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FUNCTIONAL ASSESSMENT OF THE TUMOR SUPPRESSOR ACTIVITY OF THE CUTL1 GENE

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Abstract

Mammalian Cut proteins appear to function as transcriptional repressors that play a role in determining cell type specificity. Recent evidence suggests that *cut* genes are tumour suppressor genes. This hypothesis was further examined by using functional assays to evaluate the abilities of human Cut to: a) suppress oncogene-mediated cell transformation, and b) interact with the SV40 Large T (LT) antigen. The results indicate that: a) there was a decrease in the number of transformed colonies and foci when rat embryo fibroblasts (REFs) were transfected with *ras*/c-*myc* or *ras*/*E1A* and *cut*; b) the homeodomain region of Cut interacted with SV40 LT *in vitro*; and c) Cut protein levels were elevated and Cut DNA binding was increased in cells transfected or transformed with SV40LT. These observations suggest that Cut proteins may regulate cell proliferation and that the Cut homeodomain may mediate protein interactions.

Résumé

Les protéines Cut semblent fonctionner a titre de répresseurs transcriptionnels qui sont impliqués dans la détermination de la spécificité cellulaire. De récentes résultats suggèrent que les gènes *cut* se comportent comme des gènes suppresseurs du cancer. Cette hypothèse a été examinée plus particulirement à l'aide d'essais fonctionnels évaluant la capacité de la protéine Cut humaine à: a) supprimer la transformation cellulaire induite par des oncogènes; et b) intéragir avec l'antigène grand T du virus SV40. Les résultats montrent: a) qu'il y a diminution du nombre de colonies transformées et de foyers de transformation lorsque des fibroblastes embryonnaires de rat (REF) sont transfectés avec les oncogènes c-*myc*/*ras* ou *E1A*/*ras* en présence de *cut*; b) qu'il y a intéraction entre le domaine homéo de Cut et le grand T de SV40 *in vitro*; et c) qu'il y a élévation du niveau d'expression de Cut et augmentation de sa capacité de se lier à l'ADN dans des cellules transfectées ou transformées par le grand T de SV40. Ces observations suggèrent que les protéines Cut contribuent à la régulation de la prolifération cellulaire, et que le domaine homeo de Cut est impliqué dans l'inter-action avec d'autres protéines.

Preface

The following option, as described in the "Guidelines for Thesis Preparation" from the Faculty of Graduate Studies and Research at McGill University, has been used.

> *Candidates* have *the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.*
>
> *If this option is chosen,* ***connecting texts that provide logical bridges between the different papers are mandatory.*** *The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*
>
> *The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation".* ***The thesis must include:*** *A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.*
>
> *Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.*
>
> *In the case of manuscripts co-authored by the candidate and others,* ***the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.*** *Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's best interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.*

As required by the Guidelines, Chapter 1 is a general introduction and review of the literature. The experimental functional assays performed to determine whether or not the human Cut like-1 (CUTL1) gene functions as a tumour suppressor gene are presented in

Chapters 2 and 3. I have "availed myself of the option to write Chapters 2 and 3 in the form of published or to be published papers". Therefore, both chapters contain an Introduction, Materials and Methods, Results and Discussion section. Chapter 2 describes experimental data on the ability of the human CUTL1 gene to influence oncogene-mediated cell transformation. Chapter 3 describes experimental data on the interaction between the human Cut protein and the Simian Virus 40 (SV40) Large T antigen and the consequences of this interaction on the function of Cut. Chapter 4 summarizes the main observations and conclusions of the results presented in Chapters 2 and 3 and contains suggestions for future work.

Claims to original research

1. I have presented evidence that the human *cut* gene suppresses E1A/Ras and Myc/Ras cell transformation.

2. I have shown that the homeodomain of the human Cut protein interacts with the SV40 Large T antigen *in vitro*. This study is the first report of an interaction between a homeodomain and a DNA tumour virus protein. This is also the first time that a Cut homeodomain has been found to interact with another protein.

Acknowledgements

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List of Abbreviations

CUTL1 . . . . . Human Cut like-1 gene

TBP . . . . . TATA-Binding protein

CDP . . . . . CCAAT displacement protein (or human Cut protein)

Cut . . . . . Cut proteins in general (species specified in text)

hCut, CDP/cut . . . . . Human Cut protein

cut . . . . . cut genes in general (species specified in text)

Cux/mCut . . . . . Murine Cut protein

cux/mcut . . . . . Murine cut gene

CDP2 . . . . . Rat Cut protein

CASP . . . . . Cut-alternatively-spliced-product

pRb . . . . . Retinoblastoma protein

Rb . . . . . Retinoblastoma gene

SV40 LT . . . . . Simian Virus-40 Large T antigen

REF . . . . . Rat embryo fibroblast

FBS . . . . . Fetal bovine serum

BSA . . . . . Bovine serum albumin

FP . . . . . 29 bp probe used in gel-shift assays

GST . . . . . Glutathione S-transferase

SDS . . . . . Sodium dodecyl sulfate

***Note:** Gene names are specified in the text in italics and proteins with initial capital letters.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION TO TRANSCRIPTION AND TRANSCRIPTION FACTORS

Transcription is the process by which RNA is synthesized from a DNA template and involves three steps: *initiation*, *elongation*, and *termination*. During initiation, the general (also known as basal) transcription factors bind with RNA polymerase II to a TATA element or an Initiator motif in a gene promoter, and then the first phosphodiester bond is synthesized. Initiation requires recruitment of the general transcription factor Transcription Factor IID (TFIID). Recruitment can occur by direct binding of TFIID to the TATA element through its TATA-Binding-Protein (TBP) subunit, or by tethering of TFIID components in TATA-less promoters by transcription factors such as Sp1 (1, 2). Then the other general transcription factors bind to the promoter by protein-protein interactions or by protein-DNA interactions (1). After initiation, the RNA transcript is elongated until it is full length and then transcription is terminated.

The model just described explains *basal transcription* that occurs *in vitro*. *In vivo*, transcription is regulated at the level of initiation by the binding of proteins called *activators* to cis acting DNA regulatory sequences and this is called *activated transcription*. Activators stimulate transcription when their interaction domains interact with the general transcription factors (1). Sp1, AP1, and CBP (CCAAT box binding protein) are activators.

Transcription factors that negatively regulate transcription are called *repressors*. Repressors function by inhibiting the expression of specific genes and this can occur by *indirect* (passive) or *direct* (active) mechanisms (3). Indirect repression occurs when a repressor prevents an activator from binding to DNA by: a) reversing the reorganization of chromatin structure and masking the activator binding site; b) binding directly to the activator binding site; or c) interacting with the activator and forming a complex that cannot bind DNA. Indirect repression also occurs when a repressor prevents an activator from stimulating transcription when already bound to DNA (quenching). This can occur when a repressor: a) binds to an activator thereby masking its activation domain, or b) binds to a site adjacent to an activator and interacts directly with the activator. Direct repression occurs when a repressor inhibits transcription by interfering with the general transcription factors when: a) the repressor binds to DNA and interacts with the basal transcription complex; or b) the repressor interacts with the complex via a protein-protein interaction.

1.2 HOMEODOMAIN PROTEINS AND THEIR ROLE AS TRANSCRIPTION FACTORS

1.2a Discovery of the homeodomain and homeodomain proteins.

Cut proteins are members of a family of proteins that were identified based on the presence of a DNA binding domain conserved in evolution called the *homeodomain*. The homeodomain is a 61 amino acid protein domain encoded by the 183 base pair motif called the *homeobox* (4,5) which is related to the helix-turn-helix DNA-binding domain of bacterial transcriptional repressors (6, 7).

The discovery of the homeodomain originates from the study of *Drosophila* developmental genes. *Homeotic mutations* (developmental anomalies that result in transformation of one body part to another) were identified and the developmental genes responsible for such cell type specification were called *homeotic* genes (4) since they specify segmental identity and the body plan (8). For example, mutations in the antennapedia (antp) gene result in the formation of leg structures on a segment where the antennae belong (9). Homeobox genes have also been identified in nematodes, yeasts, plants and mammals. Many of these genes function similarly to *Drosophila* homeodomain genes in that they have been shown to regulate development or cellular differentiation. For example, Hox genes are involved in the formation of the anterior-posterior axis (10). Evidence suggests that homeodomain proteins regulate development and differentiation in mammals by acting as negative and/or positive transcriptional regulators (11).

1.2b Structure of the homeodomain and DNA binding.

The three dimensional structure of the homeodomain when bound to DNA was determined for the Antp homeodomain monomer of *Drosophila* by NMR spectroscopy (6). The homeodomain consists of 4 alpha helices. The second and third helices contact the major groove of DNA and are together called the "recognition helix". This helix also determines DNA binding specificity (12). The loop between helices I and II interacts with the DNA backbone, and the flexible amino-terminal arm that precedes helix I contacts the minor groove

of DNA. In addition, the three dimensional structure of the *Drosophila* engrailed homeodomain-DNA complex was determined by X-ray crystallography (13). The two structures are almost identical except that the recognition helix in the engrailed protein is not divided into two helices.

1.2c DNA binding and the role of the homeodomain in target site specificity.

Homeodomains have been shown to bind to DNA sequences with a TAAT core although other sequences have been identified (12). The target sequences for several homeodomain proteins are similar and several homeodomains can recognize the same DNA sequence *in vitro*, however, homeodomain proteins have specific DNA binding specificities *in vivo*. The 9th amino acid of the recognition helix has been shown to determine DNA binding specificity *in vitro* since the preferred DNA binding site of a homeodomain protein can be changed when this residue is altered (12). However, other amino acids inside or outside the homeodomain may account for this specificity *in vivo*. For example, it has been suggested that specificity may depend on other conserved DNA binding domains such as the POU specific domain found in the POU class of transcription factors. Both the homeodomain and the POU-specific domains have been shown to be required for high affinity DNA binding by POU class transcription factors (14).

In some cases, the relevant amino acids may be in the amino-terminus of the homeodomain (15). When the amino-terminal amino acids of the *Drosophila* Deformed protein are replaced with those of the Ultrabithorax protein, the Deformed protein acquires Ultrabithorax DNA binding specificity. It has been proposed that residues in the amino-

terminal arm may confer specificity by way of their interactions with the minor groove of DNA. Alternatively, some of the side chains in the amino-terminal region that are directed away from DNA may confer specificity by mediating interactions with other transcription factors (16, 17).

1.2d Protein-protein interactions mediated by homeodomain proteins.

It has been suggested that interactions between transcription factors may affect the DNA binding specificity of homeodomain proteins. For example, the yeast homeodomain repressor MAT α2 binds to different DNA sequences depending on its interactions with other proteins (18). MAT α2 is able to recognize and bind to different DNA sequences by using peptides on either side of the homeodomain to mediate interactions with other proteins that bind adjacent to MAT α2 sites.

Protein-protein interactions can also affect the ability of transcription factors to activate or repress transcription. Recently a direct interaction between the TATA-binding protein (TBP) subunit of TFIID and the *Drosophila* Even Skipped (Eve) homeodomain protein was demonstrated (19). Eve is an active repressor that is involved in pattern formation during *Drosophila* embryogenesis (20). The Eve repression domain has been localized to residues 140 to 247 just after the homeodomain. Both the repression domain and the homeodomain are required for the interaction with TBP. Eve represses transcription in an active manner by interacting with TBP and interfering with its function (19).

The murine Msx-1 homeodomain repressor also interacts with TBP (21). Msx-1 is expressed during development in embryonic regions such as derivatives of the cranial neural crest and mutations in msx-1 result in craniofacial defects (22). The Msx-1 repression domain

is located in the amino-terminal arm of the homeodomain and this region is also necessary for binding to TBP. Unlike Eve, Msx-1 can repress gene transcription without binding directly to DNA. Similar to Eve, Msx-1 represses gene transcription in an active manner by interacting with TBP and interfering with its function (21).

The homeodomain and the amino acids on either side of the homeodomain have also been shown to mediate homodimerization between *Drosophila* Bicoid (Bcd) proteins (23). Furthermore, homodimerization facilitates cooperative DNA binding by Bcd proteins (24). The Bicoid protein is a transcriptional activator that is required for development of anterior structure in the embryo (25).

1.2e Homeodomain proteins and cancer.

Homeobox genes in mammals control genes for development and cell type commitment (11) and de-regulation of homeobox genes plays a role in the development of cancer. The first example providing evidence for a role of homeobox genes in cancer was the demonstration of retroviral transcriptional activation of the HOXB8 gene in a mouse myeloid leukemia cell line (26,27). Transfection of an activated HOXB8 gene transforms NIH 3T3 cells and induces fibrosarcomas in nude mice (28). Translocations involving homeobox genes have also been implicated in cancer. For example, the t(10;14)(q24,q11) chromosomal translocation in T-cell acute lymphoblastic leukemias involves the fusion of the T-cell receptor delta chain with the homebox gene tcl3. In normal T-cells, tcl3 is not expressed and over-expression of tcl3 transforms NIH 3T3 cells (29). Increased expression of homeobox genes has also been found, for example, in breast, colonic, rectal, gastric, lung, renal and testicular carcinomas (30).

1.3 THE MAMMALIAN CUT GENES AND PROTEINS

1.3a Discovery of the mammalian Cut homeodomain proteins.

The human CCAAT displacement protein (CDP) was originally identified as a protein that bound to CCAAT sites in the sperm-specific *histone H2B* gene promoter (31). Later CDP was shown to bind to CCAAT sites in the phagocyte-specific cytochrome heavy chain *gp91-phox* gene promoter in immature myeloid cells (32). The *gp91-phox* gene is required for the production of a respiratory burst and microbicidal activity of phagocytes, and expression is limited to terminally differentiated phagocytic cells of the monocyte/macrophage or granulocyte lineages. Strong CDP binding to the *gp91-phox* promoter is observed in immature myelomonocytic leukemia cells and decreases as the cells mature (32).

CDP was purified from HeLa cells by DNA-affinity chromatography using the FP oligonucleotide which contains two CCAAT sites (33). A human cDNA that was almost identical to the cDNA encoding the *Drosophila* Cut protein was isolated by screening a λgt11 cDNA library, prepared from human umbilical vein endothelial cells, with antibodies against purified CDP (33). Therefore, the human CCAAT displacement protein (CDP) is also referred to as CDP/cut, or human Cut (hCut).

A cDNA homologous to the CCAAT displacement protein was also isolated in an attempt to identify proteins involved in the transcriptional control of the c-*myc* proto-oncogene. The c-*myc* proto-oncogene codes for a transcription factor normally involved in cellular proliferation and differentiation. It is a member of a class of genes known as "immediate-early" which means that c-*myc* expression is modulated whenever cells receive a signal to proliferate or differentiate and that regulation of c-*myc* does not require *de novo* protein

synthesis (34, 35, 36). Therefore, it is believed that proteins involved in c-*myc* regulation pre-exist in cells and are modified in response to a given signal and that post-translational modification of these proteins would lead to changes in c-*myc* expression. Inhibition of c-*myc* expression arrests proliferating cells (37, 38, 39, 40), and expression is down-regulated in differentiating cells (6, 41, 42, 43, 44, 45), thereby suggesting that overexpression of c-*myc* may lead to cancer. It has been shown that co-expression of a c-*myc* plasmid and *ras* results in transformation of rat embryo fibroblasts (REFs) (46) and activation of the c-*myc* gene (resulting in overexpression) is a common occurrence in human tumors (47).

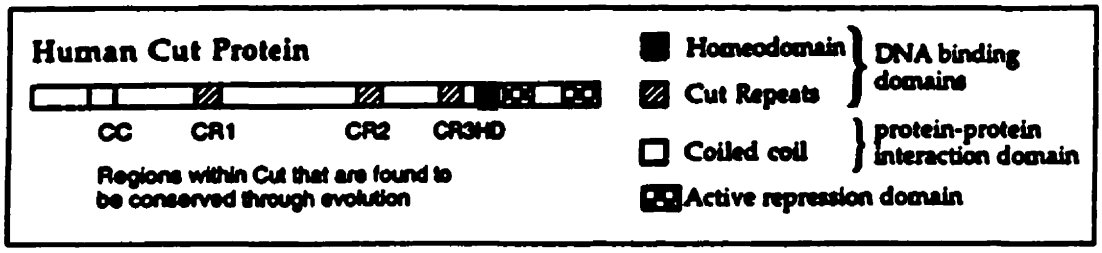
Since it is evident that overexpression of c-*myc* can lead to cancer, it is important that Myc expression be controlled in cells. A protein binding site called ME1a1 was identified that plays a role in the control of transcription initiation and elongation of the c-*myc* gene (48, 49, 50). It was shown that positively (Sp1) and negatively trans-acting regulatory proteins bind to the ME1a1 site. In order to isolate proteins binding to ME1a1, a λgt11 cDNA expression library prepared from human placenta was screened with an ME1a1 DNA probe (49). One positive clone was isolated containing a cDNA homologous to the cDNA encoding the human homolog of the *Drosophila* Cut homeodomain protein known as CDP/cut. Therefore, this gene was named CUTL1 (Cut like-1). A bacterially expressed GST/Cut protein was shown to bind to the ME1a1 site in DNA binding assays, and full length Cut repressed the c-*myc* promoter when transfected into 293 cells (49).

The cDNAs for several other mammalian homologues of the *Drosophila* Cut protein have also been isolated. The mouse Cut protein (known as mCut or Cux) was isolated in an attempt to identify proteins binding to the homeodomain consensus binding sequence ATTA

found in an *NCAM* (neural cell adhesion molecule) gene promoter regulatory element (51). Cell adhesion molecules are important in tissue physiology and morphogenesis and they can be disrupted in cancer (52). The cDNA for Cux was isolated by screening a λgt11 cDNA library prepared from RNA from an NCAM-positive neuroblastoma cell line N2a and from adult mouse brain with a probe constructed from the region of the *NCAM* promoter that contains the ATTA motif (51). The canine Cut protein (Clox), was isolated from a dog cardiac ventricle λgt11 cDNA expression library using a probe consisting of the βe2 element in the rat *β-myosin* heavy chain enhancer (53). The *β-myosin* heavy chain gene encodes the β-myosin heavy chain contractile protein which is expressed in the ventricular myocardium and in slow twitch skeletal muscle. The βe2 element in the *β-myosin* heavy chain gene enhancer is required for maximal activity of the enhancer (54). Finally, the rat Cut protein (CDP2) was isolated from a λgt11 cDNA expression library derived from a rat pheochromocytoma cell line by using a probe consisting of the *tyrosine hydroxylase* gene enhancer which contains an E-box core (55). The *tyrosine hydroxylase* gene encodes an enzyme involved in catecholamine synthesis.

These mammalian Cut proteins are almost identical to the human protein and together they form a family of homeodomain proteins that have two unique structural features (51, 53, 55, 56). First, the homeodomain has a histidine at the ninth position of the recognition helix, and second, the proteins have three 70 amino acid Cut repeats (CR1, CR2, CR3) that have 52-63% identity with each other and are conserved in evolution. Cut proteins also have a coiled-coil structure (CC) that is conserved in evolution . Figure 1.1 depicts the human Cut protein and the five conserved domains. Cut proteins are ubiquitously expressed, localized to the nucleus, and 180-190 kDa in size (51,56).

Figure 1.1 The human Cut protein. The five evolutionarily conserved domains are illustrated: the coiled-coil (CC); Cut repeats 1, 2, and 3 (CR1, CR2, CR3); and the homeodomain (HD). The first active repression domain consisting of 81 amino acids after the homeodomain, and the second active repression domain consisting of 51 amino acids at the extreme carboxy terminus are also illustrated.


Human Cut Protein
CC
CR1
CR2
CR3HD
Regions within Cut that are found to be conserved through evolution
Homeodomain
Cut Repeats
DNA binding domains
Coiled coil
protein-protein interaction domain
Active repression domain

1.3b Cut proteins bind to DNA.

Cut proteins have been isolated as proteins that bind to DNA sites in gene promoters that are also occupied by activators such as Sp1 and CBP. The human Cut protein (CDP/cut) was first identified as a protein that bound to CCAAT sites in the sea urchin testis-specific H2B gene promoter (31). Then CDP/cut was shown to bind to CCAAT sites in the *gp91-phox* gene promoter (32, 33), and to the Sp-1 like ME1a1 site in the c-*myc* promoter (49). The murine Cut protein (mCut or Cux) was isolated as a protein that binds to sites in the mouse *NCAM* gene promoter (51). Cux was shown to bind to the same sites as the activator Phox-2 which is a protein that may be involved in modulating NCAM expression and may determine neurotransmitter phenotype. The canine Cut protein (Clox) was shown to bind to the rat *β-myosin* heavy chain gene promoter (53), and rat CDP2 was shown to bind to an E-box in the rat *tyrosine hydroxylase* enhancer (55). An activator protein called rITF2 also binds to the E-box in the rat *tyrosine hydroxylase* enhancer. rITF2 is the rat homolog of the human ITF2 or E2-2 protein which binds to other basic-helix-loop-helix (bHLH) proteins such as MyoD. Binding of rITF2 to DNA was shown to be enhanced in the presence of CDP2, thereby suggesting that CDP2 and rITF2 may interact (55).

Although it was clear that mammalian Cut proteins bind to DNA sites in a number of gene promoters, next it was necessary to investigate which regions of the Cut protein were necessary for binding to DNA. In addition to the homeodomain, other conserved protein domains (such as the POU domains) have been shown to play a role in DNA binding (14). The high degree of conservation of the Cut repeats suggested that they may have important functions so their ability to bind DNA was investigated. Cut repeats were shown to bind to

sequences closely related to CCAAT and Sp-1 consensus binding sites (31, 53, 57, 58). It was shown that Cut repeats 1 and 3 function as specific DNA binding domains, and that Cut repeat 3 forms a bipartite DNA binding domain with the homeodomain (57, 58). Furthermore, although the four DNA binding domains (CR1, CR3, HD, and CR3-HD) have broad and overlapping DNA-binding specificities, a consensus sequence was identified. It consists of a core sequence ATCGAT flanked with G/C-rich sequences.

