 16-24 hours the bacteria were harvested by centrifugation. Bacteria were lysed by resuspending in 9ml of Solution I: 50mM glucose, 25mM Tris-HCl pH 8.0, and 10mM EDTA, and then adding 1ml of Solution I containing 50mg of Lysozyme. Cells were further treated with the addition of 20ml of Solution II: 0.2 M NaOH and 1% SDS, on ice for 10 minutes. The solution was neutralized by the addition of 15ml of Solution III: potassium acetate pH 4.8. Cellular debris were pelleted by another centrifugation. The supernatant was strained through gauze and the DNA within the supernatant was precipitated by the addition of 0.6 volumes of isopropanol. The nucleic acid precipitate was pelleted by centrifugation and the pellet was washed in 70% ethanol, dried, and then resuspended in 4ml of TE (pH8.0) to which 4.8g of CsCl and 200ul of ethidium bromide (50mg/ml) were added. The solution was then centrifuged for 10 minutes at room temperature to remove crude debris. The clear supernatant was then transferred to quick seal Beckman tubes, sealed and centrifuged in a Ti50 at 40,000 rpm for 48-72 hours at room temperature. After centrifugation, the closed circular plasmid DNA banded midway between the nicked circular or linear DNA, and the RNA pellet at the bottom of the tube. The closed circular plasmid DNA band was collected. Ethidium bromide contained in the DNA solution was extracted with equal volumes of NaCl saturated isopropanol until the DNA layer was clear. The plasmid solution was dialysed against TE pH8.0 for 16h. Plasmid DNA was then precipitated by adding 2 volumes of distilled H2O and 6 volumes of ethanol. The precipitated nucleic acids were collected by centrifugation at 10,000 rpm for 20 minutes at 4°C in a SS34 rotor.
Plasmid DNA used for transfection into mammalian cells was banded an additional time in CsCl, and are referred to as double banded plasmids. The concentration of plasmid DNA was calculated from the optical density at 260nm, and the preparation was stored at -20°C.

2.2.2. Preparation of radioactive Probes

Probes for DNA and RNA hybridization were radioactively labelled by random priming (Hodgson and Fisk 1987). 50-100ng of DNA was combined with 2μg of pd(N)₆ oligonucleotide primers (Pharmacia) in a total volume of 10μl of solution. The mixture was placed in a boiling water bath for 5 minutes to be denatured. The DNA was cooled on ice and 8μl of dep distilled H₂O, 25μl of 2X reaction mix (100mM KH₂PO₄ pH 6.8, 20mM Mg acetate, 0.1M DTT, 2mM dATP, 2mM dGTP, 2mM dTTP), 5μl of 3²P dCTP (3000 Ci/mMol) and 10U of Klenow fragment were added. After 60 minutes at 37°C the reaction was stopped and the probe was purified by ethanol precipitation. The probe was boiled in hybridization buffer for 2-3 minutes immediately prior to being added to the membrane to be hybridized.

Alternatively, probes were radioactively labelled using Prime-It™ Random Primer Kit as described in the instruction manual. 25 ng DNA in a sterile microcentrifuge tube was mixed with 10μl random oligonucleotide primers and water to make a total volume to 33μl. This mixture was heated to 95°C-100°C for 5 min, followed by cooling on ice. 10μl of 5x primer buffer, 5μl of labelled nucleotide and 2μl of T7 DNA polymerase were added. The mixture was incubated at 39°C for 3 min. The reaction was terminated by the addition of 2μl of stop mix. The probes were purified by ethanol precipitation as described above.

2.2.2.3. Mammalian cell transfection

Calcium phosphate transfection was essentially performed as described by Graham and van der Eb (1973). Total of 20μg of DNA was diluted with water to make up to 0.5ml. Then 10μl of calcium solution was added to the DNA solution and gently mixed. Another 50μl of calcium solution was added and also gently mixed. This mixture was
added slowly, dropwise to the gently bubbling solution in a tube which contained 0.5ml of 1x HBS and 10μl of phosphate solution. The suspension was allowed to sit at room temperature for 20 min. The suspension was added slowly and dropwise to a 9cm plate of cells containing 10 mls of complete medium while gently swirling the medium in the plate. The plate was returned to incubator and left for 16h. The medium containing the precipitate was removed and fresh, complete medium was added and cells were incubated in for the indicated time (Graham and van der Eb 1973; Parker and Stark 1979).

Alternatively, cells were transfected by a modified calcium phosphate precipitation method (Chen and Okayama 1988). 20μg of DNA was added to 500μl of 0.25M CaCl2. Then 500μl of BES-buffered saline pH 6.95 (50 mM BES, the N,N-bis-2-hydroxyethyl-2-aminoethanesulfonic acid, 280 mM NaCl and 1.5 mM Na2HPO4) was added. The mixture was left at room temperature for 10-15 minutes for a fine precipitate to be formed. The DNA precipitate was added dropwise with gentle swirling to the cell culture. The cells were then incubated in a humidified 3% CO2 chamber overnight. The following morning the cells were rinsed three times with pre-warmed medium and then left in a humidified 5% CO2 chamber.

For making a transformed cell line, cells were transfected with a total of 20μg plasmid DNA in which a proportion of them was the plasmid containing neo, the geneticin resistant gene. Three days after transfection a selection medium (DMEM) containing 0.7mg/ml of geneticin (the concentration of geneticin was determined by titration on cell's capacity to resist the effect of geneticin) was added. Selection medium was changed every 4-5 days until all of the untransfected cells died and single transformed colonies appeared. Cells were then trypsinized and transferred to 175 cm2 flask to grow for a certain time to amplify the cell number. After enough cells were obtained, part of them were used to check the expression of transfected gene with radioactive labelling and immunoprecipitation as described in section 2.2.5.1.
2.2.3 In situ hybridization

In situ hybridization was performed as described (Paeratakul et al., 1988). Cells were harvested by trypsinization and were centrifuged at 1000 rpm for 10 minutes at 4°C. Cells were washed once with cold PBS and the cell pellet was resuspended in 2ml PBS/9 cm petri dish. A single cell suspension was obtained by syringing the cells through an 18 gauge needle several times. The cells were counted and adjusted to 5x10^5 cells/ml. A Bio-Rad Hybridot apparatus was assembled using a 8x12 cm sheet of Zeta Probe which was previously soaked in PBS. A series of two-fold dilution of cells was made by dispensing 200μl, 100μl, or 50μl of cells in adjacent wells, equivalent to 1x10^5, 5x10^4, and 2.5x10^4 cells per well respectively. Vacuum suction was applied to concentrate the cells onto the filter.

2.2.3.1 DNA detection

To detect viral DNA within the cells, the cells were fixed to the Zeta Probe and lysed by the addition of 300μl of 0.5M NaOH to each well. The duration of this reaction was 30 min, at 4°C. A vacuum was applied until no liquid remained. The filter was air dried, then sucked briefly in 2xSSC immediately prior to prehybridization in 10ml of; 1.5xSSPE, 1% SDS, 0.5% Blotto, 0.5 mg/ml herring testis DNA, at 65°C for more than 2 hours. Filter was hybridized to the radioactive probe in prehybridization buffer for 16-24 hours.

2.2.3.2 RNA detection

To detect RNA within cells, the cells were fixed by adding 100μl/well of; 1% gluteraldehyde, 3% NaCl, 10 mM NaH2PO4, 40 mM Na2HPO4, for 1 hour at 4°C. The vacuum was then applied until excess liquid was removed. The fixed cells were washed three times with 100mM Tris pH 8.0/50 mM EDTA in the apparatus with the vacuum applied. The apparatus was disassembled. The cells on the filter were digested in 20mls of 100mM Tris pH 8.0/50 mM EDTA containing 40 μg/ml of proteinase K at 37°C for 30 minutes in a sealed bag. After digestion the filter was air dried and then rinsed in 2xSSC. The filter
was then prehybridized in: 50% deionized formamide, 2xSSPE, 1% SDS, 0.5% Blotto, and 0.5 mg/ml of herring testis DNA, at 43°C for more than 2 hours. The filter was hybridized in prehybridization buffer containing >10^7 cpm of the radioactive probe at 43°C for 16-24 hours (Paeratakul et al., 1988).

Both DNA and RNA filters were washed after hybridization at room temperature for 15 minutes in 2xSSC/0.1% SDS, 0.5xSSC/0.1% SDS, and 0.1xSSC/0.1% SDS. If the signal from the hand radioactive-monitor was too high after these three washes, the last wash would be repeated but at 65°C. Damp filters were sealed in bags and autoradiographed at -70°C for various length of time. The relative amount of DNA and RNA on the filter were measured by densitometric scanning of the dots which appeared on the X-ray films.

2.2.4 mRNA analysis

2.2.4.1 Isolation of polysomal RNA

Polysomal RNA was isolated by a magnesium precipitation procedure (Palmiter 1974). Cell monolayers were trypsinsized and washed once with PBS. The cells were then resuspended in 9 volumes of ice-cold buffer which contains: 25mM NaCl, 5mM MgCl2, 1mg/ml heparin, 2% Triton X-100 and 25mM Tris-HCl (pH7.5). Cells were homogenized thoroughly in a Dounce homogenizer (Kontess Glass Co.) by 10 strokes. Cell debris were pelleted by centrifuging the homogenates at 27,000g for 5 min and the supernatants were collected. An equal volume of the homogenization buffer (but containing 0.2 M MgCl2) was added to the supernatant and the mixture was incubated at 4°C for 1h. 8 ml of this sample was layered over 4 ml of 1 M sucrose in 25 mM NaCl, 0.1 M MgCl2, 25 mM Tris-HCl (pH7.5) and the polysomes in the sample were pelleted by centrifugation at 27,000g for 10 min. The supernatant and part of the sucrose layer were aspirated and discarded. The walls of the tube was rinsed with water and the remaining sucrose was aspirated and decanted. The polysomal pellets were drained briefly and resuspended in 2 ml of 20 mM Hepes buffer.
The polysomal RNA was extracted from polysomes by a proteinase K-phenol procedure (Mechler and Rabbitts 1981). The polysomal suspension was mixed with an equal volume of hot (100°C) SDS buffer containing: 1% SDS, 0.2 M NaCl, 40 mM EDTA, 20 mM Tris-HCl (pH7.4). It was incubated at 100°C for 2 min. The mixture was rapidly cooled to 30°C by putting on ice and Proteinase K was added to the final concentration of 0.5mg/ml. This mixture was incubated at 30°C for 10 min and then concentrated. Tris-HCl (pH 9.0) and SDS were added to a final concentration of 0.1 M and 1%, respectively. The solution was extracted three times with an equal volume of phenol:chloroform (1:1, v/v). The RNA was precipitated from the aqueous phase by adding 2.5 volume of ethanol and 0.1 volume of 2 M sodium acetate (pH 5.2) and storing at -20°C overnight. The precipitated RNA was recovered by centrifugation. The concentration of RNA was determined in UV spectrophotometer at 260nm.

2.2.4.2. Slot blot analysis of polysomal RNA

To glyoxylate RNA samples for slot blot analysis, 20μg of the indicated RNA was resuspended in 3.7μl of dep-treated distilled H2O to which 2.7μl of 6M deionized glyoxal, 8μl of DMSO, and 1.6μl of 0.1M sodium phosphate buffer pH 6.7, were added. RNA was glyoxylated at 50°C for 1 hour. After glyoxylation the RNA was diluted 10 fold with 10mM sodium phosphate buffer pH 6.7 and loaded onto Zeta Probe membrane which was assembled in a Bio Rad Hybri-slot Manifold. Slots were rinsed with 10mM sodium phosphate buffer under vacuum filtration. The manifold was disassembled and the filter was air dried and vacuum baked at 80°C for 2 hours to remove the glyoxal groups. The filters were then prehybridized in: 50% formamide, 0.25 M sodium phosphate buffer pH 7.2, 0.25M NaCl, 7% SDS, 1mM EDTA, for 16 hours at 43°C. Filters were hybridized in fresh prehybridization buffer containing a specific random primed probe at 43°C for 16-24 hours. The filters were then washed successively for 15 minutes in each of the following solutions at room temperature: 2xSSC/0.1% SDS; 0.5xSSC/0.1% SDS; and 0.1xSSC/0.1% SDS. If necessary, the last wash was repeated at
65°C for 15-30 min. The filters were then autoradiographed at -70°C for the indicated period of time.

2.2.4.3. Northern analysis of polysomal RNA

Cytoplasmic RNA samples and 3μl of RNA ladder (Pharmacia) were glyoxylated as described in the Slot blot protocol. After RNA was glyoxylated, four microliters of loading buffer (50% glycerol, 0.01M sodium phosphate pH6.7, and 0.4% bromophenol blue) were added and the samples were loaded immediately onto a 1% agarose gel made up in 0.01M sodium phosphate buffer pH 6.7. Electrophoresis was at 6 Volts/cm with recirculation, and was continued until the bromophenol blue had migrated approximately 2/3 of the distance of the gel. RNA was then transferred to Zeta Probe nylon membranes using the alkaline blotting method described in the Zeta Probe manual.

Prehybridization and hybridization to Zeta probe membranes were essentially performed as described in the manufacturers instruction manual. Filters were prehybridized in: 50% formamide, 2xSSPE, 7% SDS, 0.5% Blotto, and 500 μg/ml herring testis DNA at 50°C for 6 hours. Filters were then hybridized in: 50% formamide, 2 X SSPE , 1% SDS, 0.5% Blotto, 500 μg/ml herring testis DNA, and the specific random primed probe at 50°C for 16-24 hours. The filters were washed successively for 15 minutes in each of the following solutions: 2xSSC/ 0.1% SDS at room temperature; 0.5xSSC /0.1% SDS at 65°C; and 0.5xSSC/0.1% SDS at room temperature. The filters were then autoradiographed at -70°C for the indicated times.

2.2.5 Protein analysis

2.2.5.1. Immunoprecipitation

Cells in 9cm petri dishes were washed gently with PBS and were starved for methionine for 1h at 37°C. Cells were then labelled with 35S methionine (100μCi/dish) for 1.5-2.0 hours at 37°C. Unincorporated methionine was removed by gently washing the cells two times with cold PBS. The monolayers were chilled on ice and lysed with 1ml of RIPA buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA ,1% NP40, 0.1%
SDS) containing 20μl of Aprotinin. The lysate was pre-cleared by centrifugation and the supernatant was pre-adsorbed by rotating the lysate with 30μl of protein-A sepharose CL-4B beads plus 5μl of normal mouse serum (NMS) for 2 hours. The beads were pelleted by centrifugation and the supernatant was aliquoted into a number of eppendorf tubes into which 25μl of fresh beads and the specific antibody was added. After 24 hours rotation at 4°C, the beads were pelleted as described. The beads were washed three times with RIPA buffer and resuspended in 30μl of loading buffer (50mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 1% β-mercaptoethanol, 0.04% bromophenol blue). Samples were boiled for 5 min and chilled before loading onto a 9-15% SDS-PAGE gel. (Braithwaite and Jenkins 1989). Gels were fixed in 40% methanol/10% acetic acid for 30 minutes at room temperature; enhanced with Amplify (Amersham) and then dried. The dried gels were exposed to XAR film at -70°C without intensifying screens.

2.2.5.2. Western blot analysis.

Cells were lysed and the proteins in the lysates were separated by electrophoresis on SDS-PAGE gel as described in last section. The proteins on the gel were transferred to Hybond-C membrane (Amersham) overnight with BioRad mini transferring apparatus in a transferring buffer which contains 23 mM Tris-HCl, pH8.3, 192 mM glycine, 20% methanol, and 0.1% SDS. To reduce the background, the membrane was treated with 3% BSA in PBS for 3h. Then the membrane was incubated overnight at 4°C with antibody (concentration: 1-2μg/ml) in 1% BSA and 5% FCS in PBS. Membrane was extensively washed with PBS and 1% NP40 in PBS. This was followed by incubating membrane overnight at 4°C with 125I-protein A (10μCi/10ml antibody solution) in PBS solution containing 1% BSA and 5% FCS. The membrane was then thoroughly washed with PBS containing 1% NP40. Membrane was dried briefly and then exposed to XAR film with double intensifying screens (Simanis and Lane 1985).
2.2.6. Flow cytometric analysis of H-2K\textsuperscript{k} levels on the surface of target cells

The relative levels of H-2K\textsuperscript{k} on the surface of cells were determined by indirect immunofluorescence with flow cytometric analysis. Samples of 2X10\textsuperscript{6} Ad5 infected or control L929 cells were incubated for 30 min on ice with a saturating amount (0.6μg) of anti-H-2K\textsuperscript{k} monoclonal antibody (clone 11-4.1 Becton Dickinson Mountainview, CA) in 100μl medium plus 5% FCS. The cells were washed twice in medium at 4°C, resuspended in 100μl medium containing 1μg fluorescein isothiocyanate-conjugated goat anti-mouse IgG2 (Becton Dickinson), incubated for 30 min on ice, washed twice, and resuspended in 1 ml of medium. Fluorescence was analyzed on duplicate samples of 10\textsuperscript{4} or 2X10\textsuperscript{4} cells in a FACS IV (Becton Dickinson) with an argon ion laser set at 48nm, and a 515nm to 540nm bandpass filter.

2.2.7. Tc cell assay

2.2.7.1. Target cells

L929 (H-2\textsuperscript{k}) fibroblasts were infected in suspension with 20 IU per cell of adenoviruses or 20 plaque forming units of vaccinia virus for 1h prior to being replated (10\textsuperscript{7} cells) into 75cm\textsuperscript{2} Falcon flasks in 20 ml DMEM containing 5% FCS and incubated for 40h. Targets were then trypsinized, labelled with 51Cr and used in cytotoxicity assays. For super-infection, 40h-adenovirus-infected targets were infected with vaccinia virus for 1h with 20 plaque forming units per cell and labelled with 51Cr simultaneously.

2.2.7.2. Generation of effector cells

Primary virus immune splenocytes were generated by immunizing mice with 10\textsuperscript{7} IU of adenovirus or 10\textsuperscript{7} plaque forming units of vaccinia virus intravenously. Spleens were removed 6 days later. Secondary \textit{in vitro} virus-immune Tc cells were obtained by coculture of 8X10\textsuperscript{7} spleen cells from mice immunized with 10\textsuperscript{7} IU of Ad5 or 10\textsuperscript{7} plaque forming units of vaccinia virus (at least 7 days prior to the removal of the spleen), with 1X10\textsuperscript{7} syngeneic spleen cells infected with 20
IU Ad5/cell or 5 plaque forming units of vaccinia virus/cell. Cocultures were then incubated for 5 days in 40 ml of DMEM supplemented with 0.1 mM 2-mercaptoethanol plus 5% FCS in a 5% CO2 atmosphere at 37°C.

2.2.7.3. Cytotoxic assay

The $^{51}$Cr release assay has been described previously (Mullbacher et al., 1989). Briefly, $10^5$ $^{51}$Cr-labelled target cells were incubated for 6 h with effector cells at various killer:target ratios. Percent maximum releasable $^{51}$Cr was calculated by water lysis of cells and spontaneous release was about 3% per hour. The corrected percent lysis was calculated by the formula:

Specific lysis (%) =

\[
\frac{\text{Experimental release - medium release}}{\text{Maximum release - medium release}} \times 100
\]
Chapter 3

Ad5 E3 gene products interfere with the expression of the Tc cell immunodominant E1a antigen
3.1. INTRODUCTION

Adenoviruses have been used by many workers to investigate such phenomena as cell transformation and eukaryotic gene regulation (see sections 1.2.4 and 1.2.5). In both areas adenoviruses have increased our understanding of the molecular mechanisms involved in these processes. The development of a large suite of mutant viruses with defects in different early region genes has been instrumental in this regard. The availability of such mutants has made it possible also to investigate antiviral immune mechanisms using adenovirus type 5 as a model virus.

Among the most effective agents in immune defense against viral infections are cytotoxic T lymphocytes (reviewed in section 1.3.5). Such cells recognize viral products on the surface of infected cells only in the context of major histocompatibility complex class I antigens (Zinkernagel et al., 1974; reviewed in section 1.3.3). Thus, one way in which viruses may evade Tc cell surveillance is to diminish or abolish the cell-surface expression of class I MHC antigens of the infected host cell. Adenoviruses accomplish this task by two separate mechanisms. A down-regulation of mRNA encoding class I antigens, in cells transformed by highly oncogenic human adenovirus type 12, is mediated by E1a functions (Bernards et al., 1983; Schrier et al., 1983; Tanaka et al., 1985; Vasavada et al., 1986; reviewed in section 1.4.2.2.). The down-regulation of cell-surface expression of class I antigens by human adenovirus type 2 is mediated by the E3 19k protein, a glycoprotein encoded by the E3 region of adenovirus (Kvist et al., 1978; Signas et al., 1982; Paabo et al., 1983; reviewed in section 1.4.2.2). Other adenoviruses of sub-groups B, C, D, and E were also found to interfere with the intracellular transport of class I MHC antigens by the E3 19k protein (Paabo et al., 1986a). A consequence of the inhibition of expression of class I MHC antigens at the cell surface is the reduced recognition by the class I restricted Tc cell (Burgert et al., 1987; Rawle et al., 1989).

However, E3 19k glycoprotein has been shown to bind with widely varying affinity to different class I MHC molecules. For instance, in the
mouse, inhibition of cell surface expression of certain allelic forms of class I MHC antigens such as \( \text{Db} \) and \( \text{Kd} \) has been observed but others like \( \text{Kb} \), \( \text{Dd} \) and \( \text{Kk} \) molecules appear not to be affected (Burgert et al., 1987; Burgert and Kvist 1987; Tanaka and Tevethia 1988). In the human, HLA-B7 molecule was shown to bind E3 19k protein much less efficiently than HLA-A2 antigen (Severinsson et al., 1986). Moreover, reduction of Tc cell recognition caused by E3 gene products does not always coincide with decreased cell surface expression of class I MHC antigens. For example, when E3 shows no binding to class I antigens, E3 gene products can somehow still reduce Tc cell recognition. For example, Mullbacher et al (1989) noted some modulation of the immune Tc cell response to virus infected L929 target cells, although the restricting class I MHC antigen, H-2K\( ^k \), is not bound to E3 19k protein. In spite of this, mutant viruses dl\( ^{355} \) and dl\( ^{327} \) which respectively contain defects in the E3 and/or E4 genes, when used to infect target cells, showed increased susceptibility to lysis by Ad5 immune Tc cells, compared to Ad5 w.t. virus infected targets. Mutant dl\( ^{327} \) is deleted for most of the E3 coding sequence, and dl\( ^{355} \) is similarly deleted for the E3 coding sequences, with an additional 14 bp deletion in ORF6 of E4 (Halbert et al., 1985). Thus, notwithstanding a possible role played by the E4 ORF6 encoded 34kD protein, the E3 gene encodes product(s) that decrease recognition of target cells by Ad5 immune Tc cells.

These observations led me to investigate whether E3 gene products from group \( \text{C} \) adenoviruses, in addition to modulating Tc cell recognition by binding to class I MHC antigen and reducing their cell surface expression, can affect Tc cell recognition by using alternative mechanisms. In this chapter I describe experiments designed to further investigate the role of Ad5 E3 gene products in the regulation of Tc cell recognition in cells in which E3 19k does not associate with class I MHC antigen.
3.2. RESULTS

3.2.1. Ad5 E3 gene products affect adenovirus specific Tc cell recognition in L929 cells.

Mullbacher et al (1989) reported that L929 target cells infected with an E3 mutant (dl327) or an E3/E4 deletion mutant (dl355) were more sensitive to lysis by Ad5 immune Tc cells, than when infected with the w.t. Ad5 virus. To confirm this result, L929 cells were infected with 20 IU per cell of w.t. Ad5, dl327, dl355 and an E1a deletion mutant, dl312, or mock infected. Wild type Ad5 was used to immunize CBA/H (H-2K^k) mice to generate effector cells. Forty hours after infection, the target cells were labelled with 51Cr and then Tc cell lysis was measured. Figure 3.1a depicts the lysis of L929 target cells infected with w.t. Ad5, dl312, dl327 and dl355, or mock infected, by Ad5 immune Tc cells at four effector to target cell ratios. Target cells infected with dl327 and dl355 showed much higher lysability as compared to target cells infected with w.t. Ad5. However targets infected with w.t. Ad5 still showed significantly higher levels of killing than target cells infected with the E1a deletion mutant dl312, which showed nearly as low a level of lysis as for mock infected cells.

The results presented in this section confirmed the earlier report that Ad5 E3 gene products affect adenovirus specific Tc cell response in L929 cells. The result that target cells infected with dl312 showed extremely low levels of lysis by w.t. Ad5 immunized Tc cells implies that Ad5 E1a products can be efficiently recognized by adenovirus specific Tc cells and/or that they are required to activate some other viral products, such as from E1b and E2A, that may also be recognised by Tc cells.

3.2.2. Ad5 E3 gene products do not interfere with H-2K^k cell surface expression.

One possible explanation for the results mentioned above, namely that target cells infected with E3 deletion mutants were more sensitive to lysis by Ad5 immune Tc cells than those infected with w.t. Ad5, is that one or more Ad5 E3 gene products interfere with the cell surface expression of class I MHC antigen. This hypothesis was tested by

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Fig. 3.1. Adenovirus infected target cell lysis by mouse cytotoxic T cells. L929 (H-2k) fibroblasts were infected in suspension with 20 IU per cell of w.t. Ad5 and E3 deletion mutants (dl355 and dl327). The duration of infection was 40h. Target cells were then trypsinized, labelled with 51Cr and used in cytotoxicity assays. Tc cells were generated as described in section 2.2.7.2. Lysis of L929 cells infected with w.t. Ad5, dl312, dl327, dl355 or mock infected, by secondary in vitro Ad5 immune Tc cells (a), or H-2Kk specific alloreactive Tc cells (b). Assay duration was 6h.
infecting L929 target cells with w.t. Ad5, E3 deletion mutants or mock infection, and measuring lysis with Tc cells specific for H-2K^k, the restricting element of class I MHC antigen in L929 cells (Mullbacher et al., 1989). Results in Fig.3.1b showed that there is a good lysis from alloreactive, H-2K^k specific (C3HOH anti CBA/H) Tc cells at 4 E:T ratios. The alloreactive Tc cells did not differentially lyse the same panel of target cells, suggesting that all cells expressed sufficient cell surface class I H-2K^k for Tc cell recognition and lysis. Furthermore, the identical slopes strongly suggested that surface H-2K^k concentration was not altered.

As discussed in section 1.3.4., antigens recognized by Tc cells are presented as short peptides. In the experiment described above, H-2K^k would have been recognised by alloreactive Tc cells as short peptides. Therefore, the results generated from the alloreactive assay may be a "gross" measurement of functional cell surface expression of H-2K^k for Tc cell recognition, which may not really reflect the actual surface level of this class I molecule. To detect directly the target cell surface expression of H-2K^k, cells infected with w.t. Ad5, dl355 and dl312, or mock infected were stained using saturation labelling of these targets with a monoclonal antibody specific for H-2K^k and analyzed by flow cytometry. The results are shown in Fig 3.2. No changes were observed in H-2K^k surface expression of w.t. Ad5 infected as compared to uninfected L929 cells (Fig3.2a). Cells infected with dl355 (Fig 3.2b) and dl312 (Fig 3.2c) showed slightly lower H-2K^k expression in this particular experiment which is inconsistent with the results obtained with Tc cell lysis. This observation is consistent with a report that the Ad2 E3 19k protein does not bind to mouse H-2K^k (Burgert and Kvist 1987).

Thus, the results obtained from both the alloreactive Tc cell assays and flow cytometric analysis clearly show that Ad5 E3 gene products do not interfere with cell surface expression of H-2K^k during adenovirus infection.
Fig. 3.2. Flow cytometric analysis of H-2K<sup>k</sup> expression on the surface of infected cells. The relative levels of H-2K<sup>k</sup> on the surface of cells were determined by indirect immunofluorescence with flow cytometric analysis. Samples of 2x10<sup>6</sup> Ad5 infected or control L929 cells were incubated with a saturating amount of anti-H-2K<sup>k</sup> monoclonal antibody. The cells were washed twice and labelled with fluorescein isothiocyanate-conjugated goat anti-mouse IgG2. Fluorescence was then analysed with a FACS IV machine. The distribution of H-2K<sup>k</sup> in mock infected cells (-----) was compared with that 48 h after infection (——) by w.t. Ad5 (a), dl355 (b) and dl312 (c). Details of this method were described in section 2.2.6.
3.2.3. Ad5 infection does not interfere with class I antigen (H-2K<sup>k</sup>)-peptide interaction.

Although no quantitative differences in class I MHC antigen expression could be detected on L929 target cells infected with either w.t. Ad5, dl327, or dl355, another possibility is that adenovirus infection may cause a functional change in H-2K<sup>k</sup> structure. For instance, although there are normal amounts of class I antigens synthesized and expressed on the cell surface, they might not present viral peptides efficiently for Tc cell recognition. To test this possibility, presentation of vaccinia virus peptides to vaccinia virus specific Tc cell by H-2K<sup>k</sup> was investigated after the cells were infected with adenovirus.

L929 targets were infected for 40 h with either w.t. Ad5 or dl355, or mock infected, and then "super infected" for 1 h with vaccinia virus. These super infected cells were tested for lysability with both Ad5 immune Tc cells and vaccinia virus specific immune Tc cells from CBA/H mice (Fig 3.3.). Ad5 immune Tc cells (Fig 3.3a) showed the same pattern of results as shown in Fig 3.1a: targets infected with dl355 were more susceptible to lysis than w.t. Ad5 infected targets. Vaccinia virus super infection of dl355 infected targets reduced their lysis by Ad5 immune Tc cells, probably due to vaccinia virus infection causing a general "shutdown" of protein synthesis, but they were still lysed significantly more than w.t. Ad5 infected targets. Vaccinia virus immune Tc cells lysed targets infected with w.t. Ad5 and super-infected with vaccinia virus to a marginally greater extent than target cells infected with dl355 and vaccinia virus (Fig 3.3.b), thus making it unlikely that Ad5 infection altered the ability of H-2K<sup>k</sup> to present vaccinia virus peptides.

The results shown in this section suggest that the ability of H-2K<sup>k</sup> to present viral peptides to Tc cell recognition is not significantly changed during adenovirus infection.

3.2.4. Viral gene dose effects on target cell susceptibility to lysis.

The results presented in Fig.3.1a showed that, consistent with the report by Mullbacher et al (1989), E3 gene products from Ad5 reduce the
Fig 3.3. *Vaccinia virus super infected target cell lysis by cytotoxic T cells.* L929 cells were infected with w.t. Ad5, dl355 or mock infected for 40 h, and then superinfected with vaccinia virus (open symbols) or mock infected (closed symbols) for 1 h. The target cells were lysed with primary *in vivo* Ad5 immune Tc cells (a) or secondary *in vitro* vaccinia virus immune Tc cells (b) as described in Fig.3.1. Assay duration was 6 h.
sensitivity of target cells to adenovirus specific Tc cells. However, results in Fig.3.2 and 3.3 demonstrated that the products encoded in the E3 gene of Ad5 do not interfere significantly with the cell surface expression or function of H-2K^k. These observations led me to postulate that E3 gene products from group C adenoviruses, in addition to reducing Tc cell recognition by binding to class I antigen of some haplotypes and reducing their cell surface expression, may also interfere with the expression of the immunodominant E1a antigen.

In the first experiment to test this hypothesis L929 cells were infected with different doses of w.t. Ad5 and E3 deletion mutants ranging from 1 IU to 1000 IU per cell, in order to see if infection of high w.t. Ad5 multiplicity could abrogate the different sensitivity to Tc cell recognition between targets infected with w.t. Ad5 and dl355.

L929 cells were infected with different amounts of virus, ranging from 1 to 1000 IU per cell, and lysability of targets by Ad5 immune Tc cells was assessed. Fig 3.4 shows that L929 cells infected with 1 to 100 IU dl355 are more susceptible to lysis than if infected with w.t. Ad5 at equivalent multiplicities of infection. However, 1000 IU per cell of w.t. Ad5 was as effective as 100 IU per cell of dl355 (see Fig 3.4), suggesting that increased virus dose overcomes the effect of E3/E4 genes as far as presentation of early gene peptides to Tc cells is concerned. The E1a mutant dl312 showed only a slight increase over a 1000 fold range of infective virus dose, thus excluding the possibility that nonspecific factors were responsible for the disappearance of differential lysability of w.t. virus versus dl355 infected targets at high multiplicity.

The data presented in this section are consistent with the hypothesis that products of E3 and/or E4 may reduce the amount of one or more of the viral antigens recognised by Tc cells. As reviewed in section 1.4.2.1, E1a has been shown to be the immunodominant antigen for adenovirus-specific Tc cell recognition, and is thus the most likely gene whose expression may be down regulated by E3 or E4 gene products. Further experiments were done to address more directly this hypothesis.
Fig 3.4. Lysis of L929 target cells infected with increasing doses of Ad5.

L929 cells were infected with $10^0$ to $10^3$ IU per cell of w.t. Ad5, dl355 or dl312. The infection duration was 40h. The target cells were lysed by Ad5 immune Tc cells as described in Fig.3.1. The effector to target cell ratio was 10:1. Assay duration was 6 h.
3.2.5. E1a protein levels are lower in wild type than E3 deletion mutant infected cells.

To test directly the possibility that E1a may be down regulated in w.t. Ad5 infected cells by E3 gene products, comparisons of E1a protein levels were made between cells infected with w.t. Ad5 and the E3 deletion mutant dl355.

Mouse L929 cells were either infected with 20 IU per cell of w.t. Ad5 or mutant dl355 or mock infected. At 40h after infection cells were labelled with $^{35}$S-methionine for 2h. Precleared cell lysates were immunoprecipitated with the anti-E1a monoclonal antibody M73, anti-E3 19k polyclonal antibody and normal mouse serum (NMS). The immunoprecipitated proteins were analysed on a 12% SDS-acrylamide gel. Results in Fig 3.5 show a comparison of E1a protein in w.t. Ad5 and dl355 infected cells. The E1a protein level was much higher in dl355 infected cells than in cells infected with w.t. Ad5. Densitometric quantitation showed the difference to be 8-10 fold. A protein with molecular weight around 105kD was seen in the E1a track. This protein was presumed to be the retinoblastoma susceptibility gene product (P105), as E1a has been shown to form a complex with this protein (Whyte et al., 1988).

To exclude the possibility that this difference in protein level of the E1a products might be due to cells in two groups receiving different amounts of virus during viral infection, a DNA-dot blot analysis was done 3 h post-infection, before viral DNA replication started. E1a DNA was used to make a $^{32}$P-labelled probe by the random priming technique (see section 2.2.2.2). This radioactive-labelled probe was hybridized to the membranes on which equal number of cells infected with w.t. Ad5 or dl355 were spotted. The result is shown in Fig. 3.6. No difference in viral DNA copy numbers was detected when equal multiplicities (20 IU per cell) of w.t. Ad5 and dl355 were used for infection.

The results in Fig.3.5 reflect only the levels of newly synthesized E1a protein products, since cells were only labelled with $^{35}$S-methionine for a short time (2h). To see whether the total E1a protein level is also lower in w.t. Ad5 than E3/E4 deletion mutant infected cells, Western blot
analysis was employed. Again mouse L929 cells were infected with either 20 IU per cell of w.t. Ad5 or mutant dl355, or mock infected. At 40h after infection cells were lysed. The proteins from cell lysates were immunoprecipitated with saturating amounts of the E1a monoclonal antibody and analysed by Western blotting as described in section 2.2.5.2. Consistent with the results from immunoprecipitation, E1a products were found to be much lower in w.t. Ad5 infected cells, relative to the cells infected by dl355 (Fig 3.7.).

The results presented in this section show that both newly synthesized and total E1a protein levels in w.t. virus infected cells are substantially lower than in the cells infected with the E3/E4 deletion mutant dl355.

3.2.6. E3, but not E4, gene products are responsible for down regulation of E1a.

The results obtained in the above section were from studies using mutant dl355. This mutant has a large deletion in the E3 region as well as a 14 bp deletion in open reading frame 6 of E4 which encodes a 34kD protein. To examine the effect of E3 alone on E1a expression, another Ad5 derived mutant with only the E3 region deleted, dl327, was used to make a comparison with w.t. Ad5 for E1a expression. L929 cells were infected with 20 IU per cell of w.t. Ad5 or dl327. The immunoprecipitation was done as described. Results in Fig. 3.8a show the comparison of E1a proteins as well as a protein from the E1b region, E1b 55kD, in w.t. Ad5 and dl327 infected cells. Similar to the cells infected with dl355, dl327 infected cells also show much higher levels of E1a protein than in w.t. Ad5 infected cells. The E1b encoded 55kD protein was also higher in mutant infected cells.