Recently, it became apparent that Cut proteins bind to sites in histone gene promoters and may play a role in regulating histone genes. Expression of histone genes is needed for cells to progress into S phase of the cell-cycle since histone nucleosomal proteins are necessary for packaging of replicated DNA into chromatin (59). Since the original identification of CDP/cut as a protein that bound to sites in the sea urchin *histone H2B* gene promoter (31), Cut proteins from *Xenopus* nuclear extracts have been shown to bind to histone promoters (60), and CDP/cut was identified as the DNA binding subunit of the histone HiNF-D promoter complex in humans (61). The HiNF-D complex has been shown to regulate histone expression. In addition to CDP/cut, the complex contains cell cycle proteins such as cyclin A, CDC2 and either the retinoblastoma protein (pRb) or the pRb-related protein p107. CDP/cut was shown to be associated with the retinoblastoma protein (pRb) at the *histone H4* promoter and with p107 (a pRb related protein) at the *gp91-phox* promoter (61).

It was originally proposed that the DNA binding subunit of the HiNF-D complex was the transcriptional activator E2F. E2F binding sites are found in a number of genes needed for DNA synthesis including *thymidine kinase* (62) and *DNA polymerase* α (63). The activity of E2F is controlled by complexing with proteins such as pRb (64, 65, 66). pRb is

hypophosphorylated in G0 and early G1, and if conditions favour cellular proliferation, pRb is phosphorylated by cyclin-dependent kinases and cells are permitted to proceed through the cell-cycle. E2F complexes with hypophosphorylated pRb in G0 and early G1 and the pRb-E2F complex acts as a transcriptional repressor. When pRb is phosphorylated in late G1 and S phases of the cell cycle, E2F is released and genes necessary for cellular proliferation are induced (64, 65, 66). With this in mind, it has been suggested that similar to E2F, CDP/cut may also be regulated in a cell-cycle regulated manner (61).

Recent evidence suggests that DNA binding by Cut is regulated during the cell cycle. Alike the case with E2F, investigation of the cell-cycle regulation of CDP/cut may help to identify genes that are regulated by Cut proteins. DNA binding is detected only at the end of the G1 phase and during S phase (67) so it is has been suggested that two genes which are down-modulated in G1/S, *p21/WAF1/CIP1* and *MPTP*, may be physiological targets of Cut (67, 68, 69). CCAAT and Sp1 binding sites are present in the *p21(WAF1/CIP1)* and *MPTP* gene promoters, and at least in the case of p21, the Sp1 sites have been shown to be necessary for expression (70). p21 is a cyclin-dependent kinase inhibitor that is up-regulated by p53 *in vitro* in response to DNA damage. p21 blocks cell-cycle progression at G1 and suppresses growth of human brain, lung and colon tumor cells (71). MPTP is a murine T-cell protein tyrosine phosphatase (PTPase) whose function has not been determined (69).

1.3c Cut proteins repress transcription.

Cut proteins are usually described as transcriptional repressors. CDP/cut was first shown to repress the sea urchin *histone H2B* gene in nontesticular tissues (31) and the myeloid

cytochrome heavy chain gene *gp91-phox* in immature cells (32, 33, 72). Upon terminal differentiation, CDP/cut binding decreases, transcriptional repression diminishes, and the gene is activated. The human Cut protein has also been shown to repress transcription from the c-*myc* promoter (49). Murine Cut (mCut or Cux) represses transcription from the mouse *NCAM* gene promoter (51), and the canine Clox protein represses transcription from the rat *β-myosin* heavy chain gene promoter (53).

Recently it was shown that CDP/cut can repress transcription from the *histone H4* promoter (61). Recent evidence also suggests that Cut may repress the *p21* and *MPTP* gene promoters (67). mRNA levels of both genes have been shown to be decreased in S phase and this down-modulation has been suggested to occur at the level of transcription (67, 68, 69, 70, 71, 73). Since Cut is able to bind DNA and repress transcription only in G1/S, it has been suggested that Cut may play a role in this regulation.

Although Cut proteins have been shown to repress a number of gene promoters, the mechanism of repression had not been defined until recently. The mechanism of transcriptional repression was investigated using the herpes virus *thymidine kinase (tk)* promoter linked to the *CAT* gene (74). The *tk* promoter has Sp1 and CCAAT binding sites which are DNA binding targets of Cut. The human Cut protein was shown to repress transcription by both active and passive mechanisms. The carboxy-terminal region of hCut was shown to act as an active repression domain. A series of deletion mutants were constructed and two repression domains were localized to 81 amino acids after the homeodomain and to 55 amino acids at the extreme carboxy terminus (Figure 1.1). Furthermore, it was shown that each active repression domain must be tethered to DNA in order to repress transcription and that neither domain is able to

repress a minimal promoter. These findings suggest that the Cut repression domains do not affect basal transcription but instead they may hinder interactions between an activation domain and components of the pre-initiation complex (74). Passive repression by Cut was shown to occur when the composite DNA binding domain CR3-HD binds to a CCAAT or an Sp1 site in the *tk* gene promoter, thereby competing with activator proteins Sp1 and CBP (74).

1.3d Clues to Cut function from *Drosophila*, and from its role as a DNA binding protein and a transcriptional repressor.

The Cut protein has an important role in determining cell type specification in tissues in *Drosophila* (75). Viable Cut mutations result in defects in the wings, legs, external sense organs (es), Malpighian tubules, tracheal system, and central nervous system (75, 76, 77, 78, 79, 80). Defects caused by *cut* mutations seem to result from the fact that some cells follow the wrong developmental path. For example, in one such mutant the gut wall thickens where the Malpighian tubule should normally form, thereby suggesting that the cells differentiated into gut cells instead of tubule cells (77, 78, 79). Embryonic lethal mutations in *cut* result in the morphological transformation of external sense organs (es) into chorodontal (ch) organs that sense stretch (76). Conversely, ch organs are transformed into es organs when Cut is ectopically expressed in embryos (75).

Although the biological function of *cut* genes in mammals has not been determined, it is likely that the human *cut* gene also plays an important role in determining cell fate. It has been suggested that de-repression of mammalian genes controlled by CDP/cut (for example *gp91-phox*) in certain cell types may be a final step in cell differentiation (72). When cells

become terminally differentiated, Cut no longer binds and gp91-phox is expressed. It has also been suggested that Cut represses c-*myc* in differentiating cells (67). Since Cut binds to DNA only in the G1 and S phases of the cell cycle, it has been proposed that Cut may function as a cell-cycle dependent transcriptional repressor. For example, Cut can repress the *p21* and *MPTP* gene promoters, and both genes are down-modulated in G1/S phases of the cell cycle (67, 68, 69, 70, 71, 73). Further support for the role of Cut as a cell-cycle repressor comes from the recent finding that Cut complexes with cell cycle proteins such as pRb at the *histone H4* gene promoter and with p107 at the *gp-91 phox* gene promoter (61).

Although Cut proteins generally function as transcriptional repressors, it has not been ruled out that they may also act as transcriptional activators. The finding that histone gene transcription is activated in G1 and S phases of the cell cycle, and that Cut has been shown to bind to DNA and repress transcription only in G1 and S, suggests that CDP/cut may also be able to activate transcription of some genes. Since CDP/cut has been shown to form complexes with pRb and p107, it is possible that CDP/cut activates or represses transcription in a manner similar to E2F depending on whether or not it is bound to pRb or p107 (61). E2F acts as an activator when it is not bound to pRb, however, the pRb-E2F complex can repress transcription in an active manner (81, 82, 83). It was originally proposed that pRb represses transcription when bound to E2F by converting an E2F site from a positive to a negative element (81). In the presence of hypophosphorylated pRb (which binds to E2F) an E2F site acts as an enhancer that is silenced, while in the presence of phosphorylated pRb (which does not bind to E2F) an E2F site acts as an active enhancer. Recently it was shown that pRb actively represses transcription mediated by AP1, p53, and Sp1 when it is directly bound to a promoter (82). E2F

brings the pRb-E2F complex to a promoter where it actively blocks interactions with other transcription factors and the basal transcription complex (83). Further evidence supporting the idea that Cut proteins may also act as activators is that both rat CDP2 and the bHLH protein rITF2 are needed for activation of transcription from the rat *tyrosine hydroxylase* enhancer (55).

1.4 PROTO-ONCOGENES AND TUMOR SUPPRESSOR GENES AND INACTIVATION OF TUMOR SUPPRESSOR GENE PROTEINS BY DNA TUMOR VIRUS ONCOPROTEINS

1.4a Introduction to proto-oncogenes and tumor suppressor genes.

Most human cancers result from the activation of proto-oncogenes and the inactivation of tumor suppressor genes by mutation. Proto-oncogene mutations are usually dominant to their wild-type allele and are referred to as gain-of-function mutations. Tumor suppressor gene mutations are usually recessive to their wild-type allele and are therefore referred to as loss of function mutations.

1.4b Transformation by the DNA tumor viruses and inactivation of tumor suppressor gene proteins.

DNA viruses depend on the host cell to synthesize viral DNA (84). Cells in most tissues are quiescent and do not have high enough concentrations of deoxynucleotides and DNA metabolic enzymes to support many rounds of viral DNA replication. Therefore, for effective infection DNA tumor viruses must force the host cell to enter S phase and to induce expression of enzymes involved in DNA replication. Expression of the early genes of the DNA

tumor viruses (often called T antigens) is required for viral replication. The late genes are necessary for synthesis and packaging of viral DNA, cell lysis and virus release. There are generally two outcomes of viral infection that depend on whether the host is permissive or non-permissive for infection. In permissive host cells both early and late viral genes are expressed and as a result, viral particles multiply in and kill the host cells. In contrast, only the early viral genes are expressed in non-permissive host cells so viral particles do not multiply in and kill the host cells. In some cases these cells can become transformed, which generally occurs when the viral DNA is stably integrated into the host cell genome (84).

In cells that have been transformed by a DNA tumor virus, the function of tumor suppressor genes can be lost as a result of protein-protein interaction between the tumor suppressor protein and the viral protein. For example, the retinoblastoma protein (pRb) has been shown to form a complex with the SV40 Large T (LT) antigen (85, 86). When SV40 LT binds to pRb, free E2F is released, pRb-mediated transcriptional repression is blocked and cells progress through the cell cycle. p53 also forms complexes with SV40 LT, and this interaction prevents p53 from binding to DNA and activating transcription (87, 88). These results are interpreted to mean that DNA tumor viruses encode gene products that force cells into a DNA synthetic or S phase and this involves the inactivation of cellular proteins which negatively control cell proliferation. A corollary to this is that protein-protein interaction with a viral protein is taken as an indication that the interacting protein plays an important role in the control of cell growth and/or DNA replication (89).

1.4c The *p53* tumor suppressor gene and transcriptional regulation.

The *p53* gene is unusual because it appears to have properties of both a proto-oncogene and a tumor suppressor gene. *p53* was originally identified as a proto-oncogene since *p53* cDNAs isolated from human cancers transform primary cells in cooperation with the *ras* gene (90). These *p53* cDNAs were later shown to have a number of point mutations that interfere with the DNA binding and transactivation functions of p53 (91). Therefore, *p53* is now considered to be a tumor suppressor gene. Approximately 50-80% of human carcinomas have mutations in the *p53* gene making it the most commonly mutated gene in cancer (92), and the normal p53 protein is absent in most tumors. It has also been shown that high-level expression of the normal *p53* gene can suppress cell transformation (90).

Much work has focused on determining the differences between wild-type and mutant p53 in order to identify properties of p53 that are necessary for growth suppression, and to understand how mutant p53 exerts its effects. Wild-type p53 blocks cells in the G1 phase of the cell cycle (93) and recently wild-type p53 was also shown to block cells in G2/M (94). Wild-type p53 exerts its activity by binding to DNA and activating transcription from promoters containing p53 binding sites. For example, wild-type p53 induces genes that negatively regulate cell growth such as *p21* (71) and genes that promote apoptosis such as *bax* (95). Wild-type p53 also represses transcription from promoters that do not contain p53 binding sites by binding to TBP (96).

Mutant p53 acts by inhibiting the growth suppressor effects of wild-type p53 in a dominant negative fashion. In other words, mutant p53 forms complexes with and inactivates wild-type p53 (97). Mutant p53 proteins in transformed cells can sequester wild-type proteins

in a complex that is retained in the cytoplasm during G1 rendering them unable to bind to DNA and to activate transcription (98, 99). On the other hand, mutant p53 proteins present in tumors have been shown to have loss-of-function mutations. For example, some p53 mutants have lost the ability to interact with TBP (resulting in loss of ability to repress transcription), to bind to DNA specifically, and to activate transcription (100). However, some mutant p53 proteins are able to activate transcription from promoters that wild-type p53 represses such as the *PCNA* (Proliferating Cell Nuclear Antigen) promoter and the *multi-drug resistance* (MDR) gene promoter (101, 102). In this way, mutant p53 may have gain in function mutations in the absence of a trans-dominant negative mechanism of inactivation of wild-type p53 (103).

1.4d The Retinoblastoma *(Rb)* tumor suppressor gene and transcriptional regulation.

Inactivation or deletion of both alleles of the *Rb* gene causes a retinal cancer called retinoblastoma in children. Mutations in the *Rb* gene have also been observed in carcinomas of the breast, bladder and prostate; small cell carcinoma of the lung; glioblastoma; and leukemia (104, 105, 106, 107, 108, 109). These mutations are commonly found in the pocket domain of the Rb protein (pRb) which is also the region that binds to the viral oncoproteins. Injection of retinoblastoma protein (pRb) in tumor cells without functional protein suppresses tumor cell growth *in vitro* and *in vivo* (106, 110, 111).

The retinoblastoma protein plays an important role in controlling growth of normal cells in a cell-cycle regulated manner (112). In G0/G1 pRb is hypophosphorylated, and it becomes hyperphosphorylated in S,G2, and M phases of the cell cycle (113). In addition, micro-injection of pRb in normal cells blocks cell cycle progression at G1. In order to understand the function

of the retinoblastoma protein in normal cells, cellular proteins that interact with the pocket domain (which had previously been shown to interact with viral oncoproteins) were investigated (86, 115). The cellular transcription factor E2F was found to interact with the pocket domain of pRb in a cell-cycle regulated manner (64, 65, 66). During G0 and G1, pRb is hypophosphorylated and E2F complexes with pRb. This prevents E2F from activating growth regulatory genes. E2F is released when pRb is phosphorylated late in G1 by the cyclins and cyclin dependent kinases and then E2F activates transcription (64, 65, 66, 116). The pRb-E2F complex has also been shown to be an active complex involved in transcriptional repression (81, 82, 83).

1.5 THE ROLE OF THE CUTL1 GENE IN CANCER: EVIDENCE SUGGESTING THAT CUTL1 IS A TUMOR SUPPRESSOR GENE

1.5a Cut represses c-*myc* in differentiating cells and *gp91-phox* in proliferating cells.

The c-*myc* gene is activated in a number of human tumors, and when c-*myc* is overexpressed, cells can continue through the cell cycle and may not differentiate (47). The human Cut protein has been shown to repress the c-*myc* promoter (49). It was originally suspected that Cut represses c-*myc* in growth arrested cells but Cut is inactive in G0 (67). However, Cut may repress c-*myc* in differentiating cells (67) so it can be reasoned that loss of Cut-mediated repression of c-*myc* (brought about by mutation and/or deletion of the *cut* gene) could prevent cell differentiation which could in turn lead to the development of cancer.

It has been suggested that de-repression of genes controlled by CDP/cut in certain cells may be a final step in cell differentiation. CDP/cut has been shown to repress the *gp91-phox* gene promoter in proliferating myeloid leukemia cells and upon terminal differentiation Cut no longer binds to DNA or represses transcription (72). Therefore, it is possible to speculate that in the case of cancers such as leukemias where terminal differentiation fails, CDP/cut de-repression could be absent.

1.5b The CUTL1 gene locus is deleted in human cancers.

The human *cut* gene (CUTL1) has been mapped to chromosome 7q22 (117, 118), and this region is frequently deleted in human cancers such as uterine leiomyomas (119), acute myeloid leukemia (120, 121) and myelodysplastic syndrome (122, 123). Highly polymorphic markers within or close to the 5' end of the CUTL1 genes were used to show that CUTL1 is present in the chromosomal region commonly deleted in human uterine leiomyomas (124). In addition, Cut expression was decreased in some tumors.

1.5c Cellular evidence.

The inability to obtain stable transfectants expressing the CUTL1 gene in our laboratory is also consistent with the notion that CUTL1 functions as a tumor suppressor gene (67). Following co-transfection with the neomycin resistance gene, analysis of G-418 resistant clones revealed that the transfected CUTL1 gene either was deleted, or was present but not expressed. In addition, there was a decrease in the number of G-418 resistant colonies when *cut* was transfected. These results suggest that cellular proliferation may be incompatible with elevated

expression of Cut. A similar situation is observed following transfection of a wild-type p53 gene in rat embryo fibroblasts: clones that survive either have deleted the transfected *p53* transgene or express a mutated p53 protein (90).

After several attempts, another group was able to isolate one stable transformant of HL-60 myeloid cells constitutively expressing transfected CDP/cut that repressed the *gp91-phox* gene promoter (72). However, the difficulty in obtaining this stably transformed cell line suggests that these cells either express a mutated Cut protein or have suffered other genetic changes that render them able to withstand elevated Cut expression (67).

1.5d Murine models of uterine leiomyomas: Evidence that Cut interacts with Polyoma LT.

In cells that have been transformed by a DNA tumor virus, the function of a tumor suppressor gene can be lost as a result of protein-protein interaction between the tumor suppressor protein and a viral oncoprotein (83, 85, 86, 87, 88, 89, 90). Interaction with a viral oncoprotein is interpreted to mean that the interacting protein has a role in regulating cell growth and/or DNA replication (Chapter 3). Polyoma Large T (LT) was found to co-immunoprecipitate with the murine Cut protein (mCut or Cux) in leiomyomas and breast tumors from mice transgenic for the MMTV/LT (Mouse Mammary Tumor Virus/Polyoma Large T) construct (67, 125, 126, 127). This suggests that Cut may play a role in cellular differentiation and proliferation and that, similar to the inactivation of pRb and p53 by DNA tumor virus proteins, Large T may inactivate murine Cut (mCut or Cux) in uterine leiomyomas and breast tumors found in MMTV-LT transgenic mice (67).

1.6 OVERALL OBJECTIVES OF THE WORK DISCUSSED IN THIS THESIS

In summary, there is significant indirect evidence suggesting that the CUTL1 gene is a tumor suppressor gene: a) Cut represses the c-*myc* and *gp91-phox* gene promoters; b) the CUTL1 gene locus is deleted in uterine leiomyomas; c) it is not possible to efficiently obtain stable cell lines expressing transfected Cut; and d) Cut interacts with the Polyoma Large T antigen. Since Cut may play a dual role in proliferating and differentiating cells, a complete loss of function would likely be incompatible with cell viability. Instead, the function of Cut may be weakened in differentiating cells but not in proliferating cells. For example, Cut could be inactivated and/or it could become oncogenic by mutation in tumor cells. Alternatively, changes in Cut activity in tumor cells could be attributed to mutations in genes that regulate Cut.

The purpose of this thesis project was to use functional assays to determine whether or not the CUTL1 gene functions as a tumor suppressor gene by addressing two different hypotheses. First, the ability of Cut to influence oncogene-mediated transformation was studied using the rat embryo fibroblast (REF) assay. The REF assay was used to show that the *p53* gene is a tumor suppressor gene (90). When REFs were transfected with wild-type *p53* in combination with *E1A* and *ras*, there was a decrease in the number of transformed foci. Second, the ability of Cut to interact with the SV40 LT antigen and the effect of this interaction on the function of Cut was studied. An interaction between the Cut protein and a DNA tumor virus protein would suggest that the CUTL1 gene plays an important role in regulation of cellular differentiation and/or proliferation.

CHAPTER 2

EXPRESSION OF A WILD-TYPE MAMMALIAN *CUT* GENE SUPPRESSES CELL TRANSFORMATION

2.1 INTRODUCTION AND PURPOSE OF THIS STUDY

The purpose of the present study was to investigate whether or not overexpression of a mammalian *cut* gene can suppress cell transformation in the rat embryo fibroblast transformation (REF) assay. The REF assay is a commonly used model of *in vitro* transformation. Although it was originally thought that multiple steps are necessary for cell transformation, some cells such as NIH 3T3 cells can be transformed with a single oncogene such as *ras*. NIH 3T3 cells are established cells that grow indefinitely in culture, thereby suggesting that these cells have sustained alterations usually developed by a cell during its tumorigenic progression (46). Therefore, rat embryo fibroblasts (REFs) began to be used in tissue culture systems investigating transformation because they are primary cultured cells which resemble normal (non-proliferating) cells.

Expression of activated *ras* or c-*myc* oncogenes in isolation cannot transform REFs, however, both genes can cooperate to transform REFs (90, 128). It is now known that in order to become transformed, primary cells (such as rat embryo fibroblasts) must first be immortalized. Immortalized cells can grow indefinitely in culture. Oncogenes such as c-*myc* and *E1A* have been shown to immortalize REFs and a transforming oncogene such as *ras* can transform the immortalized cells.