The above results indicate that E3 may be involved in down regulation of E1a expression at some level. To exclude another possibility that E3 and E4 may act in concert, i.e. both E3 and E4 can down regulate E1a expression, a comparison of E1a protein levels was made between w.t. virus and an E4 deletion mutant, dl808. The deleted region of this mutant is from 92.0 to 97.1 m.u., and it synthesizes no E4 polypeptides
(Challberg and Ketner 1981). Results in Fig. 3.8b show that there is only marginal difference in E1a protein levels between w.t. Ad5 and dl808. Mutant dl327 showed the usual pattern: E1a levels were much higher than w.t. Ad5.

Based on results presented in this section, it appears that one or more of the products from the E3 region, down regulates expression of Ad5 E1a at some level, but that E4 has little or no effect.

3.2.7. Time course of E1a expression in w.t. Ad5 and E3 deletion mutant infected L929 cells.

The data presented above are derived from experiments which were done at a single time point: 40h after infection, the time at which Tc cell assays were done (section 3.2.1). To investigate E1a protein production over a range of time, a time course for E1a and E3 expression was performed in cells infected with w.t. Ad5 or with deletion mutant dl327 and dl355. Immunological data has shown that in the mouse cell line (L929), the specific lysis of infected targets was first detectable at 24h post-infection (Mullbacher et al., 1989). This implies that E1a is expressed prior to 24h post-infection. Therefore, the time course of E1a expression was begun at 12h post-infection.

Four sets of cells were infected at the same time with either w.t. Ad5 or mutants dl355 and dl327. The cells were labelled with $^{35}$S-methionine for 2h after different durations of infection, i.e. 12h, 24h 36h, 48h, and 72h post-infection. The proteins from the harvested cells were immunoprecipitated with anti-E1a and anti-E319k antibodies. The immunoprecipitated proteins were separated on a 12% SDS-acrylamide gel and viewed by fluorography as described in previous sections.

The relative amounts of viral proteins as determined by metabolic labelling were quantitated by densitometric measurements. The relative density of the protein bands was plotted against infection time (Fig. 3.9). At 12h after infection, E1a products from all three viruses, as well as E3 19k protein from w.t. virus were barely detectable. At 24h after infection, E1a products from dl355 and dl327 had begun to increase and reached a peak at 48h. The E1a levels at 72h post-infection still remained at about
Fig 3.5. **E1a protein products were down regulated in w.t. Ad5 infected cells.** Mouse L929 cells were infected with 20 IU per cell of w.t. Ad5 and dl355 or mock infected. Forty hours after infection cells were starved of methionine by incubation in methionine free medium for 1h, and then labelled with 100µCi of 35S methionine for 2h. Cells were then washed in cold PBS and lysed with RIPA buffer. Lysates were then immunoprecipitated with anti-E1a antibody M73 (Whyte et al., 1988), anti-E3 19k (Wold et al., 1985) or normal mouse serum (NMS). The immunoprecipitated proteins were analyzed by 12% SDS-PAGE and autoradiography as described in section 2.2.5.1.
**Fig. 3.6. Viral copy number detection by *in situ* hybridization.** L929 cells in 5cm petri dishes were infected with w.t. Ad5 and dl355 at different multiplicities (20, 4 and 0.8IU per cell) respectively. 3h after infection, cells were collected by trypsinization. Indicated numbers of cells were immobilized onto a Zeta-probe membrane. Cells on the membrane were fixed and hybridized to a $^{32}$P-labelled E1a DNA. Details of this technique were described in section 2.2.3.1.
Fig. 3.7. Western blot analysis of E1a from cells infected with w.t. and dl355. L929 cells were infected with 20 IU per cell of w.t. Ad5, mutant dl355, or were mock infected. Forty hours after infection, cells were washed and lysed with RIPA buffer. Cell lysates were precleared and immunoprecipitated with anti-E1a monoclonal antibody M73, anti-E3 19k polyclonal antibody, or NMS. The immunoprecipitated proteins were separated by 12% SDS-PAGE and transferred to a Hybond-C membrane. The membrane bound proteins were reacted with M73 and labelled with 125I-protein A.
**Fig. 3.8. Comparison of E1a levels between w.t. virus and mutants with only E3, or only E4 deleted.** Mouse L929 cells were infected for 40h with 20 IU per cell of w.t. Ad5, dl327, dl808, or mock infected. Cells were labelled with 35S-methionine for 2h. The cells were then lysed and the lysates were immunoprecipitated with M73, anti-E1b 55k, anti-E3 19k and NMS as described in Fig. 3.5. Proteins were analyzed by running a 12% SDS-PAGE and fluorography. Parts A and B were separate experiments.
Fig. 3.9. Time course of E1a and E3 19k protein expression in w.t. virus and E3 deletion mutant infected cells. L929 cells were infected with 20 IU per cell of w.t. Ad5, dl355 and dl327. Cells were labelled with $^{35}$S-methionine for 2h after an interval of 12h, 24h, 36h, 48h and 72h of infection, respectively. Cells were then lysed and the proteins from the cell lysates were immunoprecipitated with anti-E1a and anti-E3 19k antibodies as described in Fig.3.5. The immunoprecipitated proteins were analyzed by a 12% SDS-PAGE. The relative amounts of each protein were quantitated by densitometric measurements of the fluorographs. The relative density of the protein bands was plotted against the infection time.
70% of peak levels. On the other hand, E1a proteins from w.t. Ad5 remained at substantially lower levels during the whole duration of the experiment. In contrast, E3 19k protein from w.t. virus started to increase 12h after infection and immediately reached a peak level at 24h post-infection. After that, there was a slight decrease.

The time course experiment presented in this section shows that E1a products in w.t. Ad5 infected L929 cells remain at a much lower level than in E3 deletion mutant infected cells at all the time points investigated.

3.3. DISCUSSION

In some situations adenoviruses are able to reduce detection by cellular immune surveillance mechanisms by influencing the expression of cellular recognition elements (Paabo et al., 1989). In the case of Ad 12, a group A adenovirus, products encoded in the E1a gene transcriptionally down-regulate the gene encoding class I MHC proteins in some transformed rat cells (Schrier et al., 1983). On the other hand, the group C adenovirus, Ad2, does not affect class I transcription or protein synthesis, but reduces cell surface expression of some MHC class I molecules. In this instance, the E3 19k protein of Ad2 binds directly to \textit{de novo} synthesized class I proteins and inhibits their glycosylation (reviewed in section 1.4.2.2).

In the present study, another group C adenovirus, Ad5, has been used to investigate the cellular immune response to adenovirus. As was found for Ad2, it is showed that one or more E3 gene products of Ad5 influence the ability of Ad5 immune Tc cells to lyse infected target cells. Cells infected by Ad5 mutant viruses dl327 and dl355, which lack E3 coding sequences, are more efficiently lysed by Ad5 immune Tc cells than w.t. Ad5. In these target cells, the restriction element was H-2K\textsuperscript{k} (Mullbacher et al., 1989), which does not bind to the E3 19k protein of Ad2 (Burgert and Kvist 1987) or Ad5 (A.W. Braithwaite, unpublished observations). Consistent with this lack of binding to MHC class I molecules, no evidence could be found that Ad5 E3 gene products interfered with cell surface expression of MHC class I molecules, either
by gross antibody staining (Fig 3.2) or by their ability to participate in a Tc cell immune response (Fig 3.1b and Fig 3.3). Thus, in the experiments using Ad5, E3 gene products appeared to reduce Tc cell lysability by a mechanism that did not involve a reduction in expression of MHC class I molecules. This suggested that E3 gene products were affecting expression or processing of another component of the antiviral immune response, such as one or more of the viral antigens. In the case of the cytolytic T cell response to adenoviruses, E1a appears to be the most important antigen which can both elicit and be recognized by adenovirus specific Tc cells. (Bellgrau et al., 1988; Mullbacher et al., 1989; Urbanelli et al., 1989; reviewed in section 1.4.2.1).

An indication that the expression of E1a might be reduced by E3 gene products became apparent when it is found that increasing doses of w.t. virus could overcome the differences observed between w.t. Ad5 and dl355 infected cells in Tc cell lysability (Fig 3.4). This possibility was tested directly by comparing E1a gene expression in w.t. Ad5, dl327 and dl355 infected cells. It is found that at equivalent input numbers of viral DNA templates (Fig 3.6), E1a products were expressed at substantially lower levels during w.t. Ad5 infection compared with both E3 defective mutants dl355 and dl327, as determined by direct immunoprecipitation of metabolically labelled E1a proteins (Fig 3.5 and Fig 3.8) and by Western blotting (Fig 3.7). For the Western blot, quantitation showed that E1a protein expressed in w.t. Ad5 infected cells was 8-10 fold lower than for dl355. Such a difference in E1a protein levels might well account for the difference in Tc cell lysability observed between w.t. Ad5 and E3 deficient virus infected targets. Wild type Ad5 also showed lower levels of E1b 55kD protein detectable by immunoprecipitation (Fig 3.8a). One explanation for this is that E1b expression is E1a dependent, although there may be other explanations. One could see if there is some early viral protein whose expression is not affected by E3 products. In any case, relative to input viral DNA copies, E1a and E1b proteins are both clearly expressed in lower amounts from w.t. Ad5 compared to E3 deficient virus infection.
The time course experiment showed that E1a protein levels in w.t. Ad5 infected cells were substantially lower as compared with cells infected with E3 deletion mutants at all the time points examined (Fig 3.9). Theoretically, it is possible that during the period between 12h and 24h post-infection, there is a peak of E1a expression. This E1a expression stimulates E3 expression. When E3 reaches a certain level, it will act on E1a and down regulates its expression. The time course data presented here (Fig.3.9) does not show this E1a expression pattern since the time points may not be detailed enough.

Although some reports show that after viral infection E1a mRNAs are detectable within a few hours in HeLa cells or KB cells (Nevins et al., 1979; Spector et al., 1978), in our experiments the E1a proteins are barely detectable even at 12h post-infection. The reason for this difference may be due to the fact that HeLa cells and KB cells are permissive to adenoviruses, while mouse cells are non-permissive or semi-permissive (Bellett and Younghusband 1979; Braithwaite et al., 1981). The adenovirus replicative cycle is delayed in a semi-permissive infection (for review: Flint 1986)

Based on the data presented in this Chapter, it is concluded that Ad5 E3 gene products interfere with immune Tc cell lysability by reducing E1a antigen expression. How this may occur is investigated in the next chapter.
Chapter 4.

Negative regulation of E1a expression by E3 gene products may occur by translational means
4.1. INTRODUCTION

The controlled regulation of the expression of genetic information is central to all forms of life. As gene expression involves many processes, the regulation of gene expression is an extremely complex event and can occur at a number of different steps between initiation of transcription and production of the final functional protein product. To understand these regulation processes, one must first have a knowledge of the basic mechanics of gene transcription and protein translation.

The RNA of eukaryotic cells is made in the nucleus by one of three distinct RNA polymerases. The synthesis of pre-rRNA is catalysed in the nucleolus by RNA polymerase I. Heterogeneous nuclear RNA (hnRNA), which is processed into mRNA, is transcribed by RNA polymerase II. Small RNA species, containing 5S rRNAs and transfer RNA, are synthesized by RNA polymerase III. The synthesis of mRNA is carried out by RNA polymerase II and a multitude of nuclear factors. The DNA sequence of a transcription unit transcribed by polymerase II contains a number of recognition elements that play a role in initiation of transcription; 1) the DNA sequence 5' or upstream of the coding region of a typical gene contains the transcriptional regulatory sequences, which are often called promoters. Promoters are conserved sequences found most proximal to a gene and normally bind trans-acting factors including RNA polymerase II which facilitate transcription by forming stable transcriptional complexes that allow for multiple rounds of transcriptional initiation. There are two important elements within the promoter sequences. Usually a TATA box lies approximately 20-30 base pairs from the transcriptional start site. A family of general transcription factors, termed TFIID, bind to the TATA box, which facilitates the binding and correct positioning of polymerase II relative to the transcriptional start site (Simon et al., 1988). Upstream of the TATA box, a GC-containing sequence that often features a CAAT sequence, is found between -50 and -100 base pairs relative to the start site. Like the TATA box, the GC-containing sequence is recognized by a family of DNA binding proteins, which may function to stabilize the
TFIID interaction with the TATA box, and thus facilitate polymerase II transcription (Chodosh et al., 1988). It is now accepted that RNA polymerase II binds to promoter sites and begins mRNA synthesis at cap sites. 2) In addition to promoter elements, another class of DNA sequence elements that have strong effect on transcription by RNA polymerase II have also been described: enhancer elements. Enhancer elements usually lie upstream from the promoter and are DNA sequences that modulate the rate of transcription independent of position and orientation. Unlike promoters, enhancer elements vary greatly in their consensus sequences and consequently bind a large variety of trans-acting factors. Enhancers regulate the rate of transcriptional initiation, either positively or negatively, to amplify or silence the transcription from a particular promoter (Atchison 1988).

In almost all cases, the primary RNA transcript is not a functional RNA molecule; biochemical modification of the primary transcript is the rule. After RNA is synthesized, it is processed to become mature mRNA. Usually this process includes a series of modifications. The 5' end of the new RNA chain acquires a cap, a 5'-5' linkage between a methylated guanylate residue and the initiating nucleotide of the transcript (Perry and Kelly 1976). At the 3' end, a chain of 100 to 250 adenylate residues of poly A is added. This is called polyadenylation (Nevins and Darnell 1978). The final step in the mRNA processing is splicing. Splicing includes removal of one or more intervening RNA sequences, termed introns, and the joining of the remaining pieces, termed exons, which will appear in the finished mRNA product (Nevins 1983). The role of the various mRNA modifications is not completely understood but most evidence suggests that the cap plays a role in translational initiation and poly A has an effect on stabilizing mRNAs which are known to be less stable than ribosomal(r) RNA and transfer(t) RNA. After all modifications are completed, the mature mRNAs are transported to the cytoplasm to participate in protein synthesis.

The translation of genetic information carried by mRNA into protein requires the participation of enzymes and two other kinds of RNA, i.e. tRNA and rRNA. The site of translation is the ribosome. Once
ribosomes are linked to these RNAs, they are called polyribosomes or polysomes and the RNAs are called polysomal RNA (Chambliss et al., 1980). The decoding of the codons in mRNA occurs in three distinct phases. They are initiation, elongation and termination. Peptide chain initiation serves to decode the initiation codon and determine the reading frame for translation. AUG codes for methionine and is the initiation codon for all mammalian proteins. As mentioned in section 1.2.9, polypeptide chain initiation begins with formation of ternary complex consisting of initiation factor eIF2, GTP, and the initiator methionyl-tRNA(met-tRNAf). The ternary complex then binds to a 40S ribosome subunit. In the presence of a 60S ribosome, mRNA, additional factors, and ATP, an 80S initiation complex is formed setting the stage for chain elongation. Peptide chain elongation serves to translate all of the internal codons between the initiation and termination triplets. The termination phase of protein synthesis requires one of the termination codons UAA, UGA or UAG, which do not code for corresponding amino acids (Moldave 1985).

Theoretically, it is apparent that gene regulation can occur in any of these processes. At the transcriptional level, regulation events could happen at the initiation of transcription or during the complex life of RNA molecule modifications. The latter stages include the post-transcriptional production of mature cytoplasmic mRNA such as the splicing of transcripts and post-transcriptional covalent modifications (capping, polyadenylation and sequence editing), and the fate of the mature mRNA. Although little is known about the molecular mechanisms underlying regulation at these stages, it is clear that some, if not all, of these processes can be regulated (for review: Darnell et al., 1986).

The rules and processes of transcription and translation mentioned above, for most instances, are applicable to adenovirus E1a gene expression. The E1a gene has its own TATA motif (Gingeras et al., 1982) and enhancer element (Hearing and Shenk 1983b) which are involved in the initiation of RNA transcription. The mRNAs have one major cap site and a few minor initiation sites which are predominantly
used after the onset of viral DNA replication (Osborne and Berk 1983). At the end of the E1a DNA coding region there is a poly A site for all E1a mRNAs. It is preceded by the hexanucleotide sequence AAUAAA, which is specifically required for the cleavage of the precursor RNA prior to its polyadenylation (Montell et al., 1983). There is a common nuclear precursor RNA transcribed from the E1a gene. As splicing is heavily involved in E1a mRNA processing, the result is that three major mRNAs of sizes 13S, 12S and 9S are generated by differential splicing (Perricaudet et al., 1979; Tremblay et al., 1989). The mature forms of these mRNAs are transported to the cytoplasm and are translated into proteins. Adenovirus utilizes the cellular machinery for both transcription and translation.

Many sorts of gene regulation have also been reported in adenoviruses. For instance, as mentioned in section 1.2.4, the adenovirus immediate early gene E1a can transactivate other early genes. This transactivation mediated by E1a has been shown to occur at the level of transcriptional initiation, or more precisely, by acting on promoters and/or enhancers (Jones et al., 1988; Simon et al., 1988; Wu and Berk 1988). On the other hand, E1a itself is also regulated by E1b19kD protein or autoregulated at transcriptional initiation (Borrelli et al., 1984; Hearing and Shenk 1985; Jochemsen et al., 1987; White et al., 1988). Numerous changes in splice regulation and choice of polyadenylation sites during the transition from early to late phase of adenovirus infection have also been reported. These changes may greatly alter the products made from the adenovirus major late promoter (Akusjarvi and Persson 1981). The fate of a mature mRNA can be regulated by controlling either the stability or transport of mRNA from nucleus to cytoplasm. Gene regulation occurring at these steps has also been documented during adenovirus infection. For instance, E2 gene products from adenovirus type 5 have been shown to destabilize E1a mRNA and cause reduction of E1a production (Carter and Blanton 1978; Lazaridis et al., 1988).

Gene regulation is controlled to a large extent at the transcriptional level as described above. However, there is increasing
evidence for control at the level of translation. Translation may be regulated at initiation or elongation steps. Polypeptide chain initiation appears to be controlled largely by the state of phosphorylation of the initiation factor eIF2. This mechanism has also been described in adenovirus infection as discussed in section 1.2.9. VA RNAI from adenovirus has been shown to bind and inhibit the activation of double strand RNA-activated protein kinase which can phosphorylate eIF2 and prevent its recycling (Katze et al., 1987; O'Malley et al., 1989). This results in efficient recycling of eIF2 and increases the efficiency of translation. In eukaryotes, chain elongation may be controlled by mRNA degradation or by specific inhibition of capped mRNA translation. Gene regulation at the translational level may also involve the stability of protein products and protein modification (reviewed in: Ochoa 1983).

Results presented in Chapter 3 have demonstrated that E3 gene products can down regulate E1a expression. The experiments in this chapter were designed to test at what stage during adenovirus transcription and translation E1a is down regulated by E3 gene products.

4.2. RESULTS.

4.2.1. The total amount of E1a specific RNA is not changed in the presence of the E3 gene.

The first objective was to examine the E1a RNA levels to see whether they were changed in w.t. virus infected cells compared with cells infected with E3 deletion mutants. The abundance of E1a specific RNA was quantitated and compared by in situ hybridization (section 2.2.3). Meanwhile, E1a DNA copy number from the same pool of cells was also checked to see whether DNA templates were changed, as alteration in the number of E1a templates might alter the apparent level of transcription of the gene. Cells were infected at a multiplicity of 20 IU per cell and were harvested 40h after infection. The same number of cells infected with w.t. Ad5 and E3 deletion mutants, and serial two-fold dilutions of these cells, were loaded onto a Hybri-dot filtration apparatus containing a Zeta probe hybridization membrane. The membrane was
made in duplicate; one aliquot for hybridizing to DNA and one for RNA. E1a specific RNA and DNA were detected with a radioactive-labelled E1a probe as described in section 2.2.2.2.

Using this in situ hybridization technique it was found that the E1a RNA levels detected were identical between w.t. Ad5 and E3 deletion mutant infected cells when these cells were infected with equivalent multiplicities of viruses (Fig 4.1.1). Under the same conditions there was no hybridization to mock infected cells. Thus total E1a RNA levels are the same which means that it is unlikely that either RNA transcription or RNA stability could be important in accounting for the protein data presented in Chapter 3. This implies that E3 gene products may interfere with E1a expression by affecting E1a RNA processing, transport, and/or translational control.

To ensure that the in situ hybridization technique was detecting only RNA and not viral DNA on the membranes treated with proteinase K, duplicate filters were treated with RNAse prior to hybridization. In all cases tested the RNAse treated filter did not show any hybridization. To ensure that the signals from radioactive E1a probes were specific, uninfected L929 cells were used at all times as a negative control and no signals or only extremely low signals were observed in uninfected cells.

The results of in situ hybridization of DNA showed that viral DNA levels also remained basically the same between cells infected with w.t. Ad5 and E3 deletion mutants even 40h after infection, when the same dose of virus was used for infection. This is consistent with the report that naturally occurring mutants or hybrid viruses in which almost the entire E3 region is deleted still replicate like w.t. virus in cultured cells (Kelly and Lewis 1973). Furthermore, it excludes the possibility that the different level of E1a products in w.t. Ad5 and E3 deletion mutant infected cells (see section 3.2.5) are due to differences in viral DNA replication.

The above results indicate that E1a is not down regulated in w.t. virus infected cells at the level of transcriptional initiation or mRNA stability.
Fig. 4.1. E1a RNA levels are unaffected by E3 products. L929 cells were infected with 20 IU per cell of dl327, dl355, w.t. Ad5, or mock infected. Forty hours after infection, cells were collected. Indicated numbers of cells were immobilized onto a Zeta probe membrane. Cells on the membrane were fixed and hybridized for DNA and RNA (as labelled) with 32P-labelled E1a DNA in situ as described in Fig. 3.6.
4.2.2. Comparison of E1a polysomal mRNA in cells infected with w.t. Ad5 and E3 deletion mutants.

After RNAs are transcribed, only a proportion of the total population of primary transcripts are translated into proteins as there are a series of modifications of the primary transcripts. For example, if mRNAs are not properly capped, poly A tailed or spliced, then they may be improperly or less efficiently translated (Fitzgerald and Shenk 1981; Kozak 1978; Laski et al., 1986). Under certain circumstances a specific mRNA can be selectively transported from or retained in the nucleus (Newport and Forbes 1987). For instance, during the heat shock response, nuclear mRNA is selectively retained in the nucleus by an association with the nuclear matrix (Denome et al., 1989). In adenovirus infected cells, cellular mRNAs are retained in the nucleus and adenovirus mRNA is selectively transported (Schneider and Shenk 1987). Therefore, different distributions of mRNA may also affect translation. Furthermore, once the mature mRNA gets into cytoplasm, the comparative cytoplasmic mRNA level will depend on the stability of the specific mRNA. So the results presented in the above section, which showed that the total amounts of E1a RNA including nucleic and cytoplasmic RNA were not reduced in w.t. Ad5 infected cells, do not necessarily reflect the real translatable mRNA level. As the polysomes are the sites where translation takes place, therefore polysomal RNA was isolated to delineate further the mechanism of down regulation of E1a in w.t. virus infected L929 cells.

Cells were infected with 20 IU per cell of w.t. Ad5 or E3 deletion mutants, or mock infected. Thirty six hours after infection, cells were trypsinized and homogenized. The magnesium precipitation method (section 2.2.4.1) was used to isolate polysomes from the homogenates. The RNA was extracted from the purified polysomes by the proteinase K-phenol procedure (Mechler and Rabbitts 1981). The concentration of isolated polysomal RNA was quantitated by U.V. spectrophotometry. Equivalent amounts of polysomal RNA were immobilized onto a Zeta probe membrane using a slot blot apparatus. Radioactive(32P) labelled...
probes from E1a DNA, whole Ad5 DNA and α-tubulin were used to hybridize to the membrane (see section 2.2.4.2).

The results showed that E1a specific polysomal RNA as well as total Ad5 polysomal RNA were essentially the same in w.t. Ad5 and dl355 infected cells (Fig 4.2). Both E1a specific polysomal RNA and total Ad5 polysomal RNA were about two fold lower in dl327 infected cells than in cells infected with w.t. Ad5. However, this is the opposite to the expected E1a protein expression pattern. The reason of slightly lower E1a RNA in dl327 infected cells is not further pursued. Signals from α-tubulin showed approximately the same hybridizable levels of RNA in all cells, indicating that equal amounts of total polysomal RNA were loaded.

Consistent with the results obtained from total E1a RNA detection, the levels of E1a and total viral mRNAs isolated from polysomes of cells infected with w.t. Ad5 and E3 deletion mutants were shown to be essentially the same. This result indicates that down regulation of E1a in w.t. Ad5 infected L cells is not due to lack of E1a specific mRNA in the polysomes, the site where the mRNAs are translated.

4.2.3. E1a RNA is properly spliced in w.t. virus infected cells.

Another possible explanation for the low level of E1a in cells infected with w.t. Ad5 is that there is a difference in splicing control between w.t Ad5 and E3 deletion mutants. This would imply that in some way w.t. virus processes the hnRNA to the 12S and 13S poorly compared to E3 deletion mutants. A similar regulation mechanism has been reported during adenovirus infection (Yuo and Weiner 1989). To test this possibility, the polysomal RNAs isolated as described in the slot blot experiment were analysed by the Northern blot technique (section 2.2.4.3). Twenty μg of RNA was glyoxylated and loaded onto a 1% agarose gel. After electrophoresis, the RNA in the gel was transferred to a Zeta probe membrane by the alkaline transfer procedure, and a radioactive labelled E1a probe was used for detection.

The results in Figure 4.3 showed that the same classes of E1a transcripts are similar to previous reports (Nelson; PhD thesis, 1990).
Fig. 4.2. Slot blot analysis of E1a polysomal RNA from cells infected with w.t. Ad5 and E3 deletion mutants. A large number of L929 cells (1x10^8 cells) were infected with w.t. Ad5, dl327, or dl355 at 20 IU per cell, or mock infected. Polysomal RNA was isolated 40h after infection as described in section 2.2.4.1. Twenty μg of each polysomal RNA was applied to a membrane in a slot blot apparatus. Radioactive probes from E1a, whole Ad5 and α-tubulin DNA were used to hybridize the membrane (for details of making probes see section 2.2.2.2).
Fig.4.3. **Northern analysis of E1a polysomal RNA.** The polysomal RNA was isolated as described in Fig.4.2. E1a polysomal RNA from w.t. Ad5, dl327 or mock infected cells was analyzed by the Northern blot technique as described in section 2.2.4.3. Briefly, 20µg of RNA was glyoxylated and loaded onto a 1% agarose gel in 10mM phosphate buffer pH6.7. The gel was electrophoresed at 6 Volts/cm for 2h. RNA was transferred to a Zeta-probe membrane by the alkaline transfer procedure. The membrane was hybridized with an E1a DNA radioactive probe.
The patterns of E1a specific mRNA from w.t. Ad5 and dl327 infected cells are essentially the same. Consistent with the results obtained from the slot blot experiment, E1a mRNA in w.t. Ad5 infected cells showed about 2 fold less than that in dl327 infected cells. As there are 4-5 E1a transcripts with a similar migration rate, it is difficult to determine exactly which band represents which product. However, the similar patterns of the radioactive signals still gave some indication that there is no substantial difference in E1a splicing in the presence or absence of E3 products.

The similar pattern of E1a mRNA between w.t. Ad5 and E3 deletion mutant infected cells, shown by Northern blot analysis in this section, indicates that a lower level of E1a protein in w.t. Ad5 infected cells is unlikely to be explained by differential E1a mRNA splicing.

4.2.4. Stability of E1a proteins during infection.

The data presented so far in this chapter have failed to identify any clear difference in E1a mRNA transcription, splicing, stability or transport to the site of protein synthesis between w.t. Ad5 and E3 deletion mutants. It therefore seemed possible that down regulation of E1a by E3 products might occur by some translational control mechanism. The mechanism of gene regulation that occurs at translational level is also quite complex (for review see: Klausner and Harfork 1989). Like transcriptional regulation there appear to be factors which mediate both positive and negative control. Theoretically translational regulation can occur at the level of translational initiation and chain elongation, or by acting on the fate of translated proteins. Preliminary investigations were undertaken to evaluate the stability of E1a proteins by a pulse-chase labelling experiment.

To ensure that there were high enough levels of E1a proteins in w.t. virus infected L929 cells for detection in chase experiment, w.t. virus was used at 10 times higher multiplicity (200IU per cell) than dl327. Forty hours after infection, cells were labelled with 35S-methionine (200μCi per 9cm petri dish) for 30 min., then cells were washed twice and chased with DMEM medium containing 200mM unlabelled methionine. Cells
were chased for 15 min., 30 min., 60 min. and 120 min. respectively. Cells were then lysed with RIPA buffer as described. The precipitated proteins were analysed on 12% SDS-acrylamide gel. The amounts of E1a protein were quantitated by densitometric scanning. Relative E1a levels determined by densitometric scanning were directly subjected to linear regression analysis. Results in Fig.4.4 showed that although E1a levels were lower in w.t. virus infected cells at every point checked than that in cells infected with dl327, there was no significant difference in the slopes of the regression line for decay of E1a radioactivity between w.t. Ad5 and dl327 infected cells.

Another observation made in this experiment was that when 200 IU per cell of w.t. Ad5 was used for infection, the E1a protein level at zero time was still lower than the E1a level of cells infected with 20 IU per cell of dl327 (Fig.4.4). This result is consistent with the immunological data which shows that more than 10 times as much w.t. Ad5 as E3 deletion mutant was required to get the same lysability of infected target by Ad5 specific Tc cells (Fig 3.4).

The data of the pulse chase experiment presented in this section shows that there is no significant difference between the stability of E1a proteins in the presence and absence of E3 products. These results indicate that the different levels of E1a proteins between w.t. Ad5 and E3 deletion mutant infected cells cannot be explained by differential E1a protein stability.

4.2.5. The effect of E3 on E1a expression is a specific event.

The data presented in this chapter so far indicates that E3 down regulates E1a expression by affecting the ability of E1a mRNA to be translated. To study whether this down regulation is selective for E1a only, or selective for viral mRNAs, or concerns mRNA translation in general, the levels of some cellular proteins and viral proteins including some early and late viral gene products were compared between cells infected with w.t. Ad5, dl327 or mock infected.

L929 cells were infected with 20 IU per cell of w.t. Ad5, dl327, or mock infected. Forty hours after infection cells were labelled with $^{35}$S-
Fig.4.4. Linear Regression analysis of pulse-chase experimental data.
The viral infection, pulse-chase labelling and protein analysis were as described in the text. The relative E1a levels (OD) which were determined by densitometric scanning, were plotted against the chase time. The curves were determined by linear regression analysis of E1a levels.
methionine for 2h and then lysed with RIP A buffer as described. Immunoprecipitated proteins were analysed on a 12% SDS-acrylamide gel. As there are large amounts of late gene products produced at late times after adenovirus infection, a cascade detection of hexon was carried out. Fig 4.5a and Fig.4.5b show the results of immunoprecipitation of some of the viral early and late gene products. It seems that both early gene and late gene products were lower in w.t. Ad5 infected cells than in cells infected with dl327. However, quantitative measurement of these protein levels by desitometric scanning showed that the degree of down regulation of these proteins varied. Consistent with the the result shown in section 3.2.5, E1a proteins in w.t. Ad5 infected cells were about 10 times lower than in cells infected with dl327. E1b was 6 times lower, while hexon was only two times lower in w.t. Ad5 infected cells compared with that in cells infected with dl327. Fig4.6 showed the results of detection of some cellular gene products in infected and uninfected cells. The levels of cellular protein p53, a 375 amino acid nuclear phosphoprotein (Eliyahu et al., 1988) and class I MHC antigen H-2Kk were essentially the same in uninfected cells and cells infected with either w.t. Ad5 or dl327. In the anti-p53 antibody (421) track, two bands could be seen in the virus infected cells but not in the uninfected cells. This viral protein with an approximate molecular weight of 58kD is probably the E1b-55kD product, which has been reported to form a complex with p53 (Braithwaite et al., 1991a; Zantema et al., 1985).

Collectively, these results demonstrate that the down regulation effect of E3 gene products on translation is selective for viral mRNAs, and more particularly for early region mRNAs. A general down regulation of total gene expression was excluded as two cellular gene products which were detected in this experiment remain unchanged among cells infected with w.t. Ad5, dl327, or mock infected.

4.2.6. E1a proteins do not form a complex with E3 19k.

E3 19k has been shown to form complexes with some class I MHC antigens from certain haplotypes and cause reduction of class I antigen expression on the cell surface (Andersson et al., 1985; Burgert et al., 1987;
Fig. 4.5. Uneven effect of E3 on viral early and late gene expression. Cells were infected with 20 IU per cell of w.t. Ad5, dl327. Forty hours later, cells were labelled with $^{35}$S-methionine for 2h. Cells were then lysed. Cell lysates were immunoprecipitated with antibodies against some adenovirus early gene products (a) and late gene product hexon (b). The cascade precipitation of hexon was done by precipitating cell lysates overnight with anti-hexon antibody two times (marked in figure with C1 and C2 respectively). The precipitated proteins were analyzed on a 12% SDS-PAGE.
Fig.4.6. **E3 effect on mRNA translation is not a general event.** L929 cells were infected with 20 IU per cell of w.t. Ad5, dl327, or mock infected. Fourty hours after infection cells were labelled and lysed as described in Fig.4.5. Cell lysates were immunoprecipitated with anti-p53 (421), anti-H-2Kk or NMS. The immunoprecipitated proteins were analysed on a 12% SDS-PAGE.
Fig. 4.7. **E3 19k does not complex with E1a proteins.** L929 cells were infected with 20 IU per cell of w.t. Ad5 or mock infected. Fourty hours post-infection, cells were lysed with mild RIPA buffer which contains 1% NP40 but no SDS or sodium deoxycholate. Cell lysates were precipitated with anti-E1a antibody M73, anti-E3 19k or NMS. Separation of proteins and transfer of proteins to the membrane were done as described in Fig.3.7. The proteins on the membrane were blotted with M73 antibody and 125I-protein A sepharose.
reviewed in section 1.4.2.2). E1a proteins also have the ability to form complexes with some cellular proteins (Whyte et al., 1988; Whyte et al., 1989). Given this precedence, the possibility that E3 19k may form a complex with E1a proteins, which in some way regulates the ability of E1a mRNA to be translated into protein products, was investigated.

To test this possibility, the Western blot technique was employed. To make sure there were enough E1a products for analysis in w.t. Ad5 infected cells, 10 times more cells (2X10^7 cells) than usual were used. Cells were infected with 20 IU per cell of w.t. Ad5. Forty hours after infection, cells were lysed. Since there were reports that some protein complexes can only be seen when mild lysing buffer is used (Braithwaite et al., 1991a; Braithwaite and Jenkins 1989), cells were lysed with mild RIPA buffer which does not contain sodium deoxycholate or SDS. Anti-E3 19k, anti-E1a, or normal mouse serum precipitated proteins were separated on a 12% SDS-acrylamide gel. The proteins were then transferred to a Hybond-C membrane which was blotted with anti-E1a antibody plus 125I-protein A. The results are shown in Fig 4.7. In the E3 19k antibody track a band running a little bit higher than E1a could be seen. Since this band also appears in the E3 19k antibody track in mock infected cells, it presumably represents non-specific precipitation, probably actin. Otherwise no obvious band with similar molecular weight to E1a could be seen in the E319k antibody track in w.t. Ad5 infected cells, compared with mock infected cells, although E1a was clearly detectable in the anti-E1a track.

This result shows that at least with this technique (Western blot) no complexes between E1a and E3 19k were detectable in Ad5 infected L929 cells.