The REF transformation assay was used to illustrate the role of the *p53* gene as a tumor suppressor gene. It was hypothesized that if inactivation of *p53* predisposed cells to become transformed, then increasing the amount of wild-type *p53* may prevent transformation. REFs were transfected with *E1A*, and *ras* with or without murine *p53* and a significant decrease in the number of foci was seen when *p53* was cotransfected (90). Interestingly, cell lines derived from these foci did not express wild-type p53 but only expressed mutant p53. Mutant p53 cooperates with Ras to transform REFs.

The rat heat-shock protein (hsp70) expressed from the *hsc70* gene has also been shown to suppress transformation by mutant p53 and Ras, as well as c-Myc and Ras in the REF assay (129). The *hsc70* gene is induced in response to environmental or physiological stress. In contrast to the results with p53, transformed cell lines were able to proliferate in the presence of exogenous heat shock protein. Therefore, this suggests that very high threshold levels are needed for *hsc70* to exert its inhibitory effect and that this threshold is not reached in surviving cells, or that the surviving cells are derived from cells that are insensitive to suppression mediated by *hsc70*.

The REF transformation assay was also used to demonstrate that the *max* (130), *mad* and *mxi* (131) genes suppress c-Myc plus Ras transformation. Max is a basic-helix-loop-helix/leucine zipper protein that forms sequence specific DNA binding complexes with Myc proteins. Mad and Mxi are members of the Myc-related basic-region-helix-loop-helix/leucine zipper family of proteins. They associate with Max to form DNA binding hetero-dimers that

repress transactivation by Myc. Exogenous Mad and Mxi proteins were not expressed in transformed cells derived from REF foci suggesting that high levels of both proteins are incompatible with cell proliferation.

In this study, REFs were transfected with *E1A/ras*, *E1A/ras/cut*, or *E1A/ras/cux*. The resulting transformed colonies or foci were counted and the numbers were compared. Cell lines were produced from colonies or foci expressing E1A, Ras, and Cut and were analyzed for the presence of transfected human Cut RNA and protein. In addition, REFs were transfected with c-*myc/ras* or c-*myc/ras/cut*, the resulting foci were counted and the numbers were compared.

2.2 MATERIALS AND METHODS

Plasmids. The pBluescript II KS + (pKS) phagemid used as a carrier DNA for transfections is derived from pUC19 and is 2961 base pairs in length. The neo containing plasmid pLXSN (132) has the *neo* gene cloned between the SV40 early promoter and the Moloney Murine Leukaemia Virus Long Terminal Repeat (Mo-MLV LTR). The wild-type *p53* plasmid (obtained from Greg Matlashewski) contains the *p53* 1.8 kb cDNA cloned between the Hind III and Bam HI sites of plasmid pJ4Ω (133) which places *p53* under the control of the Mo-MLV LTR. The pMX *cut* plasmid constructed in our laboratory contains the 5 kb human *cut* cDNA cloned into the Sma I site of plasmid pMX-139 derived from pXMT2 which places *cut* under control of the Adenovirus Major Late Promoter (MLP). The pMX *cux* plasmid (murine *cut*) contains the 4.2 kb *cux* cDNA cloned between the Sma I and Not I sites of pMX-139. The PLE2-*E1A* plasmid (134) obtained from Richard Marcellus contains the 1.5 kb *E1A* cDNA including the promoter region inserted in the Bam HI, Hpa I site of pBR322. The CMV-*E1A* plasmid was constructed by Richard Marcellus and contains the 1 kb *E1A* cDNA from PLE2-*E1A* (excluding the promoter region) inserted between the Bam HI and Eco RV sites of pCDNA3 (Invitrogen) which places *E1A* under control of the CMV promoter. The pH06T1*ras* plasmid (135) contains the activated human *[Val^{12}] Ha-ras* gene from a bladder carcinoma cell line under control of the SV40 enhancer and the Mo-MSVLTR (Moloney Murine SarcomaVirus Long Terminal Repeat). The pSVc-*myc*1 plasmid is derived from the pSV2 *neo* plasmid and has c-*myc* under control of the SV40 early promoter (46). *myc*-2A (obtained from Alain Nepveu) is derived from a transformed clone from NIH 3T3 cells and has c-*myc* under control of the endogenous promoter. This plasmid contains all the coding

sequences of exons 2 and 3 of murine c-*myc* within the polylinker of the pMX139 vector. The gptkpn*myc* plasmid (obtained from Alain Nepveu) contains the full length (10 kb) murine c-*myc* gene and 3.5 and 1.5 Kbp of 5' and 3' flanking sequences respectively. This fragment was inserted in the Kpn1 site of the pSV2gpt vector (136) which places c-*myc* under control of the SV40 enhancer and the endogenous c-*myc* promoter.

FR3T3 ras transformation assays. FR3T3 is an established fibroblast cell line derived from Fischer rats. $5x10^5$ cells were seeded in 100-mm plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours later, cells were transfected with calcium phosphate precipitates. Cells were transfected with 1 μg of *ras* and pMX *cut* plasmids with pKS vector DNA up to a total of 10 μg. After 48 hours, cells were split 1:4 in DMEM supplemented with 10% FBS. Medium was changed every 3 days and transformed foci were counted after 2 weeks. Cells were fixed by adding 5 ml of 10% Buffered Formalin Phosphate (Fisher Scientific) to each plate for 20 minutes. After removal, 5 ml of Giemsa stain solution (Fisher Scientific) were added to each plate and incubated overnight.

E1A/Ras REF transformation assays. $5x10^5$ primary rat embryo fibroblasts (REFs) derived from Fischer rats (Bio Whittaker) were seeded in 100-mm plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours later cells were transfected with calcium phosphate precipitates containing: a) 1 μg of *EIA* and *ras* plasmids, with 1 or 5 μg of pMX *cut*, *cux*, or *p53* plasmids or b) 4 μg of *E1A* and *ras* plasmids, with 8 μg of the pMX *cut* plasmid. Each plate received 10 or 25 μg of DNA with the total

amount adjusted by the addition of pKS plasmid. For G-418 colony assays, cells were split 1:4 in DMEM supplemented with 5% FBS with 500 μg/ml of G-418 forty-eight hours after transfection. Fresh media was added to the dishes every 3 days. For focus assays, cells were split 1:3 in DMEM supplemented with 5% FBS 48 hours after transfection. Fresh media was added to the dishes every 3 days. After two weeks, transformed colonies or foci were fixed, stained, and counted as described previously, or cell lines were generated by picking colonies or foci with cloning cylinders and expanding the cells into 60mm, 100mm and 150 mm dishes.

Myc/Ras REF transformation assay. This experiment was performed essentially as described above for the E1A/Ras focus assay. However, cells were transfected with 7.5 μg of *ras* and c-*myc* plasmids with 15 μg of the *cut* plasmid. Each plate received 30 μg of DNA with the total amount adjusted by the addition of pKS plasmid.

RNA isolation. Total RNA was prepared from 150 mm tissue culture plates with acidic organic solvents based on the method of Chomczynski and Sacchi (137).

Preparation of riboprobe. A riboprobe was prepared by incubating the following for 1 hour at 37 ^{0}C: 1μg of template DNA, 4 μl of 5X transcription buffer (200 mM PIPES [pH 6.4], 2 M NaCl, 5 mM EDTA), 10 mM DTT, RNasin (40 units), 500 μM each of ATP, CTP, GTP, 12 μM of UTP, 50 μCi α-^{32}P-UTP, and T3 polymerase (15 units) in a total volume of 20 μl. After one hour 500 μM UTP was added and the sample was incubated for another 5 min at 37^{0}C. Then, the riboprobe was treated with RNase free DNase at 37^{0}C for 15 min, and passed

through a sephadex G50 spin column. The template DNA was derived from a vector developed in our laboratory that can be used to discriminate between Cut and the Cut-alternatively-spliced-product (CASP). A 296 bp RNA fragment would be protected by CASP whereas a 256 bp fragment would be protected by Cut.

RNase T2 protection analysis. Forty micrograms of RNA was mixed with 1 µl of riboprobe (50-100x100 cpm), 40 µl of formamide and 10 µl of 1X PIPES buffer (40 mM PIPES [pH 6.4], 0.4M NaCl, 1mM EDTA) and incubated overnight at 53°C. RNA-RNA hybrids were digested by adding 250 µl of RNAse T2 digestion buffer (60 mM NaAcetate [pH 5.2], 120 mM NaCl, 12 mM EDTA), containing 60 units/ml of RNase T2, for one hour at 37°C. Then hybrids were precipitated by adding 20 µg of tRNA, 4M guanidine thiocyanate and isopropanol. RNA pellets were resuspended in 80% formamide, 1xTBE and 0.1% XC+BPB dyes, boiled for 5 minutes, chilled on ice and electrophoresed on a 4% acrylamide-8M urea gel. The gel was dried and exposed to X-ray film with intensifying screens at -80 °C.

Preparation of total cell lysates for Gel-shift assays and Western blotting. Cells were harvested at 80% confluency. Cells were washed two times with PBS, scraped in 1ml of PBS and transferred to an eppendorf tube. The cells were resuspended in 20 µl of lysis buffer (50 mM HEPES-KOH [pH 7.9], 0.4 M KCl, 0.1% NP-40, 4 mM NaF, 4 mM Na_3VO_4, 0.2 mM EDTA, 0.2 mM EGTA, and 10% glycerol), with 0.5 mM DTT (Gibco #27565-41-9), 0.5 mM PMSF (Sigma #329-98-6), 1µg/ml Pepstatin A (Sigma #26305-03-3), 1µg/ml Aprotinin (Boehringer Mannheim # 981532) and 1µg/ml Leupeptin (Boehringer

Mannheim #1529048) and incubated on ice for 20 minutes. Cellular debris was removed by centrifugation for 10 minutes and the supernatant was retained. Protein concentration was determined by the method of Bradford with the Bio-Rad Protein Assay following the manufacturer's protocol.

Antibodies. Monoclonal antibodies A and W3 were generated against epitopes in the Cut repeat 3, and carboxy-terminal domains of Cut respectively, and prepared as ascetic fluids (Molecular Immunogenetics, San Andreas, CA). The W3 antibody does not bind to a Cut DNA binding domain, but incubation of this antibody with protein extracts inhibits Cut DNA binding and/or produces a supershifted band (138). Crude polyclonal amino terminal and carboxy terminal antibodies for Cut (that were prepared in our laboratory) were also used. Secondary HRP-conjugated anti-mouse, and anti-rabbit antibodies were used to detect proteins in Western blotting. The mouse monoclonal purified antibody Ab M73 that recognizes E1A (provided by Richard Marcellus) was used in gel shift assays. The secondary HRP- conjugated anti-rabbit antibody described above was used as a non-specific antibody for Cut in gel shift assays.

Western blotting for Cut expression. Total cell extracts were mixed with an equal volume of 2X Laemlli sample buffer (5X formula: 157 mM Tris [pH 6.8], 25% glycerol, 5% SDS, 12.5% β- mercaptoethanol, 0.3% Bromophenol blue) and resolved by electrophoresis on a SDS-5% polyacrylamide gel. Then the gel was transferred to a PVDF membrane overnight in transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol). The blot was blocked

overnight with shaking with 5% skim milk powder and 3% BSA (Bovine Serum Albumin) in TBS (25 mM Tris-HCl [pH] , 137 mM NaCl, 2.7 μM KCl). The blot was washed briefly with TBS-0.1%Tween 20 and incubated with an amino terminal Cut polyclonal antibody for 4 hours at a 1:1000 dilution with 5% milk and 0.5% BSA in TBS. The blot was washed four times for 10 minutes each in TBS-0.1% Tween 20 and incubated with a HRP-conjugated anti-rabbit antibody at a 1:1250 dilution in TBS for one hour. Then the blot was washed four times for 10 minutes each in TBS-0.1% Tween 20 and incubated with ECL Detection reagents (Amersham) according to the manufacturer's instructions.

Gel-shift assays. Assays were performed with 15-20 μg of total protein lysate. Protein extracts were incubated at room temperature for 5 min with 2 μl of 10X binding buffer: (100 mM Tris [pH 7.5], 250 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol [Gibco #27565-41-9], 50 mM EDTA, 50% glycerol, with 10 mg/ml BSA and 1 μg of poly(dI-dC) as a non-specific competitor in a final volume of 20 μl. In some cases, the protein samples were incubated with 1 μl of Cut or E1A antibodies for 30 minutes at room temperature prior to addition of dI-dC and/or probe. A double-stranded nucleotide with a Cut consensus binding site (upper strand, 5'-AAAAGAAGCTTATCGATACCGT-3') was end labeled with Klenow polymerase (33, 138). 1 μl of this probe was added and samples were further incubated for 20 minutes. Cold competitor DNA was not used as a control for the specificity of Cut DNA binding in these experiments because Cut DNA binding can be competed out with almost any sequence in excess (67). Therefore, antibody controls are used to demonstrate specific DNA binding by

Cut (67). Samples were loaded on a 5% polyacrylamide gel and separated by electrophoresis at 100 V for 3 hours in 1X glycine buffer (50 mM Tris, 1.4 M glycine, and 2.7 mM EDTA) The gels were dried and visualized by autoradiography.

2.3 RESULTS

The effect of human Cut on transformation of FR3T3 cells.

In order to confirm that human Cut does not transform cells, FR3T3 cells were transfected with plasmids encoding *ras*, *cut*, or *ras/cut* and the number of foci was determined. Foci were not observed when *cut* was introduced in FR3T3 cells (Table 2.1). Table 2.1 also shows that *cut* was unable to repress Ras-mediated transformation of FR3T3 cells.

Cut and Cux can inhibit E1A plus Ras transformation of rat embryo fibroblasts.

In order to determine if Cut and Cux repress oncogene-mediated cell transformation, REFs were transfected with *E1A/ras*, *E1A/ras/cut*, *E1A/ras/cux*, or *E1A/ras/wtp53* (wild-type) as a positive control. The resulting transformed colonies or foci were counted, and the numbers were compared. In accordance with the early experiments performed with p53, two different *E1A* plasmids were used in this study: a) one containing the endogenous E1A promoter (PLE2-*E1A*); and b) one containing the CMV promoter (CMV-*E1A*). First, the effect of transfecting *cut* and *cux* on the number of E1A plus Ras transformed G-418 resistant colonies was investigated. Table 2.2 shows the results of two separate experiments in which 1 μg of each plasmid was transfected. There were slight decreases in the number of transformed colonies when *cut* and *cux* were transfected with *E1A* and *ras*. There were also slight decreases when *wtp53* was transfected with *E1A* and *ras*.

Since the effect of Cut, Cux, and wild-type p53 on the number of G-418 resistant transformed colonies was minimal when 1 μg of each of these plasmids were transfected, the amount of DNA transfected was increased to 5 μg while keeping the amount of *ras* and *E1A*

Table 2.1 The effect of transfecting 1 μg of *cut* on the number of Ras transformed FR3T3 foci

	No. of foci	
Transfected DNA		Ratio to Ras control
PMX *cut*	--	--
ras	111	--
ras, PMX *cut*	131	1.18

5×10^5 cells were transfected by the calcium phosphate procedure with 1 μg of pH06T1 *ras* and 1 μg of *cut*. Each dish received 10 μg of DNA with the total amount adjusted by the addition of pKS plasmid. Transformed foci were counted after 14 days. The average number of foci from two primary plates that were not split after transfection was determined.

Table 2.2 The effect of transfecting 1 μg of *p53*, *cut* and *cux* on the number of E1A plus Ras transformed G-418 resistant REF colonies

	Experiment 5		Experiment 6		Average	
Transfected DNA	TC	Ratio to E1A+Ras	TC	Ratio to E1A + Ras	TC	Ratio to E1A+ Ras
ras	--	--	--	--	--	--
PLE2-*E1A*	--	--	--	--	--	--
ras, PLE2-*E1A*	402	--	357	--	379	--
ras, PLE2-*E1A*, *p53*	294	0.73	381	1.07	337	0.89
ras, PLE2-*E1A*, *cut*	346	0.86	280	0.78	313	0.82
ras, PLE2-*E1A*, *cux*	333	0.82	234	0.66	283	0.75
CMV-*E1A*	ND	ND	--	--	ND	ND
ras, CMV-*E1A*	ND	ND	364	--	ND	ND
ras, CMV-*E1A*, *p53*	ND	ND	428	1.17	ND	ND
ras, CMV-*E1A*, *cut*	ND	ND	457	1.25	ND	ND
ras, CMV-*E1A*, *cux*	ND	ND	315	0.86	ND	ND

5 x 10^5 cells were transfected by the calcium phosphate procedure with 1 μg of pH06T1 *ras*, 1 μg of *E1A*, and 1 μg of *p53*, *cut*, or *cux*. Plates in Experiment 5 received 10 μg of DNA with the total amount adjusted by the addition of pKS plasmid. Plates in Experiment 6 received 25 μg of DNA with the total amount adjusted by the addition of pKS plasmid. Each plate was split 1:4 and selected in 500 μg/ml of G-418. After two weeks, transformed colonies were counted. The total number of transformed colonies (TC) from the four plates was determined. ND is not done. Experiments 5 and 6 are noted in the table because the resulting cell lines were named depending on which experiment they were isolated from (for example, cell line 5-12c1, Table 2.5).

DNA constant. In fact, larger quantities of plasmid DNA have been used in REF studies performed in other laboratories (129, 130, 139). In particular, the ratio of putative suppressor DNA in comparison to the amount of oncogene DNA is usually greater than 2:1. For the sake of simplicity, further experiments focused on the effect of Cut since human Cut and murine Cux are almost identical. There was a substantial decrease in the number of transformed G-418 resistant colonies when 5 μg of *cut* was transfected with *ras* and the *PLE2-E1A* plasmid (Table 2.3). However, once again there was only a slight decrease in the number of G-418 resistant transformed colonies when *wtp53* was transfected with *E1A* and *ras*.

The effect of Cut on the number of transformed foci was also examined. Since transfection of 1 μg of *E1A* and *ras* was able to produce transformed G-418 resistant colonies (Tables 2.2 and 2.3) but unable to produce transformed REF foci (data not shown), the amount of *E1A* and *ras* transfected was increased to 4 μg. In order to keep the ratio of *cut*:*E1A*/*ras* at least 2:1, 8 μg of *cut* was transfected. There was a substantial reduction in the number of transformed foci when *cut* was transfected with *E1A* and *ras* (Table 2.4). There was also a substantial decrease in the number of foci when *wtp53* was transfected with *E1A* and *ras*.

Human Cut RNA is expressed in REF cell lines transfected with *E1A*, *ras*, and *cut*.

A minimum of 3 foci or transformed G-418 resistant colonies derived from transformed cells transfected with *E1A*, *ras*, and *cut*, were expanded into cell lines. These cell lines are described in Table 2.5. RNA and protein was isolated from each cell line shown. RNAse mapping was performed to determine if exogenous *cut* RNA was present. A human Cut probe was used that recognizes both Cut and the alternatively spliced Cut product CASP. RNA from

Table 2.3 The effect of transfecting 5 μg of *p53* and *cut* on the number of E1A plus Ras transformed G-418 resistant REF colonies

	No. of Transformed colonies	
	Experiment 7	
Transfected DNA		Ratio to E1A+Ras
ras	----	--
CMV-*E1A*	----	--
PLE2-*E1A*	----	--
ras, CMV-*E1A*	50	--
ras, CMV-*E1A*, *cut*	42	0.84
ras, PLE2-*E1A*	57	--
ras, PLE2-*E1A*, *p53*	48	0.84
ras, PLE2-*E1A*, *cut*	24	0.42

5 x 10^5 cells were transfected by the calcium phosphate procedure with 1 μg of pH06T1 *ras*, 1 μg of *E1A*, and 5 μg of *p53* or *cut*. Each plate received 25 μg of DNA with the total amount adjusted by the addition of pKS plasmid. Plates were split 1:3 and selected in 500 μg/ml of G-418. Transformed colonies were counted after 11 days. The total number of transformed colonies from three dishes was determined. Experiment 7 is noted in the table because the resulting cell lines were named depending on which experiment they were isolated from (for example, cell line 7-3a1, Table 2.5).

Table 2.4 The effect of transfecting 8 μg of *p53* and *cut* on the number of E1A plus Ras transformed REF foci

	No. of Transformed foci	
	Experiment 7	
Transfected DNA		Ratio to E1A+Ras
ras	----	--
CMV-*E1A*	----	--
PLE2-*E1A*	----	--
ras, CMV-*E1A*	74	--
ras, CMV-*E1A*, *p53*	34	0.46
ras, CMV-*E1A*, *cut*	39	0.52
ras, PLE2-*E1A*	104	--
ras, PLE2-*E1A*, *cut*	45	0.43

5 x 10^5 cells were transfected by the calcium phosphate procedure with 4 μg of pH06T1 *ras*, 4 μg of *E1A*, and 8 μg of *p53* or *cut*. Each plate received 25 μg of DNA with the total amount adjusted by the addition of pKS plasmid. Plates were split 1:3 and transformed foci were counted after 11 days. The total number of transformed foci from three dishes was determined. Experiment 7 is noted in the table because the resulting cell lines were named depending on which experiment they were isolated from (for example, cell line 7-11b4, Table 2.5).