4.3. DISCUSSION

Although the ability of the E1a gene products to regulate the expression of other viral and cellular genes has been extensively studied (reviewed in section 1.2.4), much less is known about the regulation of E1a gene expression itself. However, regulation of E1a expression by cellular proteins or other adenovirus gene products does occur.
Experiments have shown that, relative to HeLa cells, E1a and E1b genes but not other viral genes in human myeloma cells were markedly repressed by differential RNA stabilization, resulting in 20-50 fold less E1a and E1b mRNAs at steady state late in infection. The reduced E1a mRNA level corresponded to an approximately 200-fold-lower abundance of E1a polypeptides (Lavery and Chen-Kiang 1990). The best studied viral gene product which can regulate E1a gene expression is E1b-19kD protein (reviewed in section 1.2.5). E1b 19kD appears to both positively and negatively regulate E1a expression. In transformed cells, it can enhance E1a expression at the level of transcription initiation (Jochemsen et al., 1987). In HeLa cells, infection of E1b 19kD deletion mutant showed a higher steady-state level of E1a proteins than w.t. virus infection (White et al., 1988). E2a has also been shown to regulate E1a protein production by affecting E1a mRNA stability (Carter and Blanton 1978; Lazaridis et al., 1988).

The data presented in Chapter 3 showed that E3 gene products can also regulate E1a expression, and this regulation was shown to be negative. In this chapter, further experiments were designed to determine whether this down regulation of E1a broadly occurs at the transcriptional or translational level. First the total amount of E1a specific RNA was detected by a dot blot technique. The results (Fig 4.1) showed that E1a RNA abundance was essentially the same in cells infected with w.t. Ad5 and an E3 deletion mutant, at equivalent DNA template numbers. This excludes the possibility that E3 may down regulate E1a at the levels of transcriptional initiation or mRNA stability.

After mRNAs are transcribed, a series of mRNA modifications occur. The mature mRNAs are then transported to the cytoplasm. Theoretically, any change at any step of these processes will result in a qualitative or quantitative change in the mRNA, and this will be reflected by the level of synthesis of the corresponding protein. As ribosomes are the site where protein synthesis takes place, I first isolated and detected polysomal RNA to see whether down regulation of E1a by E3 occurs at the period between transcription and the start of translation. The data presented in Figure 4.2 and 4.3 showed no evidence
that the E1a polysomal RNA level was lower in w.t. virus infected cells relative to the cells infected with E3 deletion mutants, if anything, E1a mRNA from dl327 infected cells was slightly lower than that from cells infected with w.t. Ad5. Results shown in Fig 4.3 indicated that E1a mRNA was properly spliced in w.t. Ad5 infected cells at least within the limits of detection of the Northern blot technique. In summary, these data suggest that down regulation of E1a protein products by E3 may occur during mRNA translation, or by an effect on protein stability.

To determine whether E1a protein stability varied between cells infected with w.t. Ad5 or E3 deletion mutants, a pulse-chase experiment was carried out. The results (Fig.4.4) showed that there was no significant difference in the slopes of the E1a decay. Thus, E1a protein stability in cells infected with w.t. Ad5 and dl327 is essentially the same. As E1a protein stability is the same, and total amount and cytoplasmic E1a mRNA levels are also the same in cells infected with w.t. Ad5 and E3 deletion mutants, it therefore appears that E3 products may down regulate E1a expression by directly affecting the rate at which E1a proteins are translated. Since this is a novel mechanism for gene regulation in adenovirus infected cells, it would be desirable to directly test this hypothesis. However, there was in sufficient time to complete further experimental investigation of E1a translation.

There are at least two possible types of regulation at translational initiation. The first is that the overall rate of translation is controlled under some conditions. For example, when cells enter mitosis the translation initiation rate is inhibited, which causes a fall off in the rate of total protein synthesis to 30 percent of normal. One possible mechanism for the suppressed initiation of total protein synthesis is the phosphorylation of initiation factors by protein kinases which can trigger the phosphorylation of the eukaryotic initiation factor 2 (eIF2) and then inactivate it (Farrell et al., 1977). Another type of regulation at translation initiation is that the translational efficiency of specific mRNA can be controlled under some conditions. An interesting case is the one in which translational control occurs late after adenovirus infection of HeLa cells. The pre-existing cellular mRNAs are still
present but they are not translated; translation of the late viral mRNAs accounts for over 90% of the proteins made (O'Malley et al., 1989). This selective translation of adenovirus mRNAs is mediated by VA RNAI which is synthesized in large amount during late stage of adenovirus infection (reviewed in section 1.2.9). Furthermore, under certain conditions, translational initiation from only a single mRNA species is controlled. For instance, when cultured mammalian or insect cells are heated above 40°C, most translation initiation is suppressed, but the formation of heat-shock proteins is stimulated (Ashburner and Bonner 1979).

Results presented in Fig.4.6 showed that the amounts of the cellular proteins p53 and class I MHC antigen H-2K^k were essentially the same in cells infected with w.t. virus, dl327 or uninfected, whereas data in Fig.4.5a and 4.5b showed that both early and late gene products from adenovirus were down regulated in the presence of E3 gene products. Therefore, it appears that E3 does not affect mRNA translation generally, rather it is selective for viral mRNA translation.

There are two possible explanations for the general shut down of viral mRNA translation: 1) Down regulation of E1a by E3 gene products is a specific event which only affects E1a mRNA translation, or, 2) The effect of E3 gene products on mRNA translation may be an event which affects the translation of virus mRNA. For the first possibility, as E1a is required for efficient expression of other early genes as well as late genes (reviewed in section 1.2.4), the general shut down of adenoviral gene expression in w.t. Ad5 infected cells may be due to lack of enough E1a for the transactivation. However, quantitative assessment by densitometric scanning has shown that E3 does not affect translation of all viral mRNA to the same extent. E1a is the most seriously affected with about a 10 fold reduction in protein level between cells infected with w.t. Ad5 and E3 deletion mutants. Another early gene, E1b, is intermediate, showing about 6 fold difference in protein production. The late gene product hexon is only two fold lower in w.t. Ad5 infected cells compared with dl327 infected cells. The reason for this uneven effect on individual viral gene expression may be due to the fact that E1a
transactivation is a non-linear effect (Braithwaite et al., 1991b). Alternatively, the differential effect of E3 gene products on E1a, E1b and hexon might be accounted for by differences in turnover of these proteins. However, this possibility has not been investigated yet. For the second possibility, down regulation of E1a by E3 may be similar to what VA RNAI does during adenovirus infection. However, unlike VA RNAI, the effect mediated by E3 is negative.

Finally, Western blot analysis failed to show any complex formation between E3 19k and E1a proteins even under the condition of lysis with mild RIPA buffer. This result suggests that the effect of E3 on E1a translation is unlikely to involve direct contact between E1a and E3 19k, but rather occurs by an indirect mechanism such as acting on translational initiation or elongation factors.

Based on the data presented in this chapter, it is concluded that some E3 gene product(s) probably down regulates E1a expression by affecting E1a mRNA translation. In the next chapter experiments are reported in which it was determined whether down regulation of E1a by E3 also occurs in other cell lines, as well as in other serotypes of group C adenoviruses.
Chapter 5

Interference of E3 with E1a expression is a rather general phenomenon in adenovirus infected cells
5.1. INTRODUCTION.

Although humans are the natural host for adenoviruses, human adenoviruses can also infect or transform a variety of animal cell lines under experimental conditions. The interaction between adenovirus and the infected host cell is a very complicated process. The factors that govern different responses of infected cells to adenovirus infection remain mysterious, but the outcome is influenced by both the cell type and the virus serotype. For a particular serotype of adenoviruses, cells may be permissive, semipermissive or nonpermissive, depending on their species and the derivation of the tissues. The latter two categories include the cells that are susceptible to transformation by viral particles. Coincident with cells' sensitivity towards infection, adenoviruses may cause productive, or abortive infections in these cells, respectively. Moreover, the sensitivity of cells within the same species towards adenovirus infection also varies depending upon the type of cells. For example, cultures of primary human fibroblasts and most cell lines established from human fibroblasts or epithelioid tissue (e.g. HeLa cells) are permissive to adenoviruses, and viruses can grow to very high titres in these cell lines. However, not all cell lines of human origin support efficient adenovirus growth. Ad2 productively infects cultured human keratinocytes but not the basal cells from which they are derived (LaPorta and Taichman 1981). Human adenoviruses are also reported to grow poorly in a human embryo carcinoma cell line called Tera 1 (Nicolas and Levine 1981) and in cells of human lymphoid origin (Silver and Anderson 1988). Most animal cell lines are semipermissive or nonpermissive to human adenovirus infection. Rat embryo cells are semipermissive for group C adenoviruses, in which Ad2 and Ad5 can replicate but the yields of virus are decreased by several orders of magnitude compared to those obtained from fully permissive human cells (Gallimore et al., 1974; Takahashi 1972). However, cells derived from different rat embryo tissues apparently vary in their ability to support replication of viruses (Gallimore et al., 1974). Mouse cells are also shown to be semipermissive for the replication of
human group C adenoviruses under conditions of serum starvation (Bellett and Younghusband 1979; Braithwaite et al., 1981). The Simian virus 40 (SV40) transformed primate cell line, COS, however is highly permissive to adenovirus infection. That is because it expresses the large T antigen from SV40 which can release the block to adenovirus late gene expression in monkey cells, and result in a great yield of virus from each infected cell (Nakajima and Oda 1975).

Infection of mammalian cells by human adenoviruses induces dramatic alterations in the cells' abilities to continue their normal functions. However, the extent of these effects of adenovirus on infected cells also varies depending upon the cell type. When adenoviruses infect rapidly dividing, fully permissive cells, they first cause an increase, followed by a rapid decline and then virtual cessation of cellular DNA replication. Eventually, all of the infected cells die. Similarly, most semipermissive cells are eventually killed when infected by adenoviruses, but some of them may survive and become transformed (for review see Flint 1986). On the other hand, individual gene expression of adenoviruses may vary in different cell lines even though the cell lines are from the same species. For example, w.t. Ad2 infected human myeloma cells showed 20-50 fold less E1a and E1b mRNAs at late in infection, relative to the same virus-infected HeLa cells, while the other early genes remained essentially the same. These reduced levels of E1a and E1b mRNAs in Ad2 infected lymphoid cells were due to differential RNA stabilization and resulted in markedly reduced production of E1a polypeptides (Lavery and Chen-Kiang 1990).

Ad2 and Ad5 are members of group C adenoviruses. Molecular hybridization shows that their genomes are more than 90% homologous to each other (Green et al., 1979a). Specifically for the E3 region, DNA sequence studies (Cladaras and Wold 1985; Herisse et al., 1980) show that E3 of Ad2 and Ad5 are 93.7% homologous in the region -236 to 650 (relative to the cap site), which includes the sequences for pVIII, the E3A-12.5kD protein (Fig.5.1) and the E3 promoter. Homology is maintained in the remainder of E3, but it is not as pronounced as in the -
-236 to 650 region. For example, the regions which encode E3 19k are only 75.8% homologous and the E3 19k proteins from these two viruses share less than 60% homology (based on the data of DNA and protein sequences in Cladaras' (1985) and analysed by myself). As these two viruses have high DNA sequence homology, it is generally considered that they usually behave similarly in infected cells.

The data presented in Chapter 3 showed that E1a expression was down regulated by E3 gene products in the w.t. Ad5 infected L929 cell line. In Chapter 4, evidence was presented to show that this down regulation occurred by affecting E1a mRNA translation and that this translational regulation appeared to be selective for viral (possibly early) mRNAs. In this chapter, experiments were expanded by the use of different cell lines and other serotypes of adenoviruses to test whether the down regulation of E1a expression by E3 also occurs in other cell lines and with other serotypes of adenoviruses or is only restricted to Ad5 infected L929 cells.

5.2. RESULTS.

5.2.1. E1a expression is not affected when Ad5 E3 is replaced by the E3 region from Ad2.

The original aim of this experiment was to try and map the gene product(s) of E3 responsible for down regulation of E1a expression. To achieve this goal, a series of E3 deletion mutants containing single E3 gene product deletions were collected. These E3 deletion mutants were generated from a parental virus rec700, which is an Ad5-Ad2-Ad5 recombinant consisting of the Ad5 EcoRI-A fragment (map position 0-76), Ad2 EcoRI-D (76-83) and Ad5 EcoRI-B (83-100) fragments (Wold et al., 1986). Essentially the rec700 is an Ad5 serotype but its original Ad5 E3 region has been replaced by the Ad2 E3 gene. Among the deletion mutants, d1704 has part of the region encoding E3 19k deleted, but the remaining genes are the same as rec700. For d1712, the E3 11.6kD protein is not made. D1762 has the E3 14.7k gene deleted. Dl764, d1753, d1739 and d1722 have the E3 14.5kD, 10.4kD, 6.7kD and 12.5kD gene deleted, respectively (see Fig 5.1.). In general, these mutants individually contain
a deletion in each E3 gene product. Ideally, by the use of these mutants, it should be possible to precisely map the E3 gene product(s) which are responsible for down regulation of E1a expression.

In order to determine which part of the E3 gene may be involved in the down regulation of E1a expression, three mutants out of seven were first used. Among them dl722 has a deletion in the gene encoding the 12.5kD product which is located at the left-end of E3; dl704 has part of the E3 19k encoding gene deleted which is in the middle of E3 region; while dl762 has a piece of DNA deleted at the right end of E3 which encodes the 14.7k product. At the same time, Ad5 as well as rec700 were used as control. L929 cells were infected with these viruses for 40h at 20 IU per cell. Cells were then labelled with 35S-methionine for 2h and lysed. Cell lysates were immunoprecipitated with anti-E1a, anti-E3 19k, anti-E3 14.7k antibodies, or NMS. The results are shown in Fig 5.2. All the mutants showed the expected properties, i.e. no E319k band was seen in the anti-E3 19k track of dl704 infected cells and no E3 14.7k protein could be seen in the anti-E3 14.7 track of dl762 infected cells. However, E1a protein levels in cells infected with all of these mutant viruses were much higher than in cells infected with w.t. Ad5, despite the fact that the mutants had deletions at different positions within the E3 region. Moreover, rec700, which has a complete E3 region in the genome, also showed a higher level of E1a proteins than w.t. Ad5. Since these E3 deletion mutants as well as their parental virus, rec700, are from an Ad5-Ad2-Ad5 recombinant background, i.e. the E1a of these viruses is from Ad5 but the E3 region is from Ad2, these results suggest two possibilities: either down regulation of E1a expression mediated by E3 only occurs in adenovirus serotype 5, or there is a serotype specificity required for E3 to interfere with E1a expression in group C adenoviruses, i.e. E3 from Ad2 can only act on Ad2 E1a and E3 from Ad5 interferes only with Ad5 E1a expression.

Another interesting observation from the results presented in Fig 5.2. is that although the level of E1a products was substantially lower in w.t. Ad5 infected cells than in rec700, dl722 and dl762 infected cells, the E3 19k protein levels remained essentially unchanged among them. This
Fig. 5.1. Summary of the E3 deletion mutants derived from rec700. A schematic illustration of the E3 transcription unit of rec700 (see Fig.1.2) is presented in the top of the figure. The short black bars at the bottom indicate deletions in the virus mutants. The triangles mean deletion. The deleted nucleotide sequences in the virus mutants are given by the numbers next to the triangles. Figure modified from Tollefson et al (1990).
Fig. 5.2. E1a expression in cells infected with rec700 and its derivative E3 deletion mutants. L929 cells were infected with 20 IU per cell of viruses. Fourty hours after infections, cells were labelled with $^{35}$S-methionine for 2h and harvested. Extracts were immunoprecipitated with anti-E1a (M73), anti-E3 19k, and anti-E3 14.7k antibodies or NMS. The resulting proteins were separated by 12% SDS-PAGE as described in Fig 3.5.
is consistent with a report which showed that in some cell lines such as lymphoid cells, only a small amount of E1a was required for efficient transactivation of all E1a dependent early genes (Lavery and Chen-Kiang 1990). It is also obvious from the results presented in Fig. 5.2 that the E3 19k proteins from w.t. Ad5 and rec700 (in which E3 is from Ad2) show different mobilities. E3 19k protein from Ad2 runs faster than that from Ad5. This mobility difference may be due to amino acid differences or posttranslational modifications of E3 19k proteins between these two serotypes.

5.2.2. E1a expression is also decreased in w.t. Ad2 infected cells compared with an Ad2 E3 deletion mutant.

To test whether E3 gene products from Ad2 also interfere with E1a expression, dl801, an E3 deletion mutant derived from Ad2, was used. Although dl801 was originally isolated with the presence of a helper virus, electron microscopy and additional series of restriction enzyme analysis showed that this mutant only contained a single deletion at the E3 region. This virus has been found to be viable without the presence of helper virus (Challberg and Ketner 1981). The deleted region in dl801 is 78.5 to 83.5 m.u. (Challberg and Ketner 1981). This deletion is similar to that in the Ad5 E3 deletion mutant dl327 (see Chapter 3).

To ensure that equal amounts of virus were used between w.t. Ad2 and dl801, again the viruses were titrated by immunofluorescent staining and titrations were confirmed by DNA dot blot hybridization 3h after infection. L929 cells were infected with w.t. Ad2 and dl801 at 20 IU per cell for 40h. The E1a protein analysis was done by 35S-methionine labelling and immunoprecipitation as described. The results are shown in Fig 5.3. As expected, no E3 19k protein was expressed in dl801 infected cells. However, like dl327 which has a higher level of E1a expression than w.t. Ad5, dl801 expressed a much higher level of E1a products than w.t. Ad2. Densitometric scanning showed the difference to be about 10 fold.

In conclusion, these data suggest that E1a expression is also down regulated in w.t. Ad2 by Ad2 E3 gene products. However, when the Ad2
Fig. 5.3. Comparison of E1a expression in w.t. Ad2 and its E3 deletion mutant dl801. L929 cells were infected with 20 IU per cell of Ad2, dl801 or mock-infected. The duration of infection was 40h. Cells were labelled with 35S-methionine for 2h. Cell extracts were immunoprecipitated with M73 and anti-E3 19k antibodies, or NMS. The immunoprecipitated proteins were then analysed on a 12% SDS-PAGE as described in Fig. 3.5.
E3 gene was present in Ad5 infected cells instead of the Ad5 E3 gene, no down regulation of E1a was observed (see Fig.5.2). This suggests that there is no cross reactivity in E3 down regulation of E1a expression between the two members of group C adenoviruses. Considering that E1a from these two adenoviruses is highly homologous (more than 90% of homology in DNA sequence) and there are only 4 amino acid differences in 289R between these two serotypes (for review: Sussenbach 1984), it is likely that the specificity of E3 action on E1a expression is due to the greater difference in the E3 19k proteins between these two viruses.