Table 2.5 Summary of E1A+Ras+Cut REF transformed cell lines

Cell Lines	Transfected DNA (μg)	No. of Foci	Transformed Colonies	Ratio to E1A + Ras
	E1A PLE2 (1), *ras* (1)		402	0.86
5-12c1,**4**	*E1A* PLE2 (1), *ras* (1), *cut* (1)		346	
	E1A PLE2 (1), *ras* (1)		57	0.42
7-3a1,3,**5**,**6**	*E1A* PLE2 (1), *ras* (1), *cut* (5)		24	
	E1A PLE2 (4), *ras* (4)	104		0.43
7-11b4,**5**,10,**11**	*E1A* PLE2 (4), *ras* (4), *cut* (8)	45		
	E1A CMV (1), *ras* (1)		50	0.84
7-7b1,8,**10**	*E1A* CMV (1), *ras* (1), *cut* (5)		42	
	E1A CMV (4), *ras* (4)	74		0.52
7-15b2,4,**5**,**6**,7,8	*E1A* CMV (4), *ras* (4), *cut* (8)	39		

Transformed colonies and foci were picked using cloning cylinders and expanded into cell lines. A minimum of 3 foci or colonies were selected from one representative plate from each experimental condition. The numbers 5 and 7 refer to the experiment number in the preceeding tables (Tables 2.2, 2.3, 2.4) and the letters a,b,c, or d refer to the plate that the line is derived from since each primary plate was split 1:3 or 1:4 (a,b,c,d) after transfection. All cell lines were used in RNAse mapping (Figure 2.1) and cell lines in bold were used for Western blotting (Figure 2.2) or Western blotting and EMSA (Figures 2.3, 2.4).

human placenta was used as a positive control and untransfected REF RNA was used as a negative control. All cell lines tested expressed *cut* RNA at varying levels (Figure 2.1) and the magnitude of the reduction in the number of colonies or foci and the amount of RNA expressed was not correlated. Although it cannot be confirmed because the full length rat *cut* sequence has not been determined, the two lower bands may represent endogenous rat Cut (*CDP2*) and CASP RNA since these bands were present in the lanes containing RNA from REFs, but they were not present in the lane containing RNA from human placenta. The intensity of the upper band appears to be increased or decreased in comparison to the untransfected REF lane in many of the cell lines tested, suggesting that changes in expression of endogenous rat Cut (CDP2) and/or CASP may accompany cell transformation. In addition, some cell lines (for example 7-15b5, and 7-3a5) have additional RNA species of about 270 bp that could be degradation products or mutated Cut RNAs.

Human Cut protein may be expressed in REF cell lines transfected with *E1A/ras/cut*.

Cut protein was expressed in all the representative cell lines tested (Figure 2.2). The amount of Cut protein present in each cell line was approximately the same regardless of the magnitude of the reduction in the number of colonies or foci, or the amount of RNA expressed. There was much more Cut protein in extracts from transfected REF cell lines as compared to untransfected REF and FR3T3 cell extracts, thereby suggesting that these cells probably express human Cut. However, the antibody used for Western blotting does not discriminate between endogenous rat Cut (CDP2) and transfected human Cut protein, so it is not possible to rule out the possibility that the level of endogenous rat Cut (CDP2) was increased in these

Figure 2.1 RNAse protection with RNA from REF cell lines. Total RNA was prepared as described in Materials and Methods. A human riboprobe that can be used to discriminate between Cut and CASP (since a 296 bp fragment is protected by CASP and a 256 bp fragment is protected by Cut) was prepared and used. Forty micrograms of RNA was used in RNAse T2 protection analysis as described in Materials and Methods. The probe control is shown in lane 1, and the negative tRNA control is shown in lane 2. The positive control for human Cut (lane 3) is human placenta RNA and the negative control (lane 4) is untransfected REF RNA. Lanes 5-23 show RNA samples from the stable cell lines created in this study. Molecular weight markers are labelled.

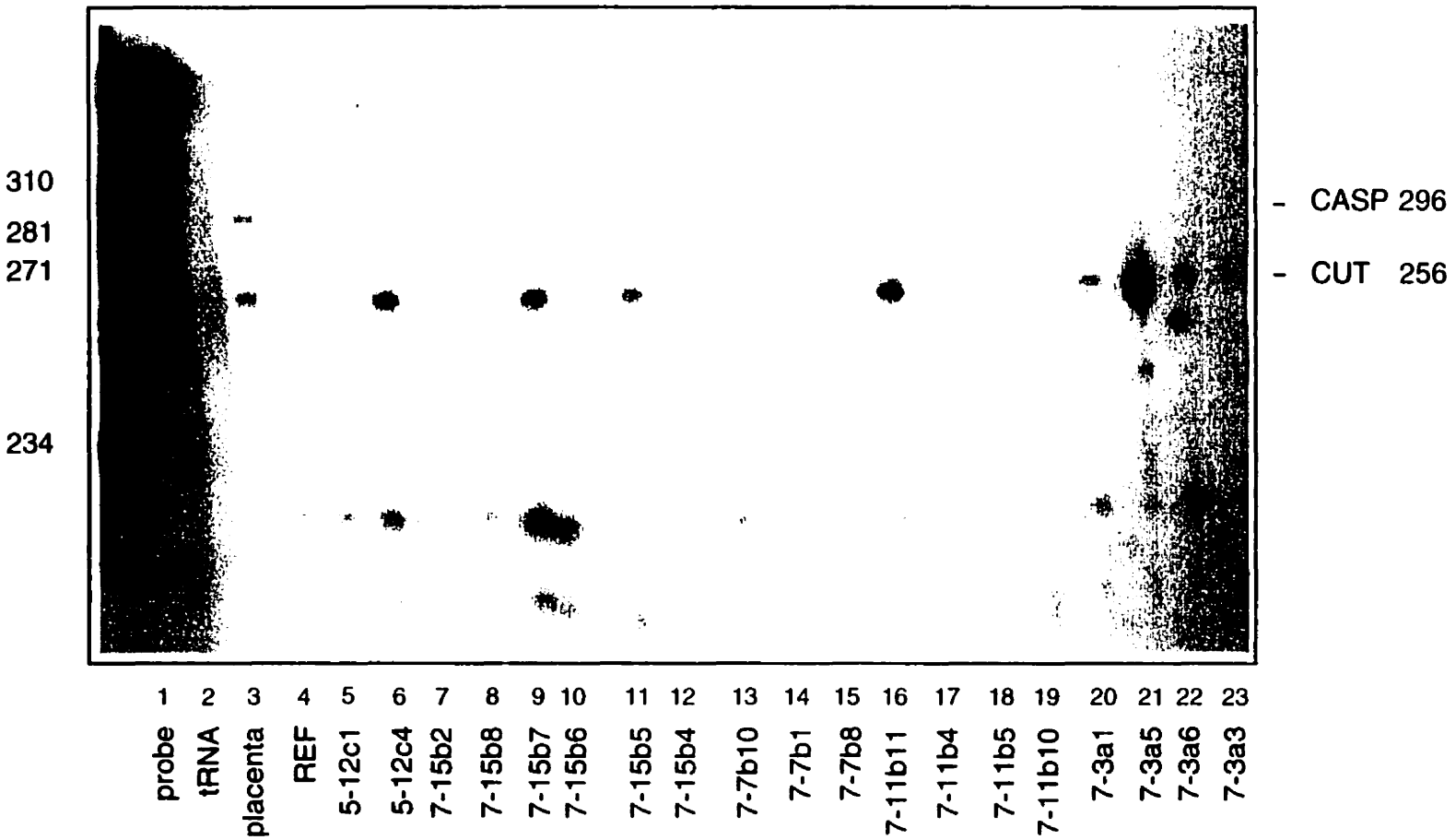
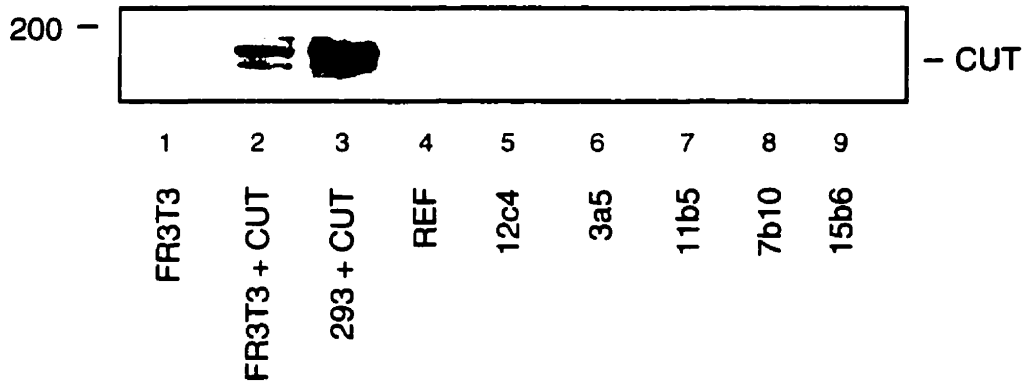
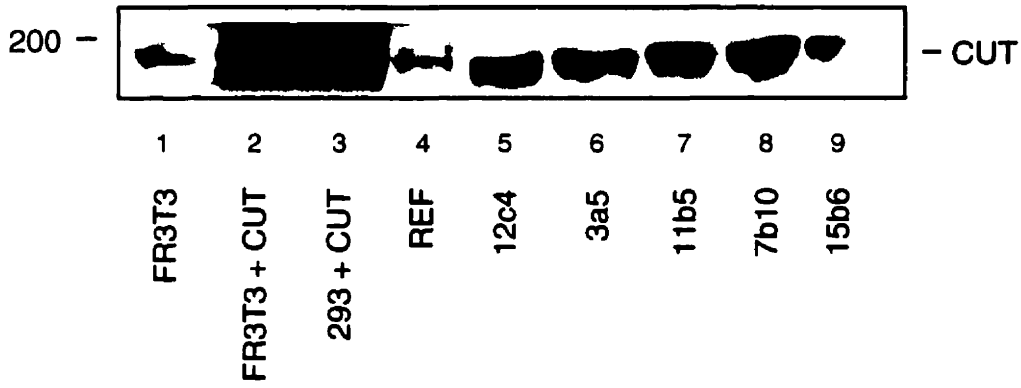

310
281
271
234
- CASP 296
- CUT 256
1 probe
2 tRNA
3 placenta
4 REF
5 5-12c1
6 5-12c4
7 7-15b2
8 7-15b8
9 7-15b7
10 7-15b6
11 7-15b5
12 7-15b4
13 7-7b10
14 7-7b1
15 7-7b8
16 7-11b11
17 7-11b4
18 7-11b5
19 7-11b10
20 7-3a1
21 7-3a5
22 7-3a6
23 7-3a3

Figure 2.2 Western blot for Cut with protein extracts from REF cell lines. Total protein extracts were prepared as described in Materials and Methods. Fifty micrograms of protein was resolved by electrophoresis, and the gel was transferred to a membrane and blotted with an amino-terminal polyclonal antibody for Cut as described in Materials and Methods. Untransfected FR3T3 (lane 1) and REF (lane 4) protein controls serve as comparisons for transiently transfected cells in lanes 2 and 3 and stable REF cell lines in lanes 5-9. Panels **(A)** and **(B)** are different exposures of the same Western blot.

A


200 –
– CUT
1 2 3 4 5 6 7 8 9
FR3T3
FR3T3 + CUT
293 + CUT
REF
12c4
3a5
11b5
7b10
15b6

B


200 –
– CUT
1 2 3 4 5 6 7 8 9
FR3T3
FR3T3 + CUT
293 + CUT
REF
12c4
3a5
11b5
7b10
15b6

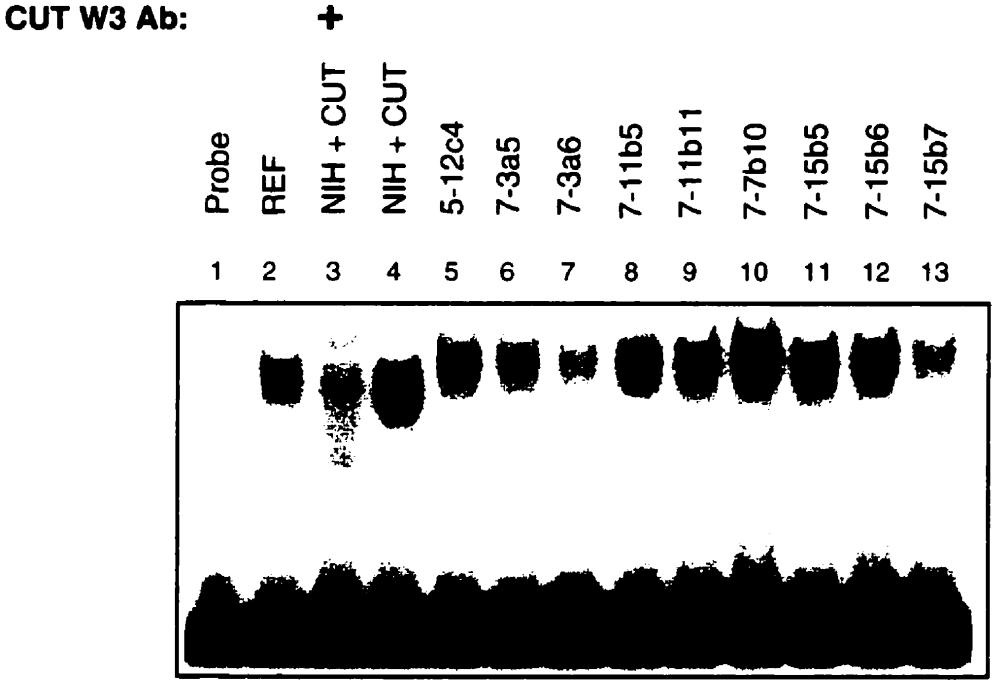
cells. Since this band co-migrated with endogenous rat CDP2 and Cut from cells transiently transfected with human Cut, and since no other major bands were recognized by the Cut antibody, it appears that the Cut protein is not grossly mutated in these cells. This suggests that at least some transformed cells can tolerate large amounts of Cut protein.

Cut DNA binding in REF cell lines transfected with *E1A*, *ras*, and *cut*.

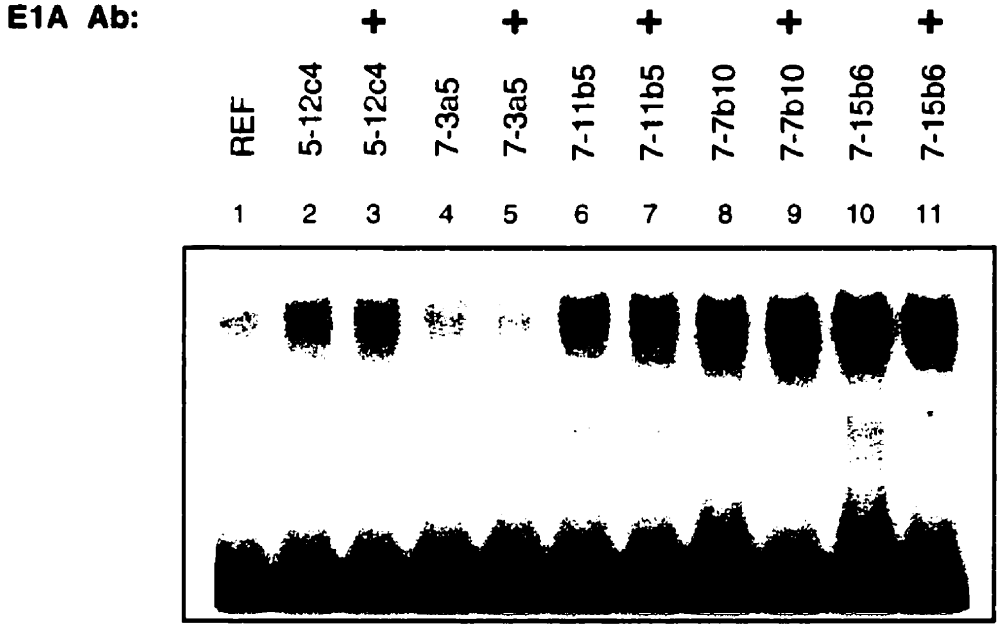
In order to determine whether or not the transfected human Cut protein was functional in the transformed cell lines generated in this study, gel-shift assays were performed. The intensity of the shifted bands and the magnitude of the reduction in the number of transformed cells were not correlated (Figure 2.3 panel A). In addition, even though approximately equal amounts of Cut protein were present in all cell lines tested (Figure 2.2), the level of DNA binding varied. There appeared to be an increase in DNA binding when compared to untransfected REFs in 5/9 cell lines tested (Figure 2.3 panel A; lanes 5, 8, 10, 11, 12) thereby suggesting that the exogenous Cut protein was functional. However, this also implies that in at least four cell lines, the exogenous Cut protein may not bind DNA and therefore may not be functional. It is important to note that it is not possible to rule out the possibility that DNA binding by endogenous rat Cut (CDP2) was increased in these cells. It is also important to keep in mind that antibody controls for DNA binding specificity of Cut were not used in the gel-shifts with the transformed REF cell lines so these results should be interpreted accordingly.

Figure 2.3 (A) Gel-shift assay for Cut DNA binding with extracts from REF cell lines. Protein extracts were prepared as described in Materials and Methods. Assays were performed with 20 μg of protein as described in Materials and Methods. The FP probe control sample shown in lane 1 was included as a negative control and did not contain protein. A negative control sample for binding by endogenous Cut (untransfected REF) is shown in lane 2. A positive control sample from NIH 3T3 cells transiently transfected with Cut and incubated with a Cut carboxy-terminal monoclonal antibody (W3) is shown in lane 3. The sample in lane 4 contains the same extract as in lane 3 without pre-incubation with the W3 antibody. The samples in lanes 5-13 are from the REF cell lines created in this study. **(B) Gel-shift assay for Cut DNA binding with proteins from REF cell lines pre-incubated with an E1A antibody.** Protein extracts were prepared, and gel-shift assays were performed with 20 μg of protein as described in Materials and Methods. The sample in lane 1 is a negative control (untransfected REFs). Samples in lanes 2,4,6,8, and 10 are from the cell lines shown in A. Lanes 3,5,7,9, and 11 are matched samples that were pre-incubated with a monoclonal antibody for E1A prior to addition of dI-dC and probe.

A


CUT W3 Ab:
+
Probe
REF
NIH + CUT
NIH + CUT
5-12c4
7-3a5
7-3a6
7-11b5
7-11b11
7-7b10
7-15b5
7-15b6
7-15b7
1 2 3 4 5 6 7 8 9 10 11 12 13

B


E1A Ab:
+ + + + +
REF
5-12c4
5-12c4
7-3a5
7-3a5
7-11b5
7-11b5
7-7b10
7-7b10
7-15b6
7-15b6
1 2 3 4 5 6 7 8 9 10 11

In order to determine if human and/or rat Cut is complexed with E1A in transformed cell lines transfected with *E1A*, *ras* and *cut*, samples were incubated with E1A antibodies prior to incubation with the DNA probe in a gel shift assay. Addition of E1A antibodies did not produce supershifted bands, thereby suggesting that human and/or rat Cut is not in complex with E1A in these cells (Figure 2.3, panel B).

Cut can inhibit Myc plus Ras Transformation of REF.

Based on the experiments investigating the effect of Max and ΔMax on Myc plus Ras transformation (130), REFs were transfected with c-*myc* and *ras*, with or without human *cut*. There was a decrease in the number of transformed foci when *cut* was transfected with c-*myc* and *ras* (Table 2.6). Attempts at making cell lines from these foci were unsuccessful. These results are interesting because of the role that human Cut has been shown to play in repressing the c-*myc* promoter.

Table 2.6 The effect of transfecting 15 µg of *cut* on the number of Myc plus Ras transformed REF foci

Transfected DNA	No. of Transformed Foci	Ratio to Myc + Ras
ras, pSVc-*myc*1	54	--
ras, pSVc-*myc*1, *cut*	8	0.14
ras, *myc*2A	31	--
ras, *myc*2A, *cut*	9	0.29
ras, gptkpn*myc*	100	--
ras, gptkpn*myc*, *cut*	30	0.30

5×10^5 cells were transfected by the calcium phosphate procedure with 7.5 µg of pH06T1 *ras*, 7.5 µg of *myc*, and 15 µg of *cut*. Each plate received 30 µg of DNA with the total amount adjusted by the addition of pKS plasmid. Plates were split 1:3 and transformed foci were counted after 12 days. The total number of transformed foci from the three dishes was determined.

2.4 DISCUSSION

The results presented here show that human Cut was able to suppress E1A plus Ras mediated transformation in the REF assay (Tables 2.2-2.4). Although there was a decrease in the number of foci when human *cut* was transfected with *E1A* and *ras* in most cases, there was a substantial suppressive effect on transformation only when 5 μg of *cut* was transfected (Tables 2.3 and 2.4). These results are similar to results previously shown for wild-type p53 which are summarized in Table 2.7. Human Cut also repressed Myc plus Ras transformation (Table 2.6). These results are similar to results previously shown for Max, Mad and Mxi1 seen in Table 2.8, and to results seen with hsp70 (129). In comparison to the results shown in Table 2.8 for Mad and Mxi (where there was no significant effect on E1A plus Ras transformation), the effect of human Cut does not seem to be specific for one transformation pathway since Cut repressed both E1A and Myc plus Ras transformation.