5.2.3. Strict relationship between the level of E1a expression and Tc cell lysability.

The experiments reported in Chapter 3 demonstrated that Tc cell killing of E3 deletion mutant infected target cells was enhanced compared with w.t. Ad5 infected targets. The data showed that the best explanation of this is that there are higher levels of E1a products present in E3 deletion mutant infected cells compared with the cells infected with w.t. Ad5. Since there are high levels of E1a expression in rec700, dl704 and dl762 infected cells, one can predict that cells infected by these viruses should be more sensitive to lysis by adenovirus specific Tc cells. To test this, w.t. Ad5 was used to generate effector Tc cells. Lysis of L929 target cells infected with w.t. Ad5, dl327 (these two viruses served as controls), as well as dl704 and dl762 by Ad5 immune Tc cell was assessed 40 h after infection. The results are presented in Fig 5.4a. Wild type Ad5 and dl327 infected cells showed the usual pattern, i.e. targets infected with dl327 were more susceptible to lysis than that of w.t. virus infected targets. As expected, rec700, as well as dl704 and dl762 infected cells showed higher susceptibility to lysis than w.t. Ad5 infected cells and this was similar to that observed with dl327 infection, although rec700 has a complete E3 gene.

Target cells infected with w.t. Ad2 and dl801 were also compared for Tc cell lysis. To test whether there is any cross reactivity in Tc cell recognition between the immunodominant antigens from Ad2 and Ad5, Ad5 immune Tc cells were used as effector cells for Ad2 infected targets.
The results presented in Fig 5.4b showed that like dl327, dl801 also caused target cells to become more susceptible to lysis than its parental w.t. Ad2. Moreover, w.t. Ad2 infected cells were lysed to a similar extent as w.t. Ad5; while dl801 infected cells showed similar extent of lysis as dl327. These results further suggest that E1a of Ad2 and E1a of Ad5 share the immunodominant peptide as recognised by Ad5 immune Tc cells.

The results presented in this section indicate that there is a direct relationship between the level of E1a products in target cells and the susceptibility of cells to Tc cell lysis. In addition, the results support the conclusion generated from the data in Chapter 3 that enhanced Tc cell response to E3 mutant infected L929 cells was due to increased immunodominant E1a antigen expression, rather than E3 interference with the cell surface expression of the restriction class I MHC antigen H-2Kk.

5.2.4. The effect of E3 on E1a expression in mouse cell lines of different H-2 haplotypes.

As reviewed in section 1.4.2.2, one of the E3 gene products from Ad2, E3 19k, has been reported to form a complex with class I MHC antigens which interferes with their terminal glycosylation (Burgert et al., 1987). This results in reduced cell surface expression of class I MHC antigens. This has been suggested to be one of the mechanisms by which adenoviruses evade immune surveillance (Andersson et al., 1985; Ginsberg et al., 1989). However, in mouse cells, Ad2 E3 19k seems to interfere with cell surface expression of only certain class I MHC antigens. For example, cell surface expression of Db and Kd has been observed to be inhibited but Kb, Dd and Kk molecules appear not to be affected (reviewed in section 1.4.2.2). The experiments presented so far have been done exclusively in L929 cells, in which the restricting element of class I MHC antigens for Ad5 specific Tc cell recognition is H-2Kk which cannot form a complex with Ad2 E3 19k protein (Burgert and Kvist 1987). Therefore, there are two questions which need to be clarified: (1) Is the down regulation of E1a expression by E3 related to a
Fig. 5.4. Relationship between E1a expression and Tc cell lysis. L929 target cells were infected with 20 IU per cell of rec700 and its derivative E3 deletion mutants (a), w.t. Ad2 and dl801 (b). Wild type Ad5 and dl327 were used as control in both a and b experiments. The duration of infection is 40h. Tc cells were prepared by immunizing mice with w.t. Ad5 and spleen cells were incubated in vitro with syngeneic cells infected with the same virus. Details are as described in the legend to Fig 3.1. Assay duration was 6h.
specific class I MHC antigen? and (2) Is there any direct or indirect relationship between the binding of E3 gene products to class I MHC antigen and down regulation of E1a expression?

To answer these questions, five mouse cell lines which express class I MHC antigens of different haplotypes were used. Since previous results (Fig. 5.2) showed that d1704, like d1327, also expressed higher levels of E1a proteins than w.t. Ad5, d1704 was used in this experiment. As usual, cells were infected with 20 IU per cell of w.t Ad5 and d1704 for 40h. Radioactive labelling of the cells and immunoprecipitation were performed as before. The bands representing E1a products were quantitated by densitometric scanning. Results are shown in Table 5.1. Around 10 fold less E1a from w.t. virus than from d1704 has been observed in L929, C3HOH, L929-Ld and HTG cells. Among these cell lines, class I MHC antigens in L929 cells (Kk and Dk) have been shown not to bind to E3 19k. Both of the class I MHC antigens in HTG cells (Kd and Db) have been shown to bind to Ad2 E3 19k. Whereas C3HOH and L929-Ld cells express class I antigens which can bind E3 19k and also class I antigens which cannot bind to E3 19k. In MC57 cells, the levels of E1a are about 3-5 fold lower in w.t. virus than in d1704 infection.

These results indicate that down regulation of E1a by E3 gene products is class I MHC antigen haplotype independent and that there is no direct relationship between the binding of E3 gene products to class I MHC antigens and down regulation of E1a expression.

5.2.5. E3 gene products interfere with E1a expression in some cell lines from species other than mice.

The above experiments demonstrated that all mouse cell lines tested showed down regulation of E1a expression when they were infected with w.t. virus, compared with equivalent cells infected by an E3 deletion mutant. For human adenoviruses, the natural host is of course human kind. It was therefore important to test if this E1a regulation by E3 also occurs in human cell lines. To determine the generality of E3 down regulation of E1a by E3, it was also necessary to test it in other species. Therefore another six cell lines including cell lines from
Comparison of w.t. Ad5 and dl704 E1a expression in different mouse cell lines

<table>
<thead>
<tr>
<th>cell lines</th>
<th>class I MHC genes</th>
<th>binding with E3 19k</th>
<th>E1a in dl704</th>
<th>E1a in w.t. Ad5</th>
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<td>8-10</td>
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<tr>
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<td>K&lt;sup&gt;d&lt;/sup&gt;, D&lt;sup&gt;k&lt;/sup&gt;</td>
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<td></td>
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<tr>
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<td>8-10</td>
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**Table 5.1** Mouse cells were infected with 20 IU per cell of w.t. Ad5 and dl704. Forty hours after infection, cells were labelled with 35S-methionine for 2h. Cells were then lysed with RIPA buffer containing 0.1% SDS. Cell extracts were immunoprecipitated with anti-E1a monoclonal antibody (M73). Immunoprecipitated proteins were analysed by a 12% SDS-PAGE. The E1a protein levels were quantitated by densitometric scanning. The levels of E1a from dl704 infected cells were divided by the levels in w.t. Ad5 infected cells.
human, rat and monkey were used. Equal multiplicities (20 IU per cell) of Ad5 and dl704 were used to infect these cells and the duration of infection was 40 h. Again E1a protein levels from 12% SDS-acrylamide gel analysis were quantitated by densitometric scanning. Results are shown in Table 5.2. Among the human cell lines, Daudi cells showed a more than 10 fold reduction of E1a expression when they were infected by w.t. virus compared with infection by dl704, whereas in 143B cells, dl704 showed only 5 fold higher E1a levels than w.t. virus infection. In HeLa and K562 cells, E1a expression was not influenced by E3 at all. No difference in E1a levels between w.t. Ad5 and dl704 could be found in one representative of monkey and rat cell lines either.

In summary, 11 cell lines (five mouse, four human, one rat and one monkey) have been tested for E1a protein levels after w.t. Ad5 and dl704 infection. The results demonstrate that down regulation of E1a by E3 occurs in more than half of the cell lines tested (7 out of 11). It appears that there is no species specificity since both mouse cell lines and human cells infected with w.t. virus showed lower levels of E1a proteins than when infected with dl704. It seems there is no direct relationship between the occurrence of down regulation of E1a and the permissiveness of cells to adenovirus, as E3 can down regulate E1a expression alike in cells which are permissive (143B) and semi-permissive (mouse cells) to adenovirus infection (Flint 1980).

5.2.6. The ratio of E3 19k to E3 14.7k is correlated with down regulation of E1a.

Due to the observation that the Ad5-Ad2-Ad5 recombinant virus rec700, and a series of E3 deletion mutants derived from it showed no reduction of E1a in infected cells, the E3 gene product(s) responsible for down regulation of E1a expression has not yet been defined. The results presented in the above section showed that E3 gene products can interfere with E1a expression in some cell lines but not in others. It was therefore of interest to detect expression of individual E3 gene products in cell lines which show or do not show down regulation of E1a in the presence of E3, as this may provide some clue as to the E3 gene
Down regulation of E1α in cell lines of species other than mouse

<table>
<thead>
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<th>cell lines</th>
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<th>down regulation of E1α*</th>
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<tr>
<td>Daudi</td>
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</tr>
<tr>
<td>143B</td>
<td>human</td>
<td>~ 5 fold</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>NRK</td>
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</tr>
</tbody>
</table>

* ratio of dl704 E1α to w.t. Ad5 E1α

Table 5.2. Human, monkey and rat cells were infected with 20 IU per cell of w.t. Ad5 and dl704. Duration of infection was 40h. E1α protein analysis was done as described in Table 5.1.

These results suggest that maintenance of a balance between E2 10k and E3 14.7k may be important in determining whether or not down regulation of E1α expression by E3 will take place.
product(s) responsible for down regulation of E1a. For instance, if any E3 gene products are reduced or not expressed in HeLa or COS cells which do not show down regulation of E1a by E3, these E3 gene products could be likely candidates for down regulating E1a expression.

L929, HeLa and COS cells were infected with 20 IU per cell of w.t. Ad5 or Ad2 for 40h. Radioactively labelled cell lysates were aliquoted and immunoprecipitated with antibodies against individual E3 gene products. The immunoprecipitated proteins were analysed on a 15% SDS-acrylamide gel. The results are shown in Fig 5.5. The most prominent feature evident in these results is that there is a large difference in the ratio of E3 19k and E3 14.7k between these two groups of cells. Densitometric quantitation showed that in L929 cells, the signal of E3 19k was three times stronger than that of E3 14.7k, whereas in HeLa and COS cells, it is the reverse. E3 14.7k showed 4-8 times stronger signals than E3 19k. Infection of these cells with both w.t. Ad2 (Fig 5.5a) and w.t. Ad5 (fig 5.5b) showed the same result.

In the anti-14.5kD antibody immunoprecipitation tracks, more than two bands can be seen. One of them has the same molecular weight as E3 19k (only could be seen in Fig 5.5a). This is not strange as it has been reported that typically five to six bands representing E3 14.5kD could be seen which ranged in apparent molecular weight from 15kD to 18kD on 10-18% SDS-PAGE, and the E3 19k has been shown to bind nonspecifically with the 14.5kD product in Ad2 infected cells (Tollefson et al., 1990). It seems that in Ad2 infection (Fig 5.5a), the 14.5kD protein is higher in L929 cells than that in COS or HeLa cells. However, in Ad5 infection (Fig 5.5b), 14.5kD protein levels from L929 and HeLa cells are essentially the same.

These results suggest that maintenance of a balance between E3 19k and E3 14.7k may be important in determining whether or not down regulation of E1a expression by E3 will take place.

5.3. DISCUSSION.

Two members of group C human adenoviruses, Ad2 and Ad5, are the best studied adenoviruses. Almost all of the viral genomes have been
Fig. 5.5. E3 gene expression in cells which show or do not show down regulation of E1a expression in w.t. virus infection. HeLa, COS and L929 cells were infected with 20 IU per cell of Ad2 (a) or Ad5 (b) for 40h. Cells were then labelled with 35S-methionine for 2h. Cell extracts were immunoprecipitated with anti-E3 19k, anti-E3 14.7k and anti-E3 14.5k antibodies. Proteins were analysed on a 15% SDS-PAGE.
sequenced. It has been shown that these two viruses are so closely related that their genomes share more than 90% of homology (for review: Sussenbach 1984). It follows that, when a function is associated with a given gene product of one serotype, it is often assumed that the corresponding gene product from the other serotype also has the similar function. Under a number of circumstances this has been the case. For instance, functions in transcriptional regulation and transformation of E1a and E1b gene products are applicable to both Ad2 and Ad5 (reviewed in 1.2.4). However, a systematic comparison of individual gene functions from these two viruses has not yet been made.

In order to determine whether the E3 gene products from Ad2 also interfered with E1a expression in mouse L929 cells, comparison of E1a protein levels was carried out using w.t. Ad2 and an Ad2 E3 deletion mutant (dl801). Consistent with the idea mentioned above, Ad2 E3 products also down regulate Ad2 E1a expression (Fig 5.3). However, the apparent finding (Fig 5.2) that the Ad5-Ad2-Ad5 recombinant, rec700, and its derivatives dl704, dl722 and dl762, with which part or all of the Ad5 E3 gene is replaced by the E3 gene from Ad2, showed no reduction of E1a expression, was surprising. Although the total genomic sequences of these two viruses share more than 90% of homology, comparative analysis of DNA sequences of the E3 region from these two viruses showed that there was less homology in this particular region. For instance, the gene which encodes E3 19k only shares about 78% homology between Ad2 and Ad5, and the homology of the corresponding proteins shares less than 60%. Actually it is often seen that Ad2 and Ad5 E3 19k have a different mobility when they are run in the same protein gel. Usually Ad2 E3 19k runs a little faster than Ad5 E3 19k (Fig 5.2). This may be due to differences in amino acid composition (Cladaras and Wold 1985) or due to different post-translational modifications. This relatively reduced homology of the E3 genes of Ad2 and Ad5 may account for the ineffectiveness of Ad2 E3 gene products in the regulation of Ad5 E1a expression.

Consistent with the inability of Ad2 E3 products to affect Ad5 E1a expression, cells infected with rec700 virus showed the same extent of
lysis by Ad5 specific Tc cells as that of E3 deletion mutants (dl704 and dl762) which are derived from it (Fig 5.4a). Cells infected with the Ad2 E3 deletion mutant, dl801, also exhibited higher susceptibility to Tc cell lysis, while higher levels of E1a was detected than in w.t. Ad2 infected cells (Fig 5.4b). These results parallel the events reported in Chapter 3. Taking all of these data together, it is apparent that the susceptibility of Ad infected cells to Tc cell lysis is correlated with E1a expression. A similar phenomenon has also been described in some virus-induced tumor cell lines (Green 1983; Vasmel et al., 1989) as well as in CMV infected cells (Del Val et al., 1989).

It has been reported that individual gene products from adenoviruses behave quite differently in different cell lines. E3 19k from Ad2 has been shown to form complexes with class I MHC antigens and reduce their expression on the cell surface. However, in a variety of other mouse cell lines tested, this was found not to be always the case. For example, inhibition of D\textsuperscript{b} and K\textsuperscript{d} has been reported but K\textsuperscript{b}, D\textsuperscript{d} and K\textsuperscript{k} molecules appear not to be affected. In human cell lines, this variation also exists. Routes and Cook (1990) have tested the generality of E3 19k reduction of class I MHC antigen cell surface expression on a variety of human cell lines which include human epithelial (293, A549, HeLa, KB), fibroblastic (TFB-1, TFB-2, WI-38 MRC-5) and lymphoid (JCB, Jurkat) cells. Only 293, an Ad5 E1 gene transformed cell line (Graham et al., 1977), showed a reduction of cell surface expression of HLA.

Another protein encoded by the E3 region, E3 14.7k, has been shown to prevent TNF-mediated cytolysis of adenovirus-infected cells (Gooding et al., 1988). Extension of this study into more cell lines showed there also exhibited exceptions. NCTC-929 cells, a derivative of mouse L cells, are spontaneously sensitive to TNF lysis but the lysis is not affected by E3 14.7k even though the protein is made in large quantities and is metabolically stable in these cells (Gooding et al., 1990). The E1a and E1b genes have been shown to be expressed quite differently in different human cell lines (Lavery and Chen-Kiang 1990). It appears that this variation depends on cell type and is a general feature of adenovirus gene functions.
Consistent with the idea discussed above, the experiments presented in this Chapter which examined the E3 gene products that interfere with E1a expression in a variety of cell lines from different species including human, mouse, rat and monkey demonstrated that four out of eleven cell lines showed no reduction in E1a products in the presence of E3. It seems that the permissiveness of cells to adenoviruses infection cannot be used to explain this cell type specificity, since ineffectiveness of E3 on E1a expression occurs in both cell lines which are permissive or semipermissive to adenovirus infection. It seems that there is no direct relationship between E3 gene product binding to class I antigens and down regulation of E1a expression either, as the lower level of E1a due to the presence of E3 was observed in mouse cell lines in which the expressed class I MHC antigens which can bind with E3 19k, as well as mouse cell lines in which the class I MHC antigens cannot bind with E3 19k protein.

The existence of cell type specificity for E3 interference with E1a expression led to a comparison of the expression of individual E3 gene products between cells that differ in the effect of E3 on E1a expression. The experiment showed that the ratio of E3 19k to E3 14.7k is reversed in cells which show or do not show down regulation of E1a in the presence of E3. In cells where E3 products can down regulate E1a, the ratio of E3 19k to E3 14.7k is greater than 3. By contrast, in cells in which E3 products show no effect on E1a expression, E3 14.7k is 4-8 times better expressed than E3 19k. This observation suggests that a balance between the levels of E3 19k and E3 14.7k might be involved in the E3 gene products regulating E1a expression.

The results of the experiments described in this chapter showed that reduced E1a expression mediated by E3 products also occurs in serotype Ad2. However, it seems that there is no cross reactivity between the two serotypes of group C adenoviruses for E3 regulation of E1a expression. Immunological studies showed that there was a strong relationship between the level of E1a proteins in infected cells and susceptibility of infected cells to lysis by Tc cells. Studies with a variety of cell lines from different species indicated that E3 gene product down
regulation of E1a expression was cell type dependent but species independent. Studies from five mouse cell lines with class I MHC antigens of different haplotypes indicated that there was no direct relationship between down regulation of E1a by E3 and the binding of the E3 19k gene product to class I MHC antigens.