Since Cut represses transcription from a number of promoters, it is possible that the human Cut protein suppressed transformation by repressing the promoters driving the oncogenes used in this study. Human Cut suppressed transformation by two *E1A* plasmids with different promoters, thereby suggesting that this effect was not specific to the promoter and enhancer elements of the *E1A* plasmids used. However, this does not rule out the possibility that Cut suppressed cell transformation by repressing the promoters. Human Cut was able to suppress transformation mediated by three c-*myc* plasmids, and two of these plasmids (*myc* 2A and gptkpn*myc*) contain the endogenous c-*myc* promoter which is known to be repressed by the human Cut protein. Therefore, this suggests that human Cut may have

Table 2.7 Effect of p53 on E1A plus Ras-mediated REF transformation

Study	Transfected DNA (μg)	No.of Foci	Ratio to E1A + Ras
Finlay (90)	EJ*ras* (1.25), SV*E1A* (1.25)	96	--
	EJ*ras* (1.25), SV*E1A* (1.25), *mp53* (1.25)	19	0.19
Finlay (90)	EJ*ras* (1.25), p*E1A* (1.25)	69	--
	EJ*ras* (1.25), p*E1A* (1.25), *mp53* (1.25)	23	0.33
Unger (139)	EJ*ras* (5), p*E1A* (5)	15	--
	EJ*ras* (5), p*E1A* (5), *hp53* (10)	2.5	0.17

EJ*ras* contains the activated human *[Val^{12}]Ha-ras* gene. SV*E1A* has the adenovirus *E1A* gene under control of the SV40 promoter/enhancer region. p*E1A* includes the *E1A* gene and the endogenous promoter. *mp53* is murine wild-type *p53* and *h*p53 is human wild-type *p53*.

Table 2.8 Effect of Max, Mad and Mxi1 on Myc plus Ras-mediated REF transformation

Study	Transfected DNA (μg)	No.of Foci	Ratio to Ras+ E1A
Mäkelä (130)	pGEJ*ras* (7.5), pSVc-*myc*1 (7.5)	37	--
	pGEJ*ras* (7.5), pSVc-*myc*1 (7.5), *max* (15)	14	0.38
Lahoz (131)	T24*ras* (2), pK0*myc* (2)	196	--
	T24*ras* (2), pK0*myc* (2), *mad* (2)	15	0.08
	T24*ras* (2), pK0*myc* (2), *mxi1* (2)	49	0.25
	T24*ras* (2), p*E1A* (2)	218	--
	T24*ras* (2), p*E1A* (2), *mad* (2)	160	0.73
	T24*ras* (2), p*E1A* (2), *mxi*1 (2)	216	0.98

pSVc-*myc*1 has c-myc under control of the SV40 promoter/enhancer region. pGEJ*ras* and T24 *ras* encode the activated human *[Val^{12}]Ha-ras* gene. pK0*myc* includes exons 2 and 3 of the mouse c-*myc* gene under control of the SV40 promoter/enhancer element.

suppressed cell transformation by repressing the endogenous c-*myc* promoter in these two cases, and in the other cases Cut may have repressed cell transformation by different mechanisms.

Although only one *ras* plasmid was used in this study, the ability of Ras to transform an established cell line (FR3T3) was not inhibited by human Cut, thereby suggesting that the Cut protein does not repress the promoter driving the *ras* gene (Table 2.1). In fact, there was a slight increase in the number of transformed foci when *cut* was transfected with *ras*. Future experiments can be designed to investigate the possibility that human Cut acted as a general repressor of the promoters controlling the oncogenes used in this study. For example, Northern and Western blotting can be performed to determine if Cut can repress expression of E1A and Ras. The possibility that other genes which repress transformation in the REF assay (*p53*, *max*, *hsc70*, *mad* and *mxi*) act by repressing transcription of the promoters controlling the oncogenes was not investigated in similar studies (90, 129, 130, 131).

The lack of an effect of human Cut on Ras transformation of FR3T3 cells is also important because it suggests that Cut did not suppress transformation by interfering with the function of Ras. This result also suggests that Cut did not repress transformation because it has a general toxic effect on cells. Similar studies showed that hsp70 did not repress Ras transformation of established Rat-1 cells (129). However, wild-type p53 was able to repress transformation by Ras in Rat-1 cells and the issue that wild-type p53 may interfere with Ras directly was not addressed (90).

Previous results in Hela cells suggest that high levels of Cut may be incompatible with cell growth (67). So far, only one stable transformant (in HL-60 myeloid leukemia cells) expressing the *cut* gene has been identified (72) and it is possible that this clone contains mutant Cut protein (67). However in this study, all transformed cell lines transfected with *E1A*, *ras*, and *cut* expressed high levels of transfected *cut* RNA and seemed to express high levels of exogenous human Cut protein (Figures 2.1, 2.2, 2.3). This is contrary to results seen with p53 where exogenous wild-type RNA or protein was absent in transformed cells, mutant p53 protein was abundant and altered mobility forms of p53 were detected (90). However, these results are similar to the results seen with hsp70 (129). The results with hsp70 were interpreted to mean that very high threshold levels of hsp70 were needed to exert this inhibitory effect and that this threshold was not reached in surviving cells. It was also suggested that surviving cells may have been derived from a small fraction of cells that were insensitive to suppression mediated by hsp70. Perhaps this explanation can also be applied to the results that were seen in this study with Cut.

Gel-shift analyses of the cell lines in this study suggest that the exogenous Cut protein was functional in DNA binding in at least 5/9 cell lines tested (Figure 2.3). However, it is important to note that these experiments were performed in unsynchronized cells and it has been shown that Cut is only active in the late G1 and S phases of the cell cycle (67). Future experiments with synchronized cells will help clarify these findings. In addition, it is also important to note that the antibody used in Western blotting did not distinguish between rat and human Cut proteins so endogenous rat protein may have been elevated in these cells. Consequently, is possible that these elevations in the levels of rat Cut could have been

responsible for the increases in DNA binding seen in some cell lines tested. Alternatively, interactions with E1A could have stabilized the endogenous rat protein in the REF cell lines, however, this does not appear to be the case since prior incubation with E1A antibodies did not induce supershifts in gel-shift assays (Figure 2.3). Immunoprecipitation experiments can be performed to further investigate if Cut proteins are complexed with E1A in these cell lines.

On the other hand, the transfected Cut protein from some cell lines seemed to be unable to bind DNA (Figure 2.3) and at least two cell lines tested expressed additional RNA species that could be mutant forms of Cut RNA (Figure 2.1), thereby suggesting that the exogenous Cut gene and/or protein may be mutated in the transformed cell lines created in this study. Although proteins with different mobilities that cross-reacted with the Cut antibody were not detected by Western blotting (Figure 2.2), the Cut protein could have small point mutations that would be indistinguishable on a gel. In the future it will be necessary to sequence the exogenous Cut genes in the transformed cell lines created in this study to identify if they are mutated and/or if they produce mutant proteins that may induce transformation. Most importantly, the suspected incompatibility of high levels of transfected Cut with proliferation must be investigated further.

CHAPTER 3

CUT INTERACTS WITH THE DNA TUMOR VIRUS SV40 LARGE T ANTIGEN

3.1 INTRODUCTION

3.1a Purpose of this study.

The purpose of this study was to investigate the potential interactions between the human Cut protein and the SV40 LT antigen and to determine the effects of this interaction on the function of Cut. Such an interaction would suggest that Cut has a role in cellular proliferation and differentiation (89) and that inactivation of Cut would be advantageous for infection and/or transformation by SV40.

3.1b The SV40 DNA tumour virus.

The SV40 DNA tumour virus is a Simian polyomavirus that was originally identified as a contaminant in rhesus monkey cell cultures used to prepare the polio vaccines in the late 1950's (141). Rhesus monkey cells are *permissive* for SV40, however, African green monkey cells are *nonpermissive* and can be *transformed* by SV40 producing a cell line called COS. Cells transformed by DNA tumour viruses express only the early viral genes which are often referred to as T antigens. The SV40 Large T antigen (SV40 LT) can *immortalize* and *transform* cells. SV40 LT is a multi-functional 94 kDa nuclear phosphoprotein that binds

specifically to the SV40 genome and plays a role in initiation of viral DNA replication. It induces the expression of host enzymes involved in DNA replication, causes a round of cellular DNA replication, has ATPase and helicase activities, binds to DNA, and activates transcription from gene promoters (84).

Originally it was suggested that SV40 is not oncogenic in humans (141), however recently the potential role of SV40 in human cancers has been addressed. SV40 has been shown to infect, immortalize and transform human cells in culture. It has also been documented that human cells from some people with a high risk of developing cancer are 10-50 times more susceptible to transformation *in vitro* by SV40 than cells obtained from normal people (141). In the 1970's, one case of malignant melanoma was attributed to SV40 infection (141). Since then, SV40 DNA sequences and protein have been found in human cerebral tumours (142, 143, 144, 145), pediatric ependymal and choroid plexus tumours (146) and human pleural mesotheliomas (147). However, SV40 DNA sequences have been detected in both normal and adenomatous human pituitary tissues which makes it difficult to determine the role that SV40 plays in human cancer (148).

In January, a workshop was held at the National Institutes of Health to review the research on the role of SV40 in human cancers. According to a recent article in Science (149), the United States Food and Drug Administration concluded that cancer incidence and mortality is not increased in people who likely received the contaminated polio vaccines. However it has not been ruled out that SV40 may play a role in human cancer. Interestingly, antibodies against SV40 have been detected in blood samples taken from people before the contaminated polio vaccine was administered as well as from others who did not receive the vaccine. Although it

is possible that these antibodies detect JCV and/or BKV viral proteins instead, this finding may suggest that SV40 has existed in humans for a number of years. In any case, further experiments are planned to better understand the role that SV40 may have in human cancer (149).

3.1c The human JC and BK viruses.

The human JC and BK viruses are human polyomaviruses that infect much of the world population during childhood, and following initial infection, anti-BK and anti-JC antibodies can be found in 70-80% of adults (150). During childhood, these viruses are dormant and can be reactivated by unknown mechanisms when immunity is suppressed (151, 152), for example, in AIDS patients (153, 154). BK viral infection can induce kidney tumours in transgenic animals (155, 156), and may be associated with brain and pancreatic islet tumours in humans (157). Infection with JC virus is associated with progressive multifocal leukoencephalopathy (PML) in humans which is a demyelinating disease of the central nervous system (158). As was mentioned with SV40, it is important to note that JCV and BKV DNA sequences have also been detected in normal tissues as well (148, 159).

3.1d The Rb and p53 tumour suppressor proteins interact with and can be inactivated by SV40, JCV, and BKV Large T antigens.

In human cancers, the *Rb* and *p53* genes are often inactivated following deletion of one allele and mutation of the other allele. However, in tumours obtained after transformation by a DNA tumour virus, the function of pRb and p53 can be lost as a result of interaction between the tumour suppressor protein and the viral protein. Inactivation of pRb by viral oncoproteins

has been studied extensively. SV40 LT binds to the pocket region of hypophosphorylated pRb (85, 86) which displaces E2F and leads to transcription of genes normally activated only during S phase. In addition, recently it was shown that binding of SV40 LT can prevent transcriptional repression by pRb (83).

Interactions between the viral oncoproteins and p53 have also been studied. In fact, p53 was first identified as a cellular protein that binds to the SV40 LT antigen in transformed cells (140). When SV40 LT binds to p53, the abilities of p53 to bind DNA (87) and activate transcription are inhibited (88). BKV LT also interacts with p53 and binding abrogates p53-mediated transactivation (160). SV40 transformation is also accompanied by hyper-phosphorylation of p53 and by an increase in the metabolic stability of p53, both of which may have an effect on p53 function (161, 162). It has been shown that stabilization is probably a consequence of the T-induced phenotype and not due to the binding of SV40 LT to p53 (162).

The biological significance of the p53 and pRb interactions with SV40 LT in SV40 transformation is unclear. p53 mutations are not selected for in SV40 T-antigen induced tumours from transgenic mice (163), thereby suggesting that SV40 LT is able to inactivate p53 in these tumours. However, binding of SV40 LT to p53 is not required for induction of choroid plexus tumours in transgenic mice (164). It is similarly unclear whether or not binding of SV40 LT to pRb is necessary for transformation by SV40 since SV40 LT mutants with deletions in the pRb binding domain can still cause tumours in nude mice (164, 165).

In any case, these interactions between tumour suppressor proteins and viral oncoproteins may provide insight into ways that cellular proteins can inactivate p53 and pRb in tumours without mutated *Rb* and *p53* genes. For example, cellular proteins (other than E2F)

have been shown to bind to the pocket domain of pRb and some of these proteins have as yet unidentified functions (114, 166). The fact that the pocket domain is commonly mutated in human tumours suggests that this region has an important role in growth suppression (114). Therefore, in tumours without mutations in the pRb pocket domain, cellular proteins could mimic the viral oncoproteins and inactivate pRb by displacing E2F. In the case of p53, a cellular protein called mdm-2 has been shown to bind to p53 in the region that SV40 LT binds and binding of mdm-2 also blocks p53-mediated transactivation (167).

Inactivation of p53 and pRb by the viral oncoproteins raises important questions with respect to the consequences of an interaction between Cut and SV40 LT. Since DNA tumour viruses try to force cells into S phase (84, 141), SV40 LT may stimulate DNA binding and transcriptional repression by Cut since Cut is only active in the G1 and S phases of the cell cycle. Similar to pRb and p53, identification of an interaction between Cut and a viral protein would give insight into protein-interaction domains in the Cut protein that may be important for growth suppression and/or normal regulation of Cut function and expression.

3.2 MATERIALS AND METHODS

Plasmids. Plasmids expressing glutathione S-transferase (GST) Cut fusion proteins (referred to hereafter as GST/Cut proteins) were previously prepared by inserting different fragments from the human *cut* cDNA into the bacterial expression vector pGEX-3X (57, 58). These plasmids (and others prepared in our laboratory) are described in Figures 3.1 and 3.2. The *p130* plasmid obtained from Hughes Corbeil contains the p130 pocket region that binds to SV40 LT inserted into the bacterial expression vector pGEX-2X. The *GST* plasmid used as a negative control is the pGEX-3X plasmid without any insert. The pMX *cut* and *cux* plasmids were described in Chapter 2. The pBL2*CAT* plasmid used in CAT assays contains the *thymidine kinase (tk)* promoter region which includes Sp1 and CCAAT binding sites (168). The wild-type *SV40 LT* plasmid SVpBR328 (obtained from Greg Matlashewski) contains the origin and early region of SV40 inserted into plasmid pBR328. Finally, pKS and pMX139 plasmids (described in Chapter 2) was used as carrier DNA in transfections.

Expression and preparation of fusion proteins. Plasmid vectors expressing GST, p130, and GST/Cut fusion proteins were introduced into the DH5 strain of *Escherichia coli*. Induction of expression and purification of GST fusion proteins was performed as follows. One hundred millilitres of GST/Cut fusion protein bacterial cultures were incubated at 37°C until an O.D. $_{595}$ of 0.6 was reached, and then induced for 1.5 hours with 0.3 mM IPTG. After spinning for 15 min at 3500 rpm at 4°C and resuspending the bacterial pellets in 6 ml of MTPBS (150 mM NaCl, 4 mM $NaH_2P0_4H_20$, 16 mM Na_2HP0_4) with 1 mM DTT (Gibco #27565-41-9) and 1 mM PMSF (Sigma #329-98-6), the bacteria were frozen overnight at -80°C. The

bacteria were thawed, sonicated 3 x 15s, and 500 µl of 10% Triton was added. The bacteria were centrifuged at 12 000g for 10 minutes and the supernatant was divided in 1 ml aliquots. Glycerol was added (to a final concentration of 30%), and aliquots were frozen at -80°C. One aliquot was thawed and 200 µl of 50% Glutathione S-transferase beads (Pharmacia) were added and incubated at 4°C with rocking for 30 minutes. The beads were washed three times for 5 minutes with NET buffer (1% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 8.0]) and resuspended in 200 µl of NET buffer. Ten microlitres of beads were mixed with sample buffer and boiled, and then the proteins were resolved by electrophoresis on a SDS-10% polyacrylamide gel. The approximate concentration of each protein was determined by staining with Coomassie Brilliant Blue. For elution of proteins used in gel-shift assays, 300 µl of 25 mM reduced glutathione in 50 mM Tris [pH 8.0], containing 1 mM DTT, was incubated with the protein-bound beads for 20 minutes at 4°C. The beads were centrifuged for 3 min at 10 000g and the supernatant was retained. Glycerol was added to a concentration of 20% and the proteins were stored at -80°C. Protein concentration was determined by the method of Bradford using the Bio-Rad Protein Assay according to the manufacturer's protocol.

Antibodies. Two SV40 LT antibodies were used: the amino terminal mouse monoclonal purified antibody PAb 416 (Oncogene Science # DP02), and the carboxy terminal mouse monoclonal purified antibody PAb 101 (Santa Cruz Biotechnology # sc-147). The unpurified polyclonal Cut amino terminal antibody was described in Chapter 2.

Cell culture of SV40 LT-containing cell lines and control cell lines for GST pull-down assays, Gel-shift assays, Western blotting, and immunoprecipitations. The Balb/c/3T3 clone A31 (ATCC CCL-163) is one of several cell lines derived from Balb/c mouse embryos. It is a permanent, non-tumorigenic, contact-inhibited cell line which was used as a negative control for the SV-T2 cell line. SV-T2 cells (ATCC CCL-163.1) are derived from a clone of SV40 transformed cells obtained by SV40 infection of a cell culture that gave rise to Balb/c/3T3 cells. SV-T2 cells have a high saturation density, lack contact inhibition and are able to induce tumours. The CV-1 (ATCC CCL-70) cell line is derived from the kidney of a male adult African green monkey and it was used as a negative control for the COS-1 cell line. CV-1 cells grow rapidly and form monolayers of fibroblast-like cells. The COS-1 cell line is a fibroblast cell line established from CV-1 cells which were transformed by an origin-defective mutant of SV40 which codes for wild-type T antigen. 293 cells are human embryonal kidney cells that have been transformed with sheared adenovirus type 5 (Ad 5) DNA and contain both E1A and E1B transforming proteins. 293 cells were used as negative controls for 293T cells. 293T cells are 293 cells that have been transformed with the SV40 Large T antigen (obtained from N. Sonenberg). Cells were plated at a 1:8-1:12 ratio and harvested when 80% confluent. For immunoprecipitation experiments involving 293T cells transiently transfected with *cut*, *cux* and *SV40 LT*, cells were transfected with 6 µg of each plasmid as described in the section: Transient transfections and cell culture for gel-shift assays.

GST pull-down assay. GST pull-down assays with purified SV40 LT were performed as follows. Unless stated otherwise, approximately 500 ng of each GST fusion protein bound to GST beads was incubated with 500 ng of a 2 μg /μl or a diluted 100 ng/μl solution of baculovirus-produced purified SV40 Large T antigen (CHIMERx # 5800-01). The original 2 μg/μl solution was diluted to 100 ng/μl in storage buffer: (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 100 mM DTT, 1 mM EDTA, and 50% glycerol). The assay was performed in 1 ml of TNT buffer (200 mM NaCl, 0.5% NP40, 20 mM Tris [pH 7.5]), containing 0.5 mM PMSF (Sigma #329-98-6), 1μg/ml Pepstatin A (Sigma #26305-03-3), 1μg/ml Aprotinin (Boehringer Mannheim # 981532), and 1μg/ml Leupeptin (Boehringer Mannheim #1529048) for 1 hour at 4°C with rocking. In experiments where DNAse was used, 1, 2, or 5 μl of DNAse was added to TNT buffer before addition of SV40 LT and protein bound beads. GST beads (up to a total of 40 μl) were added to each tube in order to facilitate washing, and the samples were centrifuged briefly. The beads were washed three times with TNT buffer for 5 minutes, resuspended in 25μl of sample buffer and boiled for 8 minutes. Samples were resolved by electrophoresis on a SDS-6% polyacrylamide gel, and the gel was transferred to a PVDF membrane overnight in transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol). The blot was washed briefly in TBS-0.5% Tween 20 and blocked for two hours with shaking with 5% BSA in TBS-1% Tween 20. The blot was washed briefly with TBS-0.5% Tween 20 and incubated with amino and carboxy terminal antibodies for SV40 LT at 1:1000 dilutions in TBS-0.5% Tween 20 containing 1% filtered calf serum, and 1% BSA overnight at 4°C with rocking. The blot was washed two times for 5 minutes each with TBS-0.5% Tween 20, and four times for 5 minutes each with TBS-0.5% Tween 20 containing

1% filtered calf serum, and 1% BSA. Then the membrane was incubated with a HRP-conjugated anti-mouse antibody at a 1:4000 dilution in TBS-0.5% Tween 20 containing 1% filtered calf serum, and 1% BSA for one hour. The blot was washed as described above, incubated with ECL Detection reagents (Amersham) for one minute and exposed to film.

The GST pull-down experiments with COS-1 and CV-1, and Balb/c/3T3 and SV-T2 cell extracts were performed as follows. Cells were harvested at 80% confluency. Cells were washed with PBS and incubated with 500 µl of NET buffer (0.5% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 8.0]) containing 0.5 mM PMSF, 1µg/ml Pepstatin A, 1µg/ml Aprotinin and 1µg/ml Leupeptin. The plates were incubated on ice for 30 minutes. Cells were scraped, transferred into tubes and centrifuged at 12 000 g for 10 minutes. The supernatants were retained. GST/fusion proteins (that were bound to beads) were added to the supernatant from 2 x 100 mm plates of cells (1 ml), and incubated as described above. The remainder of the protocol was the same as described for the pull-down experiments with purified SV40 LT.

Silver Stain. SV40 LT protein (10, 50, and 100 ng) was mixed with Laemlli sample buffer, boiled and electrophoresed on a SDS-6% polyacrylamide gel. The gel was placed in a 30% ethanol, 10% acetic acid solution overnight at room temperature with shaking. The next day, the solution was removed and the gel was incubated twice in 30% ethanol with shaking for 30 minutes. Then the gel was washed three times with deionized water for 10 minutes with shaking. Next, a 0.1% solution of $AgNO_3$ was added for 30 minutes with shaking and then the gel was washed under a stream of deionized water for 20 seconds. After washing, a

2.5% sodium carbonate, 0.02% formaldehyde solution was added for several minutes (3-5) until bands appeared and the background of the gel began to darken. Finally, the reaction was terminated by washing the gel in 1% acetic acid for a three minutes. The gel was washed several times with deionized water for 10 minutes each and photographed.