Further attempts to map individual E3 gene products involved in regulation of E1a expression are described in the following chapter.
Chapter 6

E1a expression in E3 transformed mouse cells

The ability to introduce exogenous DNA into cultured mammalian cells (DNA-mediated gene transfer) has proved to be one of the most powerful tools for analysing molecular mechanisms of gene expression. The technique is currently being used to identify and analyse transcriptional control signals, analyse mechanisms of gene modulation by regulatory and oncogenes implicated in carcinogenesis (see a review of Fareseck et al., 1989).

There are several methods currently used for the introduction of DNA into cultured mammalian cells (Bogo et al., 1986; Caponchi 1988, Kawai and Nishizawa 1994). The most widely used procedure by far is the calcium phosphate transfection technique (Chen and Whitesell 1984; Graham and van der Eb 1973; Parker and Slack 1980), although other methods may be preferred for particular applications. Using the calcium phosphate technique, virtually any source of donor DNA can be used and gene expression can be determined in transfection cells either after a short adaption period or, as is more important, after the establishment of long-term cell lines transformed cells (Handbook et al., 1988). When donor DNA is co-transfected with a plasmid containing a gene which encodes a selectable marker, long-term transfected cells are readily obtained by growth in suitable selective media.

In the previous chapter, evidence was presented that when the Ad5 E3 region replaced the Ad5 E3 region in B22O, this E3 gene did not down regulate Ad5 E1a expression even though there was obvious expression of E3 gene product during polyI:C induction. Thus, it was not possible to determine which E3 region gene products were responsible for down regulation of E1a. Instead, additional findings suggest that the ratio of E2-19k to E2-1A protein might be important in determining whether E3 products could affect E1a expression in infected host cells. Currently studies which lack individual Ad5 E3 gene products are unavailable.
6.1. INTRODUCTION.

The ability to introduce isolated DNA into cultured mammalian cells (DNA-mediated gene transfer) has proved to be one of the most powerful tools for analysing eukaryotic gene expression. The technique is currently being used to identify and analyse transcription control signals in eukaryote genomes; to investigate RNA splicing; to analyse mechanisms of gene modulation by regulators; and to identify and analyse cellular oncogenes implicated in carcinogenesis (for a review: Sambrook et al., 1989).

There are several methods in current use for the introduction of DNA into cultured mammalian cells (Boggs et al., 1986; Capecchi 1980; Kawai and Nishizawa 1984). The most widely used procedure by far is the calcium phosphate transfection technique (Chen and Okayama 1988; Graham and van der Eb 1973; Parker and Stark 1979), although other methods may be preferred for particular applications. Using the calcium phosphate technique, virtually any source of donor DNA can be used and gene expression can be determined in recipient cells either after a short equilibration period (transient or short-term expression) or after the establishment of long-term cell lines (transformed cells) (Sambrook et al., 1989). When donor DNA is co-transfected with a plasmid containing a gene which encodes a selectable marker, long-term transfected cells are readily obtained by growth in suitable selective media.

In the previous chapter, evidence was presented that when the Ad2 E3 region replaced the Ad5 E3 region in rec700, this E3 gene did not down regulate Ad5 E1a expression, even though there was obvious expression of E3 gene product during rec700 infection. Thus, it was not possible to determine which E3 region gene product(s) were responsible for down regulation of E1a. Instead, additional findings suggest that the ratio of E3 19k to E3 14.7k products might be important in determining whether E3 products could affect E1a expression in infected host cells. Currently mutants which lack individual Ad5 E3 gene products are unavailable.
An alternative approach might be to make cell lines which express individual E3 gene products and to test E1a expression by infecting these cells with mutant virus in which the whole E3 gene is deleted, such as dl327. That is, to ask if an individual E3 gene product could complement the defect in dl327 to decrease E1a expression. Therefore, it was of interest to investigate E1a expression in E3 transformed cell lines for two reasons: first, to determine whether endogenously expressed E3 gene products can also affect E1a expression in the similar way to that which occurs in infection, and secondly, to explore the possibility of using a DNA mediated gene transfer system to map the E3 gene product(s) responsible for down regulating E1a, or using this system to confirm the idea that E3 down regulation of E1a requires a certain ratio of E3 19k to E3 14.7k, as was suggested from the preliminary data in the previous chapter.

6.2. RESULTS.

6.2.1. Construction of E3 plasmids

The construction of the recombinant plasmids is outlined in Figure 6.1. Briefly, whole Ad5 DNA was digested with Hind III and the fragments generated from this digestion were separated by running a 0.7% agarose gel. The HindIII-B fragment (the second largest fragment) which contains Ad5 DNA from 73.2-89.1 m.u. was isolated from the agarose gel and purified by ethanol precipitation. This fragment was ligated into a PBCBO7 (Ramshaw et al., 1987), which was also digested with HindIII. This recombinant plasmid was named PBCBE3. After amplification of this plasmid in E. coli, purified PBCBE3 DNA was further digested with SmaI plus HindIII, SmaI plus EcoRI, and EcoRI plus HindIII, respectively. These digestions generated three fragments which contained Ad5 DNA 76.6 to 89.1, 76.6 to 83.5 and 83.5 to 89.1 m.u., respectively. The cohesive ends of these fragments were filled in with Klenow fragment and then blunt-end ligated into another plasmid vector called PBC12CMV (Cullen 1986), which contains the cytomegalovirus (CMV) immediate early promoter. The orientation of inserts in the new recombinant plasmids was checked by digestion of plasmid DNA with a
variety of endonucleases and electrophoresis on agarose gel. The plasmids which contained the inserts in the correct orientations were selected. These recombinant plasmids were named CMVSH, CMVSE and CMVEH. The plasmid CMVSH contains the whole E3 region. CMVSE and CMVEH respectively contain the E3A and E3B transcription units. As CMVSH and CMVSE have their own E3 promoter in the DNA fragments, presumably the E3 genes in these two plasmids will be driven by the E3 promoter, while CMVEH will be driven by the CMV immediate early promoter.

6.2.2. Expression of the constructed E3 plasmids in COS cells.

To test whether the E3 genes in the recombinant plasmids were efficiently transcribed in vivo, 20μg of DNA from each plasmid was used to transfect COS cells in 9cm petri dishes (5x10⁵ cells per dish) with the BBS transfection technique (see section 2.2.2.3 and Chen and Okayama, 1988). In order to determine when gene expression was optimal, a time course experiment was set up. Transfected cells were harvested 24h, 48h, and 72h after transfection respectively by trypsinizing the cells. Cells were loaded onto Zeta probe membrane, and the E3 DNA and RNA in the cells were detected by hybridizing the membrane with a ³²P-labelled E3 DNA as described in section 2.2.2.2. The results in Fig 6.2 showed that there were specific E3 mRNAs transcribed from these plasmids after they were transfected into COS cells. The time course experiment showed that the mRNA synthesis was at its highest at 24h, but declined by 48h and 72h after transfection. This profile of expression is typical of transient transfection assays, presumably because the introduced DNA is destroyed by host endonucleases. Consistant with this, Fig.6.2 shows a loss of hybridizable DNA at 48h and 72h after transfection.

In order to test whether the mRNAs transcribed from E3 plasmids were properly translated, E3 plasmid DNA transfected cells were checked for E3 protein production. Twenty-four hours after transfection, cells were labelled with ³⁵S-methionine for 2h and then lysed. The cell lysates were immunoprecipitated with anti-E3 19k, an
FIG. 6.1. Schematic illustration of construction of E3 plasmids. Whole Ad5 DNA was digested with HindIII. The B fragment from HindIII digestion was isolated and ligated to PBCB07, which was also cut with HindIII. The new plasmid, named PBCBE3, was further digested with SmaI plus HindIII, SmaI plus EcoRI and EcoRI plus HindIII, respectively. Three fragments which contain different E3 genes were generated from this digestion. They were isolated and filled in with Klenow fragment. Another plasmid called PBC12CMV which contains the CMV immediate early promoter and the SV40 origin, was also linearized and filled in. The three blunt-ended fragments were cloned into PBC12CMV and three new recombinant plasmids were generated. They were named CMVSH, CMVSE and CMVEH.
Fig. 6.2 Detection of E3 DNA and RNA from cells transfected by E3 plasmids. Twenty μg of CMVSH, CMVSE and CMVEH DNA were transfected into COS cells by the BBS transfection technique (section 2.2.2.3). Transfected cells were incubated for 24h, 48h and 72h after transfection. Cells were then trypsinized and the same number of cells from each transfection were spotted onto a Zeta probe membrane. The E3 DNA and RNA were detected with a $^{32}$P-labelled E3 DNA as described in Fig. 3.6.
Fig.6.3. Detection of E3 19k protein from the cells transfected by E3 plasmids. Twenty μg of CMVSH and CMVSE DNA were transfected into COS cells as described in Fig.6.2. Twenty-four hours after transfection, cells were labelled with $^{35}$S-methionine for 2h. Cells were then lysed with RIPA buffer and the cell lysates were immunoprecipitated with anti-E1a (M73), anti-E3 19k antibodies or NMS. The precipitated proteins were analysed on a 12% SDS-PAGE.
unrelated antibody (anti-E1a) and NMS. The immunoprecipitated proteins were analysed by 12% SDS-PAGE. The results in Fig.6.3 show that E3 19k was produced by both E3 plasmids tested (CMVSH and CMVSE). This indicates that mRNAs expressed from these plasmids are properly processed and translated. However, E3 19k was higher in CMVSH transfected cells than in cells transfected with CMVSE at 24h after transfection. This difference may be due to a higher mRNA copy number in CMVSH transfected cells than in CMVSE transfected at 24h after transfection, as shown in Fig.6.2.

6.2.3. Establishment of a mouse cell line expressing Ad5 E3.

In order to determine whether a transfected E3 gene can down regulate E1a in infected cells, the recombinant plasmid CMVSH (which contains the entire E3 region of Ad5) was first used to make a cell line. L929 cells were seeded at 0.5x10^6 per 9cm petri dish one day before transfection. Cells in each dish were transfected with a total of 20μg of DNA, which included 15μg of CMVSH and 5μg of CMVneo DNA (Braithwaite et al., 1987). The neo gene in the CMVneo plasmid is a bacterial gene which can confer resistance to the neomycin-kanamycin family of antibiotics when it is introduced into cultured mammalian cells (Southern and Berg 1982). Therefore neo serves as a selection marker which can render the transfected cells resistant to the neomycin analogue, geneticin (Gibco). One dish which was transfected with 20μg of CMVSH DNA without CMVneo was used as a control. The transfected cells were allowed to grow for 48h in normal medium until the transfected neo gene was expressed. Then cells were grown under geneticin selection medium (0.7mg/ml of DMEM medium). One week later, cells began to die. Two weeks later, colonies began to appear in the dishes transfected with CMVSH plus CMVneo DNA, while all of the cells died in the dishes transfected with CMVSH DNA alone. Colonies were maintained in petri dishes for another a week. When colonies were counted, there were about 100 colonies in the E3 transfected dish. Cells were then trypsinized and transferred to 175 cm² flasks for
amplification. The mouse L929 cell line transformed with the whole Ad5 E3 gene was named LE3.

6.2.4. E1a expression is not down regulated in LE3 cells.

The transformed LE3 cells were checked for E3 19k expression. In order to see whether the endogenously produced E3 gene products can affect E1a expression, both LE3 and L929 cells were used for an infection experiment. The same number of cells was seeded (2x10^6 per 9cm petri dish) and infected with the same multiplicity of E3 deletion mutants dl327 and dl801 (20 IU/cell). Wild type Ad5 was used as a control in L929 cells. Thirty hours after infection, cells were labelled with 35S-methionine for 2h and then cells were lysed. The cell lysates were immunoprecipitated with anti-E1a antibody, anti-E3 19k antibody or NMS. The immunoprecipitated proteins were analyzed on a 12% SDS-acrylamide gel. The results were shown in Fig 6.4. As usual, in L929 cells, w.t. Ad5 showed a much lower level of E1a compared with dl327. In uninfected LE3 cells, E3 19k band could be seen but at very low levels. However, after dl327 and dl801 infection E3 gene expression in LE3 cells was markedly increased. This is consistent with the report that E1a transactivates the E3 promoter (Zajchowski et al., 1988). However, in spite of the large increase in E3 gene expression after infection, both dl327 and dl801 showed no obvious reduction in E1a expression in LE3 cells compared with that in dl327 and dl801 infected L929 cells.

In the experiment described above, E1a and E3 genes were introduced separately into the cells, that is E1a was from a virus infection and E3 was from DNA transfection. Thus it is likely that there are more E1a gene copies than E3 gene copies in each cell. Therefore, one possible explanation for the lack of effect of endogenous E3 products on E1a expression may be that there is not enough E3 expressed in LE3 cells to restrict the expression of E1a. To test this hypothesis, both LE3 cells and L929 cells were infected with 1 IU/cell of dl327 and E1a expression was compared using immunoprecipitation analysis. Results in Fig.6.5 show that although E1a levels were much lower than when infected with 20 IU/cell, they remained at the same levels between LE3
**Fig. 6.4** E1a expression is not changed in the LE3 cell line after dl327 infection. LE3 cell line (A) was infected with 20 IU per cell of E3 deletion mutant dl327 or dl801, or left uninfected. L929 cells (B) were infected with 20 IU per cell of w.t. Ad5, dl327 and dl801. Fourty hours after infection, cells were labelled with 35S-methionine for 2h. Cells were then lysed with RIPA buffer and cell lysates were immunoprecipitated with anti-E1a, anti-E3 19k antibodies, or NMS. The immunoprecipitated proteins were separated on a 12% SDS-PAGE.
Fig. 6.5. E1a expression in LE3 cell line infected with low multiplicity of dl327. LE3 cell line (A) and L929 cells (B) were infected 1 IU per cell of dl327. The infection period was 40h. Cells were labelled with $^{35}$S-methionine for 2h and lysed. The cell lysates were immunoprecipitated with anti-E1a (M73) and anti-E319k antibodies or NMS. The immunoprecipitated proteins were analysed on a 12% SDS-PAGE.
and L929 cells. These results indicate that the ineffectiveness of transfected E3 on viral E1a expression is not due to the lack of sufficient E3 products relative to expressed E1a. Instead, there may be some other factor which contributes to the phenomenon occurring during the transfection of cells.

6.2.5. E3 gene expression pattern is changed in LE3 cells.

The preliminary results presented in Chapter 5 (Fig 5.5) showed that the ratio of E3 19k and E3 14.7k was different between cells in which E3 does or does not affect E1a expression. In L929 cells, where E3 19k signals are stronger than those of E3 14.7k, down regulation of E1a expression mediated by E3 occurred when cells were infected with w.t. virus. In HeLa or COS cells where the ratio of E3 19k to E3 14.7k is reversed to that in L929 cells, E3 products showed no effect on E1a expression. Thus a possible reason why E3 products in LE3 cells had no effect on E1a expression may be that somehow the E3 gene is not completely expressed; for example, if only E3A is expressed but not E3B; or the ratio between E3 19k and E3 14.7k is altered so that it is more like HeLa cells and COS cells than in the parental L929 cells.

To test this possibility, both LE3 and L929 cells were infected with a multiplicity of 20 IU per cell of dl327 and w.t. Ad5, respectively. The same amount of antibodies against E3 products or E1a were used to precipitate the lysates from these two groups of cells. The results in Fig.6.6 show that both E3A and E3B DNA are integrated into the cellular genome and expressed in LE3 cells, as the corresponding protein bands from both E3 19k which is encoded in E3A and E3 14.7k which is encoded in E3B, could be observed on a SDS-acrylamide gel. However, the ratio between the signals from E3 19k and E3 14.7k in the LE3 cells is similar to that observed in HeLa cells or COS cells, where E3 has no effect on E1a levels. These results suggest that some molecular alterations have occurred to the E3 gene during its integration into the cellular genome. These results also support the conclusion that cell type specificity of E3 gene products interference with E1a expression is at least in part due to the balance between E3 19k and E3 14.7k.
Fig. 6.6. Individual E3 gene product expression in LE3 cells. L929 cells (A) were infected with 20 IU per cell of w.t. Ad5 or mock infected. LE3 cells (B) were infected with 20 IU per cell of dl327. Forty hours after infection, cells were labelled for 2h with 35S-methionine and lysed with RIPA buffer. The cell lysates were immunoprecipitated with anti-E3 19k, anti-E3 14.7k and anti-E3 14.5k antibodies. The immunoprecipitated proteins were separated on a 15% SDS-PAGE. As mentioned in the text, the E3 14.5k appeared as more than two bands with molecular weights ranging from 15kD to 18kD on 10-18% SDS-PAGE. E3 14.7k is also reported to be doublet or triplet on immunoprecipitation gels and Western blots (Tollefson and Wold 1988).
6.3 DISCUSSION.

The data presented in Chapter 3 clearly showed that when E3 gene products were present, E1a expression was reduced. Therefore it is concluded that E3 gene products can down regulate E1a expression during group C adenovirus infection of mouse L929 cells. The experiments described in this chapter aimed to investigate whether introduction of an E3 gene into L929 cells by gene transfer techniques could complement the deleted E3 gene in dl327 to produce a w.t. virus like infection. If E3 gene products do affect E1a expression, it was hoped that this system could be used to map the product(s) from the E3 transcription unit that are responsible for the down regulation of E1a.

A series of recombinant plasmids which contain different parts of Ad5 E3 gene were constructed using PBC12CMV as a vector. When these plasmids were transiently transfected into COS cells, E3 genes in these plasmids were properly transcribed and the mRNAs were translated as shown by both RNA and protein analyses (Fig. 6.2 and 6.3).

Then Ad5 E3 gene transformed mouse cell line (LE3) was made to see whether the endogenously expressed E3 gene could compensate for deleted E3 region in dl327. When LE3 cell lines were tested for E3 gene expression, it was found that the transfected E3 gene was expressed but at a very low level. However, infection of transformed cells with dl327 enhanced this E3 expression dramatically (Fig. 6.4). This result is not surprising since E1a can transactivate E3 expression by acting on the E3 promoter (Jones and Shenk 1979a; Leff et al., 1984; Weeks and Jones 1985). Although there is a CMV immediate early promoter in plasmid CMVSH, the E3 gene in this plasmid contains its own promoter as well. Presumably the E3 gene is driven by its own promoter since E3 transcription can be significantly stimulated.

A surprising finding is the results shown in Fig. 6.4 which shows no reduction of E1a expression when LE3 cells were infected with dl327, compared to the E1a level in dl327 infected L929 cells. It seems unlikely that the lack of effect of E3 on E1a expression in LE3 cells is due to lack of E3 protein production, as the results in Fig. 6.5 showed that the E1a level still remained unchanged between LE3 and L929 cells when the cells
were infected with 1 IU/ per cell of dl327. Furthermore, after E1a was expressed during dl327 infection, E3 expression was stimulated to a comparatively high level as shown in Fig.6.5.

The data in Chapter 5 indicated that the maintenance of a certain E3 19k/E3 14.7k ratio might be involved in determining whether E3-mediated down regulation of E1a occurs in some cell lines. Therefore another possible reason for these results was that the ratio between these two E3 products might somehow be changed in LE3 cells. When this possibility was tested, it turned out to be the case. Results in Fig.6.6 show that in LE3 cells, the ratio of E3 19k to E3 14.7k is greatly increased, which is different to the situation in w.t. virus infected parental L929 cells but similar to the situation in HeLa or COS cells.

Why the E3 gene, when it is integrated into the mammalian cell genome, functions differently from that obtained by virus infection, is not clear. However, there are reports that deletions, point mutations and complex rearrangements of transfected genes frequently occur during DNA transfection (Calos et al., 1983; Razzaque et al., 1984; Razzaque et al., 1983). It seems that these deletions, point mutations and rearrangements of the input plasmids inside animal cells have cell specificity as well. For instance, in mouse L cells, genes in plasmids are changed more frequently than that in human KB-cells (Biomonti et al., 1985). If deletions and rearrangements do happen in CMVSH transformed cells, it may well account for the differential E3 expression pattern seen in these cells, as there are reports that even a small single deletion in the E3 region can cause a dramatic change in individual E3 product production (Bhat and Wold 1987). For instance, a 64 base pair deletion in the E3 region causes mainly mRNAs f and h to be formed, from which E3 14.7k is mainly translated, while mRNAs a and c, from which E3 19k is mainly translated, are barely made. This deletion does not contain either a splice site or a polyadenylation site. Thus, the deleted sequence must function in alternative pre-mRNA processing independently of the signal at the actual splice and polyadenylation sites (Bhat and Wold 1986).
The results in this chapter showed that even in L929 cells, when the E3 expression pattern was changed, E1a expression could not be down regulated by E3 gene products. These data further confirmed the conclusion that the relative amounts of E3 19k and E3 14.7k may be the critical determinants of down regulation of E1a by E3 gene products.
Chapter 7

General Discussion
7.1. THE DIVERSE WAYS IN WHICH ADENOVIRUSES EVADE IMMUNE SURVEILLANCE.

The consequences of virus infection are commonly considered to be destruction of the infected cells by the virus or recognition of viral antigens within or on the cell membranes and subsequent destruction by the host’s immune system. However, as the field of virology continues to unfold, it is becoming increasingly clear that long-term infection of the host is a highly significant phenomenon in the natural history of many virus groups. Termination of early symptoms and disease is not always accompanied by elimination of the virus, but by the continuous presence of viral genomic material in some form, i.e. either a persistent (viral DNA replication continues, but at a low level) or a latent (viral genome is present but does not allow replication) infection. This can result in recurrence of the acute form of disease or a more slowly progressing disease, such as cell transformation or cancer, which may or may not resemble the original condition (for review: Haywood 1986; Mims and White 1984; Oldstone and Notkins 1986).

The ability to establish and sustain a persistent infection is a well-recognized property of adenoviruses. Actually adenoviruses were initially discovered because of the spontaneous expression of persistent virus in human tonsillar tissues (Rowe et al., 1953). Group C human adenoviruses (Ad2 and Ad5) commonly cause self-limited upper respiratory tract infections that may lead to a state of prolonged viral persistence. The evidence that group C adenoviruses cause persistent infections comes from the following observations: 1) Production of virus in long term cultures of tonsillar fibroblasts and tonsillar lymphocytes (Andiman and Miller 1981; van der Veen and Lambriex 1973); 2) Detection of adenovirus DNA sequences in cultured tonsillar tissue and blood mononuclear cells (Green et al., 1979b; Horvath et al., 1986; Neumann et al., 1987); 3) Intermittent shedding of virus months or even years after the original human adenovirus infection (Fox et al., 1969).

Although the observation of persistent adenovirus infection was made about 40 years ago, the reasons for adenovirus persistence in
humans remain unknown long after the original description of this phenomenon. Clinical studies indicate the importance of an intact cellular immune response in recovery from adenovirus infection, as the vast majority of fatal adenovirus infections occur in patients with impaired cellular immunity (Koneru et al., 1987; Shields et al., 1985; Zahradnik et al., 1980). There are few reported cases of fatal adenovirus infection in patients with antibody deficiency states (Lederman and Winkelstein 1985). Recent studies have shown that indeed adenoviruses can evade cellular immune surveillance under certain conditions.

Among the components of the cellular immune response that appear to be important in the host response to viral infections are NK cells, macrophages, and CD8+ and CD4+ T lymphocytes. E1a gene products from group C adenoviruses have been shown to induce susceptibility of infected rodent cells to lysis by NK cells and activated macrophages (Cook et al., 1987; Cook et al., 1986). However, in contrast to infected rodent cells, group C adenovirus infection of human fibroblasts and epithelial cells does not result in increased susceptibility to either human or rodent NK cell-mediated killing, despite high levels of E1a protein expression (Routes and Cook 1989). This functional inactivity of E1a gene products in rendering NK cells responsive to adenovirus infected human cells has been considered to be one of the mechanisms for adenovirus to establish a persistent infection in humans (Routes and Cook 1989).

One of the other mechanisms proposed to explain adenovirus persistence is the effect of the E3 19k proteins from group C (Ad2 and Ad5) adenoviruses on host cell surface class I MHC antigen expression (section 1.4.2.2). E3 19k can bind the newly synthesized class I MHC molecules in the endoplasmic reticulum and results in inhibition of terminal glycosylation of class I molecules and the blocking of their transport to the cell surface (Andersson et al., 1985; Burgert et al., 1987; Kvist et al., 1978; Paabo et al., 1986a; reviewed in section 1.4.2.2). As Tc cell recognition requires that viral antigenic determinants be bound by and co-expressed with class I MHC antigens on the cell surface, a marked decrease in surface class I MHC antigen expression early after infection
would theoretically favour maintenance of adenovirus infection by rendering infected cells resistant to elimination by Tc cells. Consistent with this expectation, reduced Tc cell lysis has been observed in some cell types when they are infected with w.t. adenovirus, compared with lysis of E3 deletion mutant infected targets (Burgert et al., 1987; Rawle et al., 1989; Tanaka and Tevethia 1988).

However, more thorough investigations into the interactions between E3 19k and class I MHC antigens showed that E3 19k interference with class I antigen surface expression is not a universal event during adenovirus infection, in either mouse or human cell lines. Class I antigens from mouse cells of different haplotypes demonstrate variable susceptibility to being affected by Ad2 E3 19k. Inhibition of cell surface expression of Db and Kd has been observed but Kk, Dd, and Kb appear not to be affected. In the study by Routes and Cook (1990) it was shown that most types of human cells were resistant to the E3 19k glycoprotein effect on cell surface expression of class I MHC antigens. Moreover, Mullbacher et al (1989) reported that the susceptibility of L929 cells infected with w.t. group C adenoviruses to Tc cells was reduced compared with the targets infected with E3 deletion mutants, although the restricting element in this cell line, H-2Kk, does not bind with E3 19k.

All these observations suggest that there exists at least one other mechanism for group C adenoviruses to evade Tc cell recognition.

Results of experiments presented in Chapter 3 showed that E3 gene products from Ad5 could neither interfere with cell surface expression of H-2Kk, nor affect its ability to present viral antigenic determinants for Tc cell recognition. On the other hand, data presented in this thesis based on a series of protein analyses showed that E1a gene products, the immunodominant antigen for adenovirus Tc cell recognition, were greatly reduced during w.t. group C adenovirus infection, compared with the E1a protein levels in cells infected with their corresponding E3 deletion mutants. Moreover, an immunological study in Chapter 5 showed that there is a strong relationship between the E1a protein levels and the Tc cell killing of target cells. Thus, these observations provide evidence that in some cell types group C
adenoviruses can escape Tc cell recognition and thus elimination by expressing extremely low levels of the Tc cell immunodominant antigen, the E1a protein products.

7.2. THE GENERALITY OF E3 GENE PRODUCT DOWN REGULATION OF E1A EXPRESSION.

Experiments presented in Chapter 5, in which comparison of E1a protein levels was made from 11 cell lines which were infected with w.t. group C adenoviruses and their E3 deletion mutants, extended the observations made in Chapter 3. Results of these experiments indicated that:

1. E3 down regulation of E1a expression occurs in the majority of the cell lines tested but not all of them (7 out of 11 cell lines).

2. E3 gene products interfere with E1a expression in cell lines which are from more than one species (both mouse and human).

3. In mouse cell lines, down regulation of E1a by E3 gene products is class I antigen haplotype independent and it seems there is no direct relationship between binding of E3 gene products to class I MHC antigen and down regulation of E1a expression.

4. E1a expression is also down regulated during w.t. Ad2 infection of L929 cells, as compared with the E1a protein level in dl801 infected cell; but E3 19k from Ad2 cannot down regulate Ad5 E1a expression.

Results from experiments presented in Chapter 5 and Chapter 6 provided evidence that the expression of some of the individual E3 gene products vary in different cell types, and that this might explain, at least in part the cell specificity of the E3 regulation of E1a expression. In cells where down regulation of E1a occurs, the ratio of E3 19k to E3 14.7k is greater than 1; whereas in cells in which down regulation does not occur, this ratio is reversed. Moreover, even in L929 cells where down regulation of E1a by E3 does occur during virus infection, when the E3 19k to E3 14.7k ratio is changed in an E3 gene transformed cell line (LE3), E3 gene products showed no effect on E1a expression. These results suggest that whether E3 gene products can affect E1a expression in
certain cell types depends on the pattern of expression of individual E3 gene products.

Both Ad2 and Ad5 are members of the group C adenoviruses. Their genomes are more than 90% homologous. However, direct comparisons of the E3 region from these two viruses showed that the homology in this region is less pronounced than in other regions (Cladaras and Wold 1985). Some sequences exist in the genome of one serotype but not the other. These "gaps" occur in intergenic regions, or at the 5' or 3' ends of the genes. Consistent with these sequence differences, some gene products from these two viruses such as the E3 19k protein showed different mobilities when they were run on the same gel. Moreover, E3 gene product(s) from Ad2 showed no effect on Ad5 E1a expression.

Adenoviruses have been used by many workers as vectors to carry foreign genes for the development of potential live vaccines (Alkhatib and Briedis 1988; Haj-Ahmad and Graham 1986; Johnson et al., 1988; Prevec et al., 1989). Studies on packaging constraints in adenovirus type 5 suggested that a maximum of about 2 kilobases of extra DNA can be inserted into the wild type viral genome (Ghosh-Choudhury et al., 1987). In order to increase this limit and enhance the usefulness of adenovirus as a vector, some parts of the adenovirus gene were deleted to offer more room for foreign genes to be cloned. As the E3 gene of adenovirus has been considered to be nonessential for virus growth in most types of cultured cells (Kelly and Lewis 1973; Saito et al., 1985), most of these deletion mutants are those with E3 region deleted (Dewar et al., 1989; Morin et al., 1987; Zhu et al., 1988). However, the experimental results presented in this thesis showed that it is a rather general phenomenon that with the E3 gene deleted, E1a expression is greatly enhanced. As E1a gene products have a lot of important biological effects on both viral and cellular processes such as gene regulation and cellular transformation (section 1.2.4), the strategy of using adenovirus E3 deletion mutants as potential live vaccine vectors may need reconsideration. Consistent with this prediction, a preliminary observation made by Mullbacher et al has found that an Ad5 E3 deletion
mutant, dl327, was more "toxic" to the infected mice than w.t. Ad5 (Mullbacher et al; unpublished observations).

7.3. POSSIBLE MECHANISMS BY WHICH E3 INTERFERES WITH E1A EXPRESSION.

Experiments discussed in Chapter 4, aimed to investigate possible mechanisms for the interference of E1a expression by E3. The results showed that in L929 cells:

(1) E3 gene products from Ad5 do not interfere with E1a transcriptional initiation or transcription efficiency as showed by the normal abundance of E1a mRNA.

(2) E3 gene products do not affect E1a RNA splicing, as Northern analysis showed a similar pattern of E1a mRNAs from w.t. Ad5 and dl327 infected cells.

(3) E1a polysomal RNA levels are unchanged or if anything, higher in w.t. Ad5 infected cells than that in cells infected with dl327, which is opposite to the protein levels.

(4) Pulse-chase experiments show that there is no significant difference in E1a stability in cells infected with w.t. virus and E3 deletion mutant.

These results provide evidence that the E3 gene products interfere with E1a by some translational control mechanism.

De novo synthesis of proteins in eukaryotes is a complex process involving multiple steps and numerous components. The basic machinery is provided by the ribosomal particles with which the translational enzymes, mRNA and aminoacyl-tRNA interact during protein synthesis. It has become clear during the last decade that the protein synthesis machinery is not only capable of synthesizing proteins with a high degree of accuracy, but the level of translation of mRNA can be regulated both quantitatively and qualitatively. Most studies concerning translational regulation have dealt with regulatory mechanisms influencing the rate of initiation, but it has become increasingly evident that the elongation and termination processes are

The initiation process of translation serves to decode the proper initiation signal on the mRNA thereby positioning the ribosome on the mRNA in the correct reading frame. The first step in the initiation is the dissociation of the 80S ribosomes into two subunits. In the following reaction, initiator tRNA, Met-tRNA\textsubscript{f} in a ternary complex with GTP and initiation factor eIF2 are bound to the 40S subunit and are then ready to pick up the mRNA. After all of these processes are finished, this complex is bound to the 60S subunit to form a programmed 80S ribosome, which is then ready for chain elongation. Since the data from Chapter 4 showed that there is no significant difference in the E1a polysomal mRNA levels from the cells infected with w.t. Ad5 and dl327, it may be argued that E3 gene products do not interfere with E1a expression at translational initiation. However, as the mRNA was isolated from unfractionated polysomes in this thesis, this polysomal RNA detection may not completely reflect the E1a mRNA levels at the point before the chain elongation starts.

As discussed in section 1.2.9, one of the adenovirus gene products, VA RNAI, has been shown to regulate gene expression at translational initiation. VA RNAI can bind to the double-stranded RNA activated inhibitor which phosphorylates eIF2, thus resulting in efficient recycling of eIF2 and efficient translation. Moreover, VA RNAI seems to selectively up regulate viral mRNA translation. One hypothesis proposed by O'Malley et al (1989) for this selective action is that there may be segregation of host and viral RNAs into different translational compartments. The viral compartment would contain viral mRNAs and VA RNAI, which would prevent activation of double-stranded RNA activated inhibitor and thus permit active protein synthesis. Whereas in the host mRNA compartment there would be no VA RNAI and the cellular mRNA translation would be inhibited. Consistent with the observation from VA RNAI, E3 gene products also showed a selective effect on viral mRNA translation (although there may be another explanation, such as that E3 specifically interferes with E1a expression.
and the low level of products from other genes may be due to lack of E1a transactivation). Unlike VA RNAI, E3 gene products inhibit the viral mRNA translation. Therefore one possibility would be that E3 gene products interfere with VA RNAI and abrogate its function on translational regulation.

Chain elongation during protein synthesis requires participation of two factors, eEF-1 and eEF-2. During purification of these factors it was discovered that the eEF-1 activity occurred in multiple forms with molecular masses from 50kD to more than 500kD (Nombela et al., 1976; Slobin and Moller 1975). A polypeptide called eEF-1a of low-molecular-mass is the functional form and the high-molecular-mass form is a storage form (Slobin and Moller 1975). The activity of the eEF-1a seems to correlate with the extent of methylation (Fisher and Moldave 1980). It has been reported that in Simian-virus-40 transformed 3T3 cells, eEF-1a is methylated to a much higher extent than in non-transformed cells (Coppard et al., 1983). It has been suggested that methylation may account for differences in growth properties for the different cell types. If E3 gene products interfere with E1a expression at elongation instead of translational initiation, one possible mechanism would be that they inhibit eEF-1a methylation. Another possible mechanism for E3 gene products to down regulate nascent E1a chain elongation would be that they bind with a few newly synthesized E1a amino acids and thereby prevent further elongation.

7.4. FUTURE DIRECTIONS.

In order to identify the E3 gene product(s) which is responsible for down regulation of E1a expression, two approaches could be taken. The first would be to generate a series of deletion mutants of group C adenoviruses, each with a single E3 gene deleted, like the derivative mutants of rec700, but with the E1a region from the same serotype as the E3 region. By comparing E1a expression in L929 cells infected with these mutants, it would be possible to identify which E3 gene product(s) is involved in affecting E1a expression. Alternatively, plasmids containing individual E3 genes and E1a region from the same serotype could be
constructed. The E3 plasmids could be constructed by building an E3 cDNA library. By comparing E1a expression from cells cotransfected with an E1a plasmid and plasmids containing individual E3 genes, it should also be possible to map the E3 gene products which are responsible for interfering with E1a expression.

Additional studies on translational regulation might help to clarify the detailed mechanisms of the E3 gene product(s) involved in regulation of E1a expression. Studies with the in vitro translation technique should be able to determine whether E3 gene products interfere with E1a expression at translational initiation or elongation. Tests of activities of some initiation and elongation factors such as eIF2 and eEF-2 could also shed light on this problem. By examining whether E3 gene products affect expression and function of VA RNA, which has been shown to be involved in gene regulation at the level of translational control, it might be possible to identify whether E3 indirectly interferes with E1a expression in this way.

Finally, studies on the effect of E3 deletions in vivo, such as checking the pathological and immunological differences between animals infected with w.t. Ad5 and dl327, might provide more information about the significance of the down regulation of E1a expression by E3 gene products.


Hearing, P. and Shenk, T. (1986). The adenovirus type 5 E1A enhancer contains two functionally distinct domains: One is specific for E1A and the other modulates all early units in cis. *Cell.* 45: 229-236.