[^{35}S]methionine-labelling. Cells were washed two times with PBS and incubated with 200 µCi of [^{35}S]methionine Transmix (Amersham) in 2 ml of DMEM without methionine for 4 hours. Each hour the plates were rocked briefly to distribute the medium.

Preparation of cell lysates for immunoprecipitation. Two immunoprecipitation protocols were utilized and protein lysates were prepared differently for each protocol. The first protocol was used for the [^{35}S]methionine-labelled immunoprecipitation and the co-immunoprecipitation-Western experiment in 293T cells. Plates were washed once with DMEM, and twice with PBS. One ml of PBS was added to each plate and the cells were scraped into tubes. After brief centrifugation, the cell pellets were frozen at -80 ^{0}C overnight. Cell pellets were thawed and resuspended in 1 ml of TNT buffer (200 mM NaCl, 0.5% NP40, 20 mM Tris [pH 7.5]) containing 0.5 mM PMSF, 1µg/ml Pepstatin A, 1µg/ml Aprotinin and 1µg/ml Leupeptin and incubated on ice for 15 minutes. Samples were centrifuged and the supernatant was transferred to a new tube.

For the second immunoprecipitation protocol used in all other experiments, plates were washed two times with PBS, and 800 µl of NET buffer (0.5% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 8.0]) containing 0.5 mM PMSF, 1µg/ml Pepstatin A, 1µg/ml

Aprotinin, and 1μg/ml Leupeptin were added to each plate and the plates were incubated on ice for 30 minutes. The cells were scraped into tubes, centrifuged at 12 000g for 10 minutes, and the supernatant was retained.

Immunoprecipitations. Two immunoprecipitation protocols were utilized. The first protocol was used for the [^{35}S]methionine-labelled immunoprecipitation and the co-immunoprecipitation western experiments with 293T cells. Five microlitres of each antibody were added to the protein lysates and incubated on ice for 1 hour. Then, 100 μl of a 10% slurry of protein G beads were added and incubated for one hour with rocking at 4^{0}C. After centrifugation, the beads were washed three times for 10 minutes with TNT buffer. The beads were resuspended in 50 μl of sample buffer and boiled for 5 minutes. Samples were resolved by electrophoresis on a SDS-6% polyacrylamide gel. [^{35}S]methionine immunoprecipitation gels were dried and exposed to film. Immunoprecipitation gels to be used for Western blotting were transferred to a PVDF membrane overnight in transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol). The blot was washed and incubated with SV40 LT antibodies as described above (see GST pull-down). The membrane was stripped by submerging it in 50 ml of stripping buffer (100 mM 2-β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.8]) for 30 minutes at 50^{0}C with occasional agitation. Then it was washed with TBS-0.1% Tween 20 extensively and incubated with the amino terminal Cut antibody as described in Chapter 2.

The second immunoprecipitation procedure used for all other immunoprecipitations was performed as follows (169). One millilitre of protein lysate was pre-cleared with 40 μl of a 20% slurry of Protein A Sepharose beads at 4^{0}C with rocking for 30 minutes. Pre-cleared

lysates were incubated with 5 µl of Cut or SV40 LT antibodies at 4°C with rocking for 1 hour. Then, 40 µl of a 20% slurry of Protein A Sepharose beads were added and samples were incubated at 4°C with rocking for 15 minutes. The beads were washed three times for 5 minutes with NET buffer, resuspended in 25µl of sample buffer, and boiled for 8 minutes. Samples were resolved by electrophoresis on a SDS-6% polyacrylamide gel. [^{35}S]methionine-immunoprecipitation gels were dried and exposed to film. Immunoprecipitation gels to be used for Western blotting were and transferred to a PVDF membrane overnight, and blotted with antibodies for SV40 LT and Cut as described above.

Western blotting. Western blotting for SV40 LT was described in the section: GST pull-down assay. Western blotting for Cut was described in Chapter 2.

Transient transfections for CAT assays. NIH 3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum. 3 x 10^5 cells were seeded in 100 mm plates 24 hours before transfection. Cells were transfected with 6 µg of *cut*, *cux*, and *SV40 LT* plasmids, and 4 µg of the *tkCAT* plasmid. When necessary, pMX-139 plasmid DNA was added to make the total amount of DNA in each plate equal to 16 µg. Cells were washed two times with PBS 18 hours after transfection. Cells were harvested 24 hours later.

CAT assays. Cells were incubated with 1 ml of TEN buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl) for 5 minutes on ice. After centrifugation, cells were resuspended in 100 µl of 0.25 M Tris-HCl [pH 7.5], and subjected to three cycles of freeze-

thaw with dry ice-ethanol and a 37°C water bath. After centrifugation, the cytoplasmic extracts were recovered, and protein concentration was determined by the method of Bradford using the Bio-Rad Protein Assay according to the manufacturer's protocol. CAT assays were performed with 20 μg of protein extract as previously described (170).

Transient transfections and cell culture for Gel-shift assays. NIH 3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum. 3 x 10^5 cells were seeded in 100 mm plates 24 hours prior to transfection. Cells were transfected with 6 μg of *cut*, *cux*, or *SV40 LT* plasmids. When necessary, pMX-139 plasmid DNA was added to make the total amount of DNA in each plate equal to 16 μg. Cells were washed two times with PBS 18 hours after transfection. Cells were harvested 24 hours later. Where noted, cells were serum-starved for 24 or 48 hours in media containing 0.4% serum prior to harvesting.

Preparation of protein extracts for Gel-shift assays and Western blotting. Extracts were prepared as described in Chapter 2.

Gel Mobility Shift Assays (Gel-shift assays). For experiments investigating the effect of SV40 LT on transiently transfected *cut* and *cux* in NIH 3T3 cells, assays were performed as described in Chapter 2. In order to examine endogenous Cut DNA binding activity in NIH 3T3 cells transfected with *SV40 LT* and in cell lines expressing SV40 LT, assays were performed as described in Chapter 2, except poly(dI-dC) was not added before incubation with the probe. Assays with GST/Cut fusion proteins were performed as described in Chapter 2 with

10-100 ng of Cut fusion proteins and an equal amount of purified SV40 LT. One hundred nanograms of poly(dI-dC) was added as described in Chapter 2. W3, non-specific (HRP-conjugated anti-rabbit), and carboxy-terminal SV40 LT antibodies were used as described in Chapter 2. Cold competitor DNA was not used as a control for the specificity of Cut DNA binding in these experiments because Cut DNA binding can be competed out with almost any sequence in excess (67). Therefore, antibody controls are used to demonstrate specific DNA binding by Cut (67).

3.3 RESULTS

I. THE HUMAN CUT PROTEIN INTERACTS WITH THE SV40 LT ANTIGEN

The homeodomain region of the human Cut protein interacts with SV40 LT *in vitro*.

Figure 3.1 is a diagrammatic representation of some of the Cut fusion proteins that were used in the experiments investigating the interactions between human Cut and SV40 LT. Figure 3.2 shows a GST-pull down assay that was performed using baculovirus-produced SV40 LT and Cut fusion proteins. The negative control for SV40 LT binding in this experiment (GST) was the pGEX-3X vector that expresses GST (Panel A Figure 3.2, lane 1). The positive control for SV40 LT binding in this experiment was a fusion protein containing the pocket domain of the retinoblastoma-related p130 protein (Panel A Figure 3.2, lane 2). These results show that the coiled-coil (CC); the Cut repeats 1, 2, and 3 (CR1, CR2, CR3); and the Cut repeat plus Homeodomain (CR3-HD) regions of the Cut protein interacted with 500 ng (0.25 µl) of a high concentration of SV40 LT (2 µg/ µl). Furthermore, the CR3-HD region of the Cut protein appeared to interact with SV40 LT most efficiently (lane 11). The only region of the Cut protein that did not interact with SV40 LT was the carboxy terminus (lane 12).

Further experiments were performed to determine if binding between the Cut fusion proteins and SV40 LT would be more specific using lower concentrations of SV40 LT. Only the CR3-HD and HD regions of Cut interacted with SV40 LT when 500 ng (5 µl) of a lower concentration of SV40 LT (100 ng/µl) was used (Figure 3.2, panel B). These interactions were relatively inefficient since only about 1% of the SV40 LT protein bound to the CR3-HD

Figure 3.1 Diagrammatic representation of GST/Cut fusion proteins. Five of the GST/Cut fusion proteins used in GST pull-down assays are shown. Plasmids encoding these protein domains contain the corresponding human Cut cDNA sequences cloned into the pGEX-3X plasmid. Proteins were prepared as described in Materials and Methods.

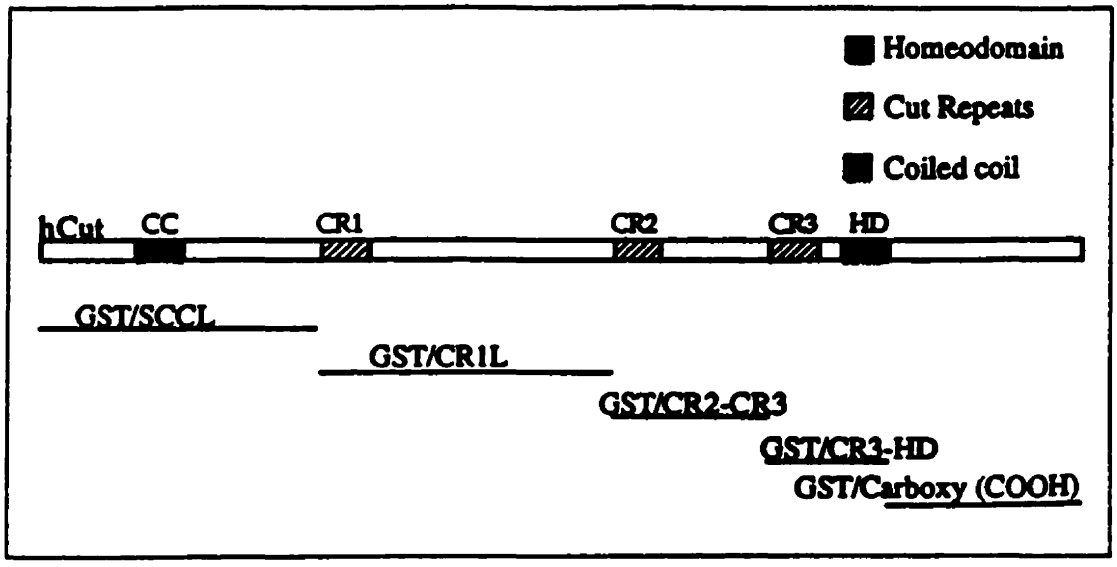
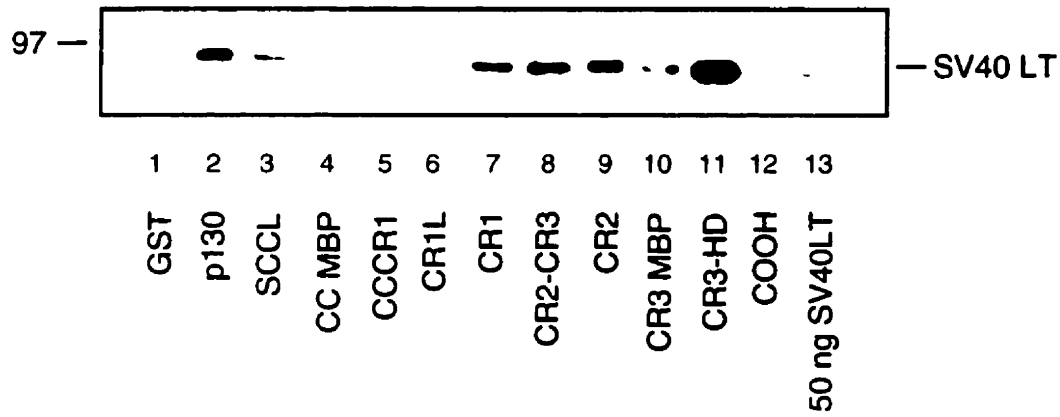
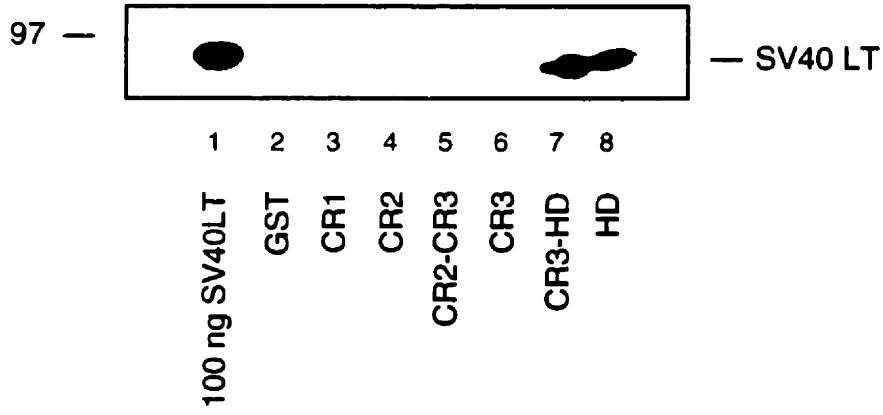

Homeodomain
Cut Repeats
Coiled coil
hCut
CC
CR1
CR2
CR3
HD
GST/SCCL
GST/CR1L
GST/CR2-CR3
GST/CR3-HD
GST/Carboxy (COOH)

Figure 3.2 (A) GST pull-down assay with Cut fusion proteins and concentrated purified baculovirus-produced SV40 LT. Five hundred nanograms (0.25 µl) of a 2 µg/µl solution of purified SV40 LT antigen was incubated with approximately 500 ng of each protein bound to GST beads, and the amount of SV40 LT bound to the beads was determined by Western blotting. SCCL, CR1L, CR2-CR3, CR3-HD and COOH fusion proteins are represented in Figure 3.1. CCCR1 , CR1 and CR2 are GST-fusion proteins derived from the proteins shown in Figure 3.1. CCCR1 contains the coiled-coil and the Cut repeat 1 regions, CR1 contains the Cut repeat 1 region and CR2 contains the Cut repeat 2 region. CCMBP, and CR3MBP are maltose binding fusion proteins derived from the proteins shown in Figure 3.1. CCMBP contains the coiled-coil region, and CR3MBP contains the Cut repeat 3 region. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. The p130 fusion protein was used as a positive control for SV40 LT binding. It contains the pocket region of the p130 protein that binds to SV40 LT. Fifty nanograms of purified SV40 LT protein was included as a positive control for Western blotting. **(B) GST pull-down assay with Cut fusion proteins and diluted purified baculovirus-produced SV40 LT.** Five hundred nanograms (5 µl) of a 100 ng/µl solution of purified SV40 LT antigen was incubated with approximately 500 ng of each protein bound to GST beads, and the amount of SV40LT bound to the beads was determined by Western blotting. The CR2-CR3 and CR3-HD fusion proteins are described in Figure 3.1, and the CR1 and CR2 GST fusion proteins are described in panel A (above). The CR3 and HD GST fusion proteins are derived from the proteins shown in Figure 3.1. CR3 contains the Cut repeat 3 region, and HD contains the Homeodomain region of Cut. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. One hundred nanograms of purified SV40 LT was included as a positive control for Western blotting.

A


97
SV40 LT
1 2 3 4 5 6 7 8 9 10 11 12 13
GST
p130
SCCL
CC MBP
CCCR1
CR1L
CR1
CR2-CR3
CR2
CR3 MBP
CR3-HD
COOH
50 ng SV40LT

B


97
SV40 LT
1 2 3 4 5 6 7 8
100 ng SV40LT
GST
CR1
CR2
CR2-CR3
CR3
CR3-HD
HD

and HD protein-bound beads. Since the CR3 and CR2-CR3 fusion proteins did not interact with SV40 LT, this indicates that the interaction between Cut and SV40 LT was mediated by the homeodomain. Furthermore, the amount of SV40 LT bound to the HD and the CR3-HD regions was similar thereby suggesting that the CR3 region did not stabilize or destabilize the interaction mediated by the homeodomain.

The interaction between the CR3-HD region and SV40 LT shown in Figure 3.3 was not as efficient as the interactions shown in Figure 3.2. A possible explanation for this finding is that with the exception of the experiments illustrated in Figure 3.2, all experiments were performed with SV40 LT protein that had been stored at -20^{0}C (according to the manufacturer's recommendations) for over a week. The inefficiency of the interaction between the CR3-HD and HD fusion proteins and SV40 LT is also demonstrated in Figure 3.3 since 500 ng of SV40 LT and CR3-HD proteins were needed to detect binding by Western blotting. Figure 3.3 also shows that addition of 500 ng of a competitor protein extract from FR3T3 cells did not inhibit binding between the CR3-HD and SV40 LT.

The interaction between the Cut homeodomain and SV40 LT is stabilized by DNA.

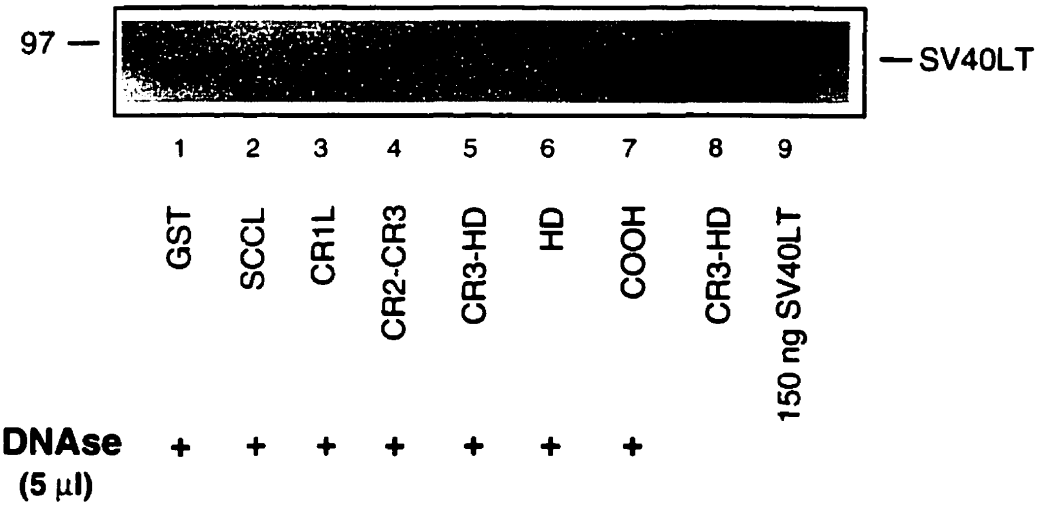
Next it was necessary to rule out the possibility that the interaction between the Cut homeodomain and SV40 LT could be attributed to both proteins binding to contaminating DNA present in the SV40 LT or GST fusion protein preparations (21, 23, 24, 171). Figure 3.4, panel A shows that the CR3-HD and HD fusion proteins still interacted with SV40 LT in the presence of DNAse. However, the interaction between Cut and SV40 LT was stabilized by DNA since there was at least a 75% reduction in the amount of SV40 LT that

Figure 3.3 GST pull-down assay with a 2:1 ratio between CR3-HD and SV40 LT, and addition of FR3T3 competitor protein. Purified SV40 LT antigen (250 or 500 ng) was incubated with approximately 500 ng of the CR3-HD protein bound to GST beads and the amount of SV40 LT bound to the beads was determined by Western blotting. In addition, 500 ng of a FR3T3 cell extract was incubated with SV40 LT and the CR3-HD protein-bound beads where indicated. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. Purified SV40 LT (150ng) was included as a positive control for Western blotting.

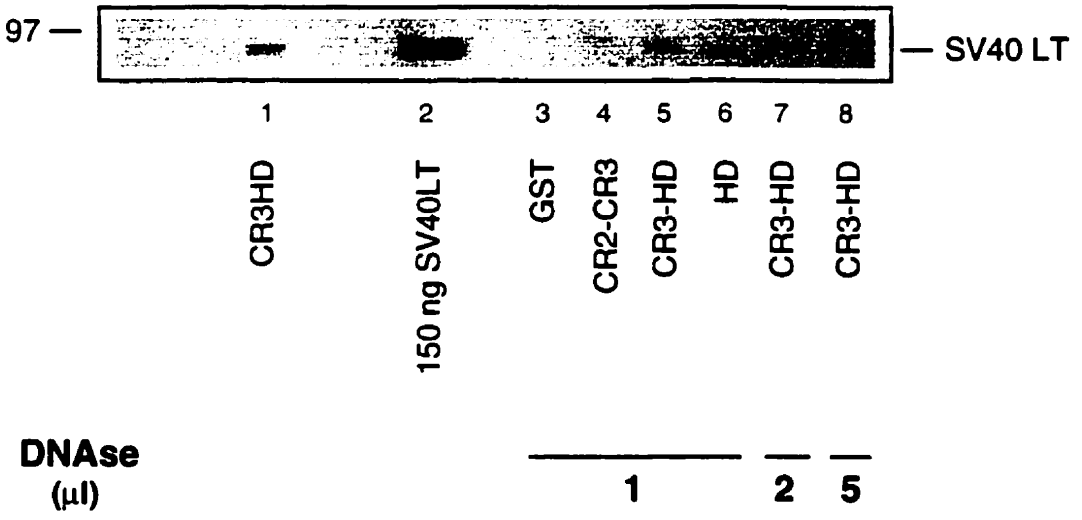
	1	2	3	4	5
	GST	50ng SV40LT	CR3-HD	CR3-HD	CR3-HD
SV40LT (ng)			250	500	500
FR3T3 (ng)					500

Figure 3.4 (A) GST pull-down assay with Cut fusion proteins and purified baculovirus-produced SV40 LT in the presence of DNAse. Five hundred nanograms (5 µl) of a 100 ng/µl solution of purified SV40 LT antigen was incubated with approximately 500 ng of each protein bound to GST beads. The amount of SV40 LT bound to the beads was determined by Western blotting. RNAse free DNAse (5 µl) was added to the reaction mixtures where indicated as described in Materials and Methods. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. Purified SV40 LT (150 ng) was included as a positive control for Western blotting. **(B) GST pull-down assay with CR3-HD and HD fusion proteins and purified baculovirus-produced SV40 LT in the presence of increasing amounts of DNAse.** Five hundred nanograms (5 µl) of a 100 ng/µl solution of purified SV40 LT antigen was incubated with approximately 500 ng of each protein bound to GST beads. The amount of SV40 LT bound to the beads was determined by Western blotting. RNAse free DNAse (1, 2, or 5 µl) was added to the reaction mixtures where indicated as described in Materials and Methods. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. Purified SV40 LT (150 ng) was included as a positive control for Western blotting.

A


97
SV40LT
1
2
3
4
5
6
7
8
9
GST
SCCL
CR1L
CR2-CR3
CR3-HD
HD
COOH
CR3-HD
150 ng SV40LT
DNAse
(5 µl)
+
+
+
+
+
+
+

B


97
SV40 LT
1
2
3
4
5
6
7
8
CR3HD
150 ng SV40LT
GST
CR2-CR3
CR3-HD
HD
CR3-HD
CR3-HD
DNAse
(µl)
1
2
5

bound to the CR3-HD protein when it was pre-incubated with DNAse. This stabilization effect is confirmed in Figure 3.4, panel B since the amount of SV40 LT bound to the CR3-HD region decreased as the volume of DNAse increased.

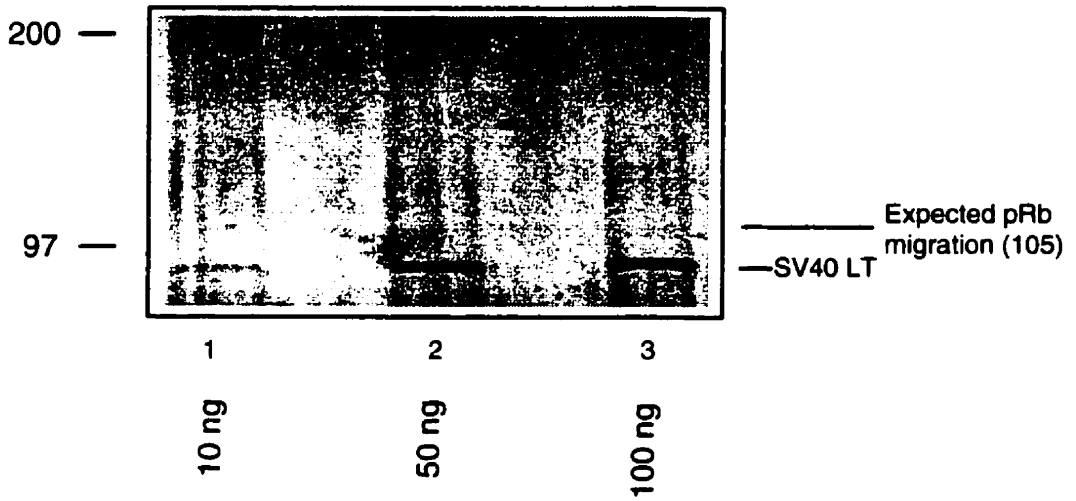
The interaction between Cut and SV40 LT is not mediated by the retinoblastoma protein.

It was also necessary to consider the possibility that the interaction between the Cut homeodomain and SV40 LT might be mediated by the retinoblastoma protein (pRb) since an *in vitro* interaction between Cut and pRb was identified recently (61). More specifically, the homeodomain may bind to pRb that is bound to SV40 LT. Therefore, silver staining was performed to determine if pRb was present in the purified baculovirus-produced SV40 LT used in these experiments. Silver staining is able to detect as little as 1-10 ng of a protein (172). Figure 3.5 shows a silver stain of increasing amounts of purified SV40 LT. A 105 kDa protein band corresponding to pRb was not evident in any lane thereby confirming that the interaction between Cut and SV40 LT is not mediated by pRb.

The CR3-HD and HD Cut fusion proteins can also interact with SV40 LT from COS-1 and SV-T2 cells.

Next, GST pull-down experiments were performed to determine if the CR3-HD and HD fusion proteins interact with SV40 LT from two SV40 transformed cell lines. These experiments are more physiologically relevant and they simulate the interaction *in vivo* since the CR3-HD and HD proteins must interact with SV40 LT in the presence of many other proteins. Evidence of such interactions would also rule out the possibility that the CR3-HD

Figure 3.5 Silver stain of purified baculovirus-produced SV40 LT antigen. Purified SV40 LT antigen (10, 50, and 100 ng) was mixed with Laemlli sample buffer and electrophoresed on a SDS-6% polyacrylamide gel. The gel was silver stained as described in Materials and Methods. Briefly, the gel was fixed, washed and then incubated with a 0.1% solution of $AgNO_3$. The image was developed by washing with a solution containing sodium carbonate and formaldehyde, and the reaction was terminated by washing in an acetic acid solution. The gel was photographed immediately afterwards. The expected position of pRb migration is noted in the figure.


200
97
Expected pRb migration (105)
SV40 LT
1
2
3
10 ng
50 ng
100 ng

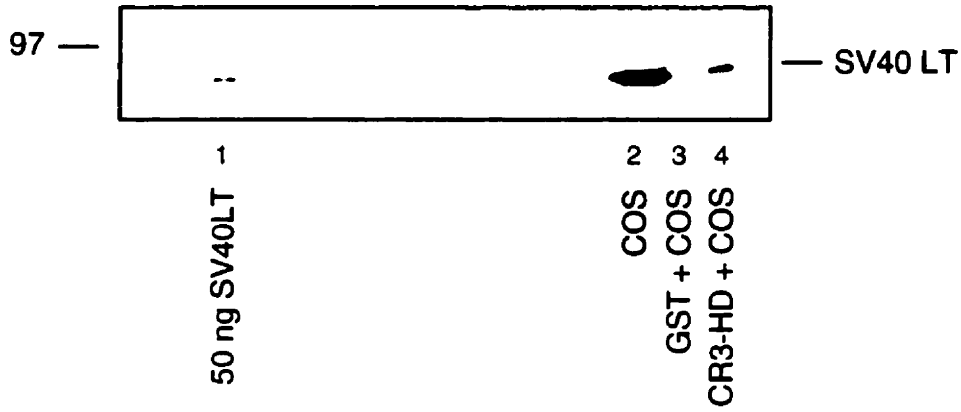
and HD proteins could only interact with baculovirus-produced SV40 LT. Panel A of Figure 3.6 shows that the CR3-HD fusion protein interacted with SV40 LT from COS-1 cells. Panel B of Figure 3.6, shows that the CR3-HD and HD fusion proteins interacted with SV40 LT from SV-T2 cells. However, these interactions were rather weak since only about 1% of the SV40 LT protein that was originally incubated with the beads interacted with the CR3-HD and HD fusion proteins.

Cut proteins may bind to SV40 LT *in vivo*.

In order to determine if Cut proteins interact with SV40 LT *in vivo*, a number of immunoprecipitation experiments were performed. Panel A of Figure 3.7 shows a co-immunoprecipitation experiment with extracts from 293T cells that were transfected with *cut* and *SV40 LT* and labelled with [^{35}S]-methionine. A faint band that co-migrates with Cut was immunoprecipitated by the carboxy-terminal SV40 LT antibody in extracts from cells transfected with *cut* (lane 9), and *cut/SV40 LT* (lane 10). However, the SV40 LT antibody immunoprecipitated two proteins migrating at approximately 94 kDa from un-transfected (lane 6), and transfected (lanes 8-10) cell extracts, so it was unclear which of these proteins was SV40 LT. Higher levels of the faster migrating (or smaller) protein were detected in extracts from cells transfected with *cut* and *SV40 LT* (lane 10), and higher levels of the slower migrating (or larger) protein were detected in extracts from cells transfected with *SV40 LT* (lane 8) or *cut* (lane 9). The slower migrating (or larger) protein was also detected in extracts from untransfected or transfected cells that were immunoprecipitated with the Cut antibody (lanes 1-5). Previous experiments have shown that SV40 LT is present in

Figure 3.6 (A) GST pull-down assay with the CR3-HD fusion protein and COS-1 cell extracts. Protein extracts prepared from 2 x 100 mm plates of COS-1 cells were incubated with approximately 500 ng of CR3-HD and GST proteins bound to GST beads. The amount of SV40 LT bound to the beads was determined by Western blotting. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. Fifty nanograms of purified SV40 LT and approximately 1/4 of COS-1 cell protein extract from a 100 mm tissue culture dish of cells were included as positive controls for Western blotting. **(B) GST pull-down assay with CR3-HD and HD fusion proteins and Balb/c/3T3 and SV-T2 cell extracts.** Protein extracts were prepared from 2 x 100 mm plates of Balb/c/3T3 and SV-T2 cells and were incubated with approximately 500 ng of CR3-HD, HD and GST proteins bound to GST beads as described in Materials and Methods. The amount of SV40 LT bound to the beads was determined by Western blotting. Balb/c/3T3 cell extracts were included as negative controls for extracts from SV-T2 cells. The GST protein expressed from pGEX-3X was used as a negative control for SV40 LT binding. Approximately 1/4 of SV-T2 protein extract from a 100 mm tissue culture dish of cells was included as a positive control for Western blotting. The two panels represent two exposures of the same Western blot.

A


97
SV40 LT
1
2
3
4
50 ng SV40LT
COS
GST + COS
CR3-HD + COS

B

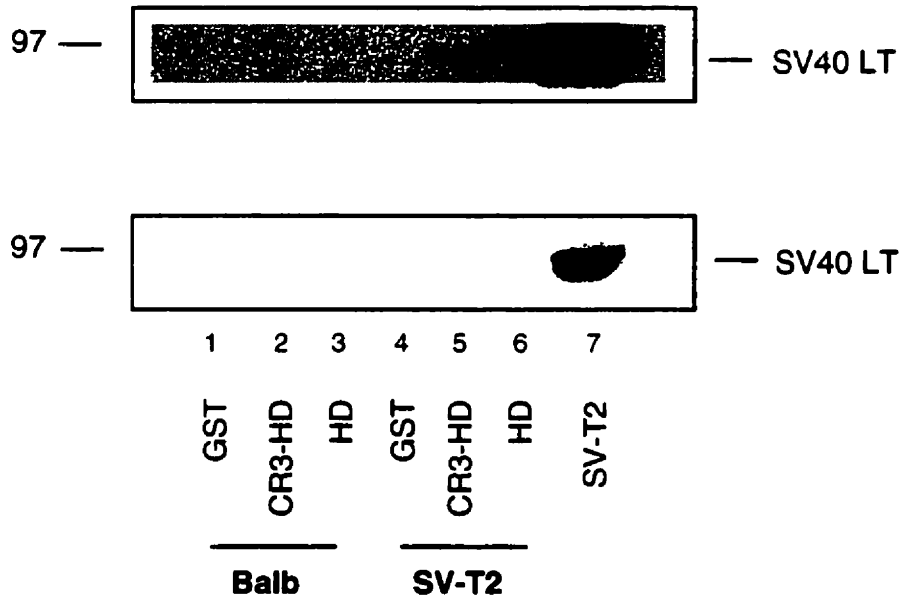
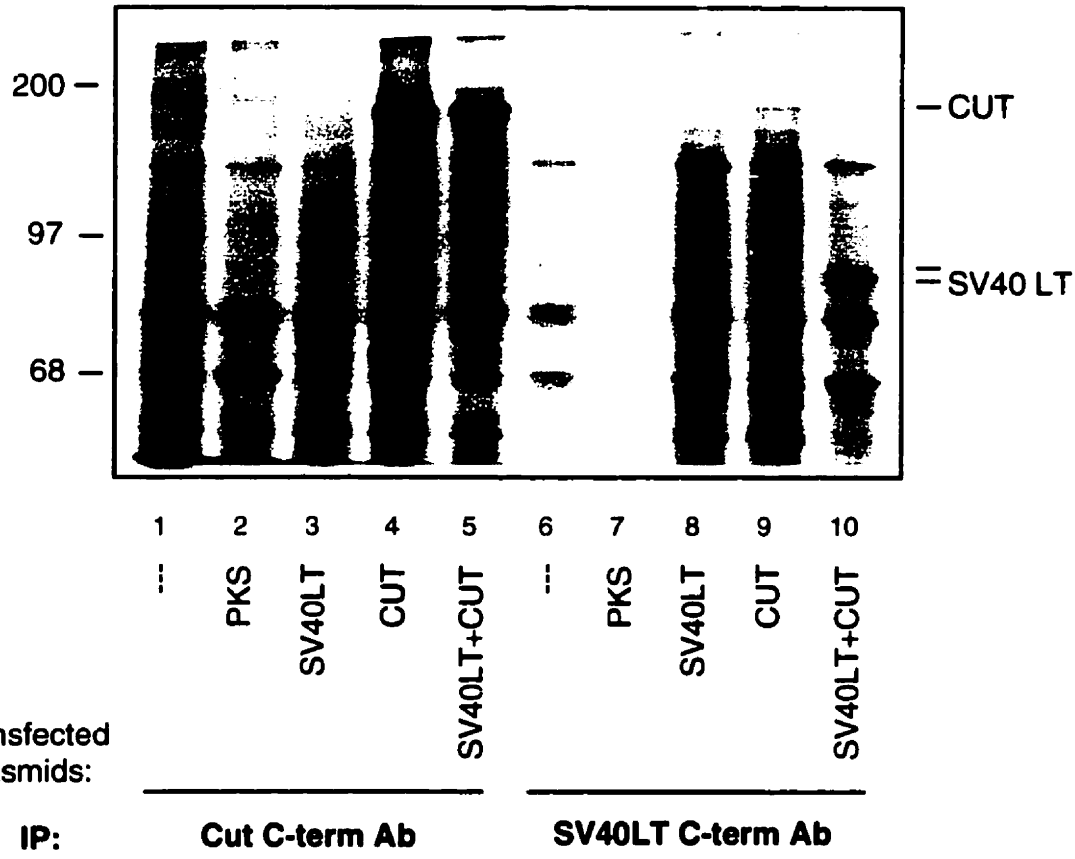

97
SV40 LT
97
SV40 LT
1
2
3
4
5
6
7
GST
CR3-HD
HD
GST
CR3-HD
HD
SV-T2
Balb
SV-T2

Figure 3.7 (A) [^{35}S]methionine-labelling and co-immunoprecipitation of 293T cell extracts with SV40 LT and Cut antibodies. 293T cells were transfected with plasmids encoding SV40 LT and Cut with the total kept constant with pKS plasmid DNA. Immunoprecipitations were performed as described in Materials and Methods. Cells were labelled with [^{35}S]methionine for four hours and protein extracts were prepared. Extracts were immunoprecipitated with a carboxy terminal antibody for Cut (Cut C-term Ab) or a carboxy-terminal antibody for SV40 LT (SV40LT C-term Ab), and proteins were electrophoresed on a SDS-6% polyacrylamide gel. The gel was dried and exposed to film. **(B) Co-immunoprecipitation of 293T cell extract with SV40 LT antibodies.** 293T cells were transfected with plasmids encoding SV40 LT antigen and Cut as above, protein extracts were prepared, and immunoprecipitations using an SV40 LT carboxy-terminal antibody (SV40 LT C-term Ab) were performed as described in Materials and Methods. Proteins were electrophoresed on a SDS-6% polyacrylamide gel. The gel was transferred and Western blotted for SV40 LT as described in Materials and Methods.

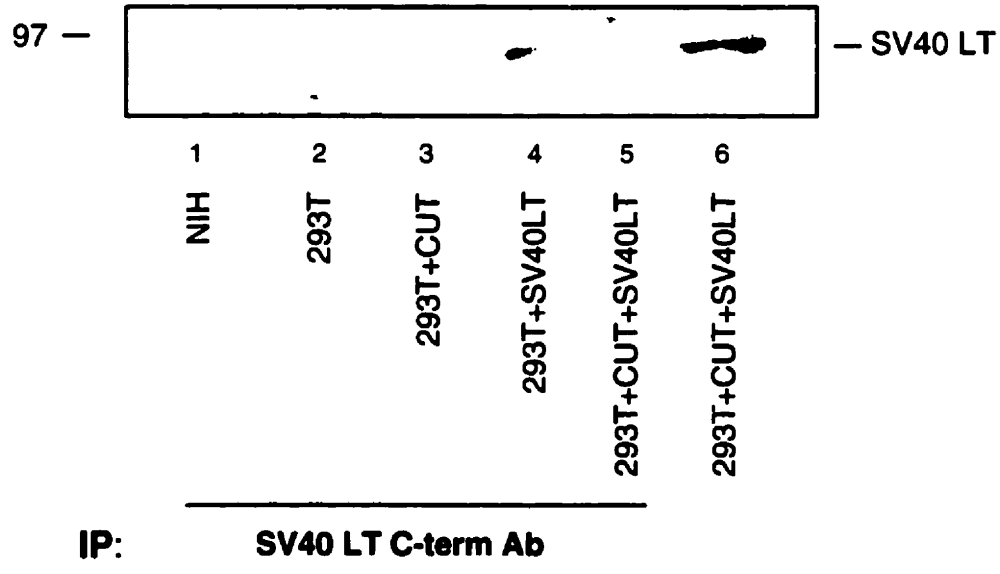
A


200
97
68
CUT
SV40 LT
1 2 3 4 5 6 7 8 9 10

PKS
SV40LT
CUT
SV40LT+CUT

PKS
SV40LT
CUT
SV40LT+CUT
Transfected plasmids:
IP:
Cut C-term Ab
SV40LT C-term Ab

B


97
SV40 LT
1 2 3 4 5 6
NIH
293T
293T+CUT
293T+SV40LT
293T+CUT+SV40LT
293T+CUT+SV40LT
IP:
SV40 LT C-term Ab

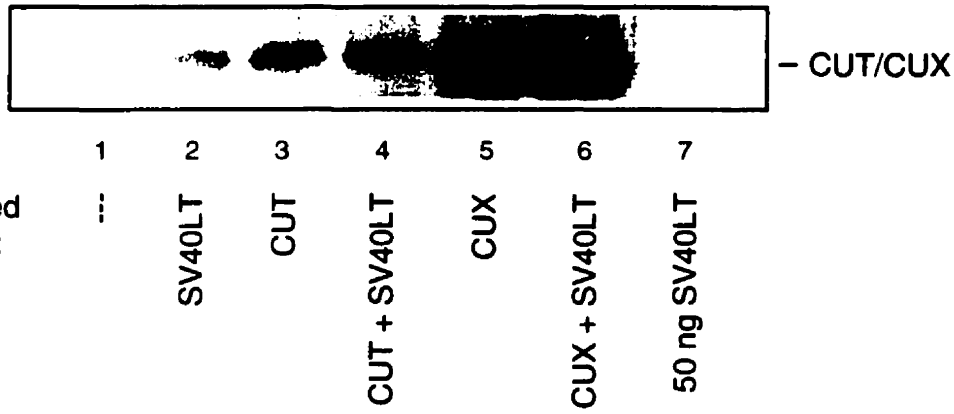
immunoprecipitates in unphosphorylated (faster migrating) and phosphorylated (slower migrating) forms (173), so it is possible that there was an increase in the unphosphorylated form in extracts from cells transfected with *cut* and *SV40 LT* (lane 10). On the other hand, the slower migrating protein present in extracts from cells transfected with *cut* and *SV40 LT* may have been the transfected SV40 LT protein.

In order to determine whether or not these two proteins represented unphosphorylated and phosphorylated forms of SV40 LT, or transfected SV40 LT, a co-immunoprecipitation-Western experiment was performed with 293T cell extracts. Panel B in Figure 3.7 shows a co-immunoprecipitation-Western experiment with extracts from cells that were transfected with *SV40 LT*, and *cut*. Neither endogenous nor transfected SV40 LT protein was immunoprecipitated from 293T cell extracts (lanes 2-5). Transfected SV40 LT protein was detected in extracts from cells transfected with *cut* and *SV40 LT* (lane 6), thereby suggesting that the abundant protein present in lane 10 of panel A (Figure 3.7) was the transfected SV40 LT protein.

In order to investigate this further, extracts from NIH 3T3 cells transfected with *SV40 LT*, *cut*, and *cux* were subjected to Western blotting with an SV40 LT antibody. Panel A of Figure 3.8 depicts a Western blot for Cut/Cux showing that both transfected Cut and Cux proteins were expressed, however Cux expression was stronger. Panel B of Figure 3.8 is a Western blot for SV40 LT indicating that SV40 LT was detected only when co-transfected with *cux*. The reason that SV40 LT was not detected when transfected with *cut* is unknown, but it may be related to the fact that Cux expression was stronger in these cells. A possible explanation that may account for the finding that transfected SV40 LT could only be detected

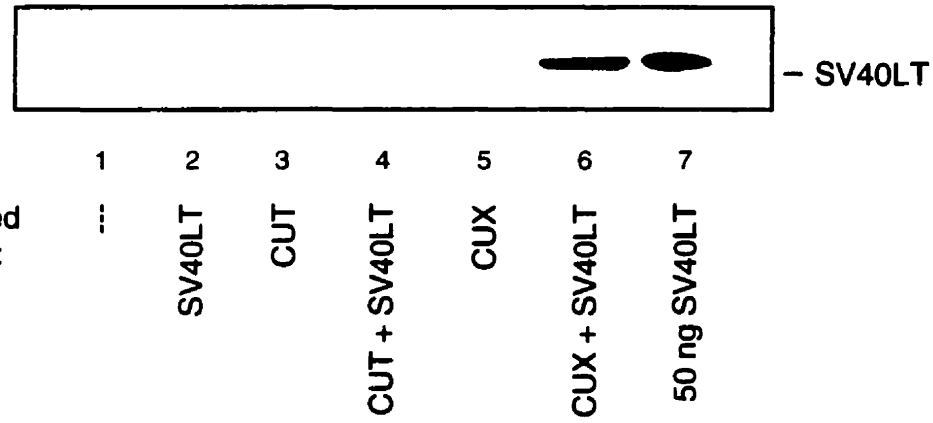
Figure 3.8 (A) Western blot for Cut with extracts from NIH 3T3 cells transfected with *SV40 LT*, *cut*, and *cux*. Cells were transfected with *SV40 LT*, *cut*, and *cux* and extracts were prepared as described in Materials and Methods. Proteins were electrophoresed and Western blotted for Cut as described in Materials and Methods. **(B) Western blot for SV40 LT with extracts from NIH 3T3 cells transfected with *SV40 LT*, *cut*, and *cux*.** Cells were transfected with *SV40 LT*, *cut*, and *cux* and extracts were prepared as described in Materials and Methods. Proteins were electrophoresed and Western blotted for SV40 LT as described in Materials and Methods.

A


– CUT/CUX
1
2
3
4
5
6
7
Transfected plasmids:

SV40LT
CUT
CUT + SV40LT
CUX
CUX + SV40LT
50 ng SV40LT

B


– SV40LT
1
2
3
4
5
6
7
Transfected plasmids:

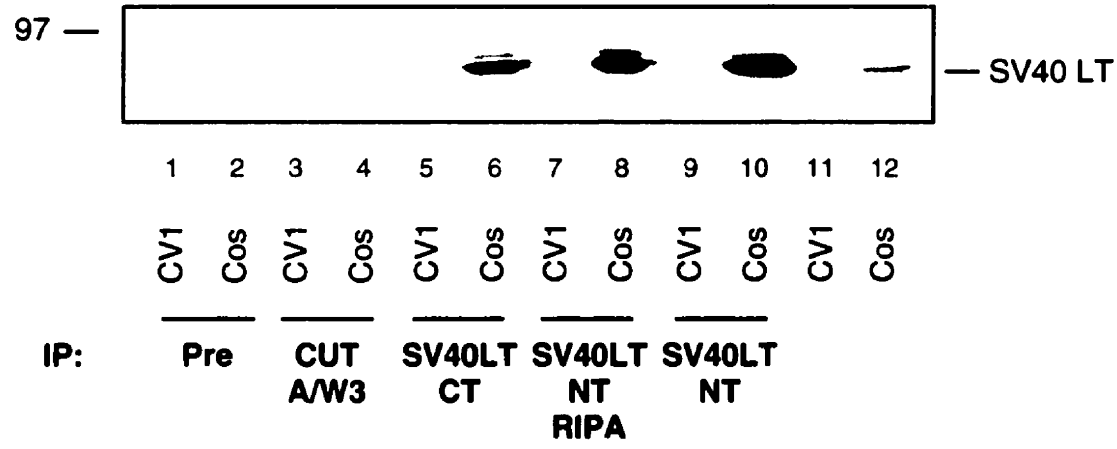
SV40LT
CUT
CUT + SV40LT
CUX
CUX + SV40LT
50 ng SV40LT

when transfected with *cux* may be that binding of Cux stabilized the transfected protein in some way that enabled it to be detected. Future experiments should be repeated with control cells that do not express SV40 LT (293 cells) and new batches of 293T cells in order to clarify whether the 94 kDa proteins detected were unphosphorylated and phosphorylated forms of SV40 LT. In addition, these experiments should be repeated with different SV40 LT plasmids in order to determine if this effect is specific to the SV40 LT plasmid used in this study.

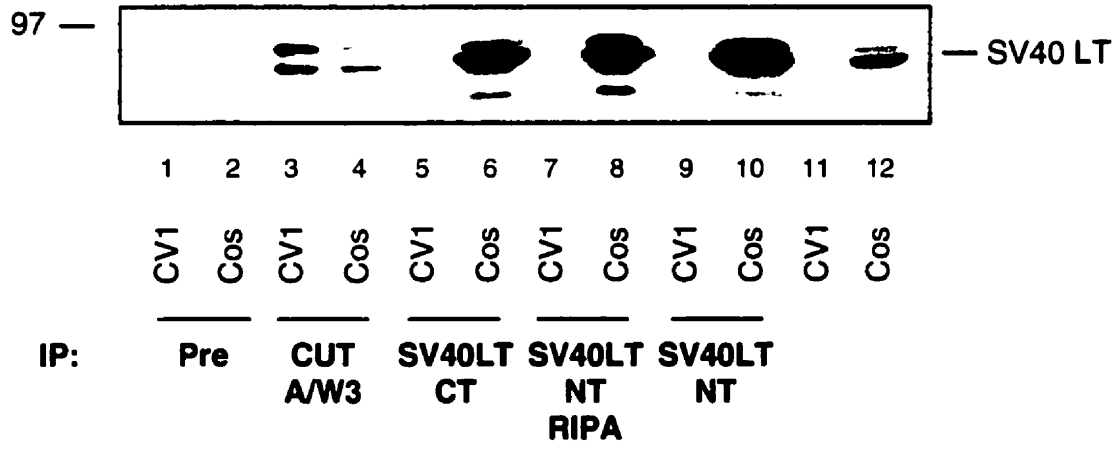
Further co-immunoprecipitation experiments were performed with established cell lines that were transformed with SV40 or SV40 LT and express large amounts of the SV40 LT antigen. Initial experiments were performed with COS-1 cells (that are transformed with SV40 LT) and with CV-1 cells that were used as negative controls since they do not express SV40 LT. Cut antibodies A and W3 (which immunoprecipitated LT from MMTV-Polyoma LT transgenic mice) immunoprecipitated proteins that co-migrated with and were recognized by SV40 LT antibodies (Panel B of Figure 3.9, lane 4). However, these proteins were also detected when CV-1 extracts were immunoprecipitated with A and W3 (lane 3) suggesting that these proteins were not SV40 LT. In addition, SV40 LT antibodies did not immunoprecipitate Cut from COS-1 cell extracts. The corresponding Western blot for Cut is not shown since endogenous Cut was not immunoprecipitated well by Cut antibodies from these cell extracts. The choice of lysis buffer, and the fact that protease inhibitors were not used in the washes (perhaps resulting in protein degradation) may have prevented efficient immunoprecipitation of Cut.

Figure 3.9 Co-immunoprecipitation of CV-1 and COS-1 cell extracts with Cut and SV40 LT antibodies. Protein extracts were prepared as described in Materials and Methods. All cells were lysed and immunoprecipitated with NET buffer with protease inhibitors except as noted where RIPA buffer was used (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris [pH 8.0]). CV-1 cell extracts were included as negative controls since these cells do not express SV40 LT. Extracts were immunoprecipitated with CR3 (A), and carboxy-terminal (W3) monoclonal antibodies for Cut; or with carboxy-terminal (SVLT CT) and amino-terminal (SVLT NT) monoclonal antibodies for SV40 LT. Protein extracts from COS-1 and CV-1 cells were included as controls. Proteins were electrophoresed on a SDS-6% polyacrylamide gel. The gel was transferred and Western blotted for Cut (not shown) and SV40 LT. The two panels (**A** and **B**) show different exposures of the same Western blot.

A


97 —
— SV40 LT
1 2 3 4 5 6 7 8 9 10 11 12
CV1 Cos CV1 Cos CV1 Cos CV1 Cos CV1 Cos CV1 Cos
IP:
Pre
CUT A/W3
SV40LT CT
SV40LT NT RIPA
SV40LT NT

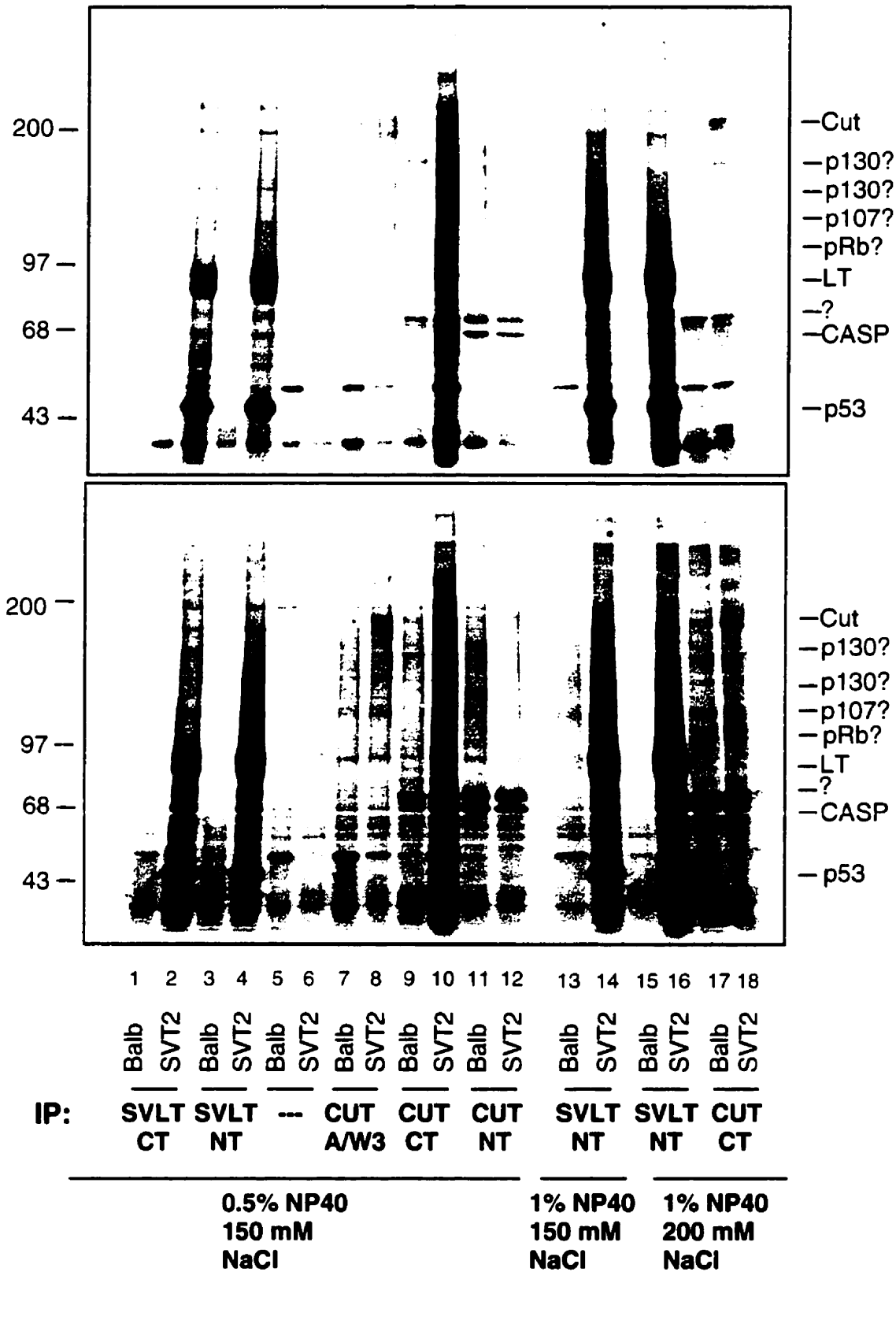
B


97 —
— SV40 LT
1 2 3 4 5 6 7 8 9 10 11 12
CV1 Cos CV1 Cos CV1 Cos CV1 Cos CV1 Cos CV1 Cos
IP:
Pre
CUT A/W3
SV40LT CT
SV40LT NT RIPA
SV40LT NT

Figures 3.10 and 3.11 show similar co-immunoprecipitation experiments with SV-T2 cells that have been transformed with SV40 and with Balb/c/3T3 cells which were used as negative controls because these cells do not express SV40 LT. SV-T2 cells were used because SV40 LT antibodies have been shown to immunoprecipitate an unidentified 193 kDa protein from SV-T2 cells labelled with [^{35}S]-methionine (169). Figure 3.10 shows a [^{35}S]-methionine labelled co-immunoprecipitation experiment with SV-T2 and Balb/c/3T3 cells. Cut was immunoprecipitated by Cut antibodies in all buffer conditions, however, the protein band was most prominent when the lysis buffer with 1% NP40 and 200 mM NaCl was used. A faint 200 kDa protein band was also immunoprecipitated by the amino-terminal SV40 LT antibody that co-migrated with Cut in extracts from SV-T2 cells when lysis buffers with 1% NP40 and either 150 mM or 200 mM NaCl were used. This protein was not detected when the lysis buffer with 0.5% NP40 and 150 mM NaCl was used. However, Cut antibodies did not immunoprecipitate SV40 LT from SV-T2 cell extracts. Several other proteins were immunoprecipitated by Cut antibodies from both Balb/c/3T3 and SV-T2 cell extracts, and these proteins may be important to understanding the function of Cut. Some of these proteins have molecular weights corresponding to the pRb-family proteins which, in light of the recent finding that Cut interacts with pRb and p130 *in vitro* and that the Cut homeodomain may mediate protein interactions, would be worthwhile to investigate further.

Panel A of Figure 3.11 shows a co-immunoprecipitation-Western experiment with Balb/c/3T3 and SV-T2 cell extracts. Cut antibodies immunoprecipitated SV40 LT (lanes 10 and 12 in the two lower panels in A), however SV40 LT antibodies did not immunoprecipitate Cut (lanes 2, 4 in the upper panel in A). The Cut protein was immunoprecipitated by Cut

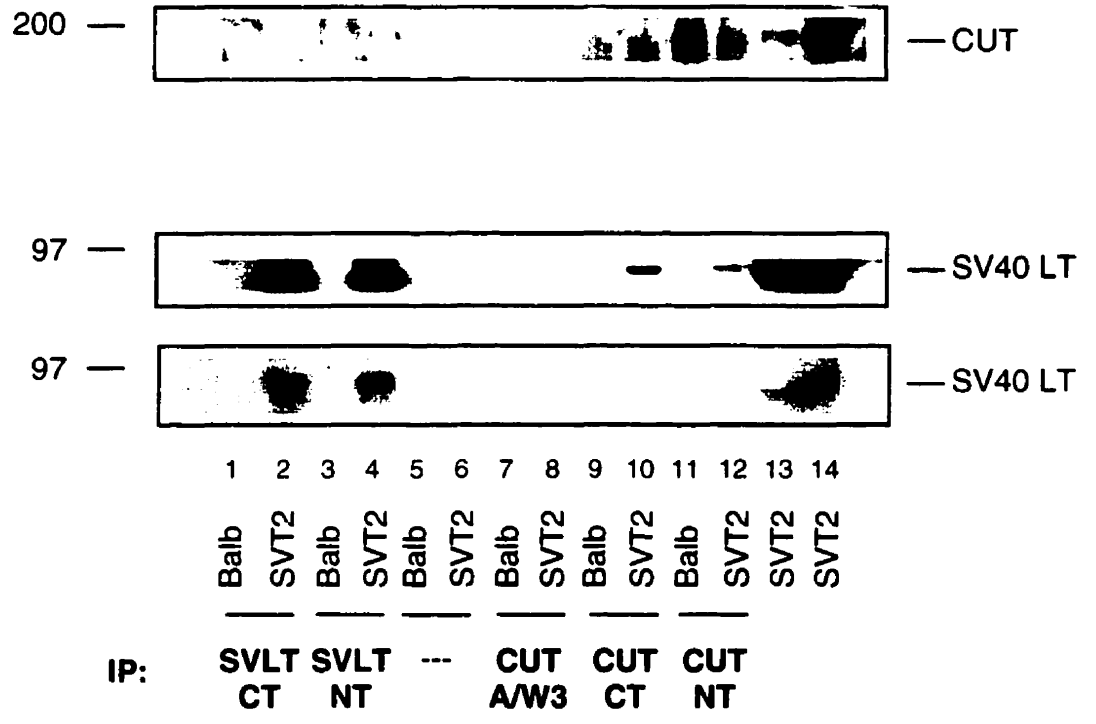
Figure 3.10 [^{35}S]methionine-labelling and co-immunoprecipitation of extracts from Balb/c/3T3 and SV-T2 cells with SV40 LT and Cut antibodies. Immunoprecipitations were performed as described in Materials and Methods. Cells were labelled with [^{35}S]methionine for four hours, protein extracts were prepared and then immunoprecipitated with the NET buffers described in the figure. Extracts were immunoprecipitated with CR3 (A) and carboxy-terminal (W3) monoclonal antibodies for Cut; carboxy-terminal (CT) and amino-terminal (NT) polyclonal antibodies for Cut; or with carboxy-terminal (SVLT CT) and amino-terminal (SVLT NT) monoclonal antibodies for SV40 LT. Balb/c/3T3 cell extracts were included as negative controls for extracts from SV-T2 cells. Proteins were electrophoresed on a SDS-6% polyacrylamide gel, and the gel was dried and exposed to film. The two panels show different exposures.


200
97
68
43
—Cut
—p130?
—p130?
—p107?
—pRb?
—LT
—?
—CASP
—p53
200
97
68
43
—Cut
—p130?
—p130?
—p107?
—pRb?
—LT
—?
—CASP
—p53
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2
IP:
SVLT CT
SVLT NT

CUT A/W3
CUT CT
CUT NT
SVLT NT
SVLT NT
CUT CT
0.5% NP40 150 mM NaCl
1% NP40 150 mM NaCl
1% NP40 200 mM NaCl

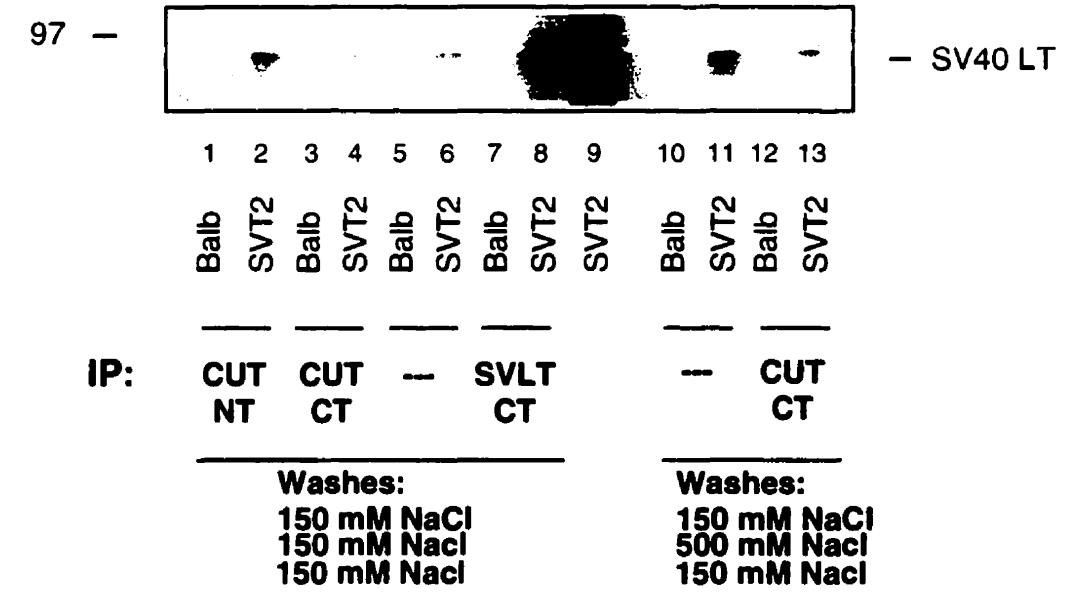
Figure 3.11 Co-immunoprecipitation of Balb/c/3T3 and SV-T2 cell extracts with Cut and SV40 LT antibodies. Protein extracts were prepared and immunoprecipitations were performed as described in Materials and Methods. Balb/c/3T3 cell extracts were included as negative controls for extracts from SV-T2 cells. Extracts were immunoprecipitated with CR3 (A) and carboxy-terminal (W3) monoclonal antibodies for Cut; carboxy-terminal (CT) and amino-terminal (NT) polyclonal antibodies for Cut; or with carboxy-terminal (SVLT CT) and amino-terminal (SVLT NT) monoclonal antibodies for SV40 LT. Proteins were electrophoresed on a SDS-6% polyacrylamide gel. The gel was transferred and Western blotted for Cut in the top panel of **(A)** and for SV40 LT in the lower panel of **(A)** as described in Materials and Methods. **(B) Co-immunoprecipitation of Balb/c/3T3 and SV-T2 cell extracts with Cut and SV40 LT antibodies using more stringent washings.** Protein extracts were prepared and immunoprecipitations were performed as described in Materials and Methods. Balb/c/3T3 cell extracts were included as negative controls for extracts from SV-T2 cells. Extracts were immunoprecipitated with carboxy-terminal (CT) and amino-terminal (NT) polyclonal antibodies for Cut; or with carboxy-terminal (SVLT CT) and amino-terminal (SVLT NT) monoclonal antibodies for SV40 LT. Beads were washed with buffers described in the figure. Proteins were electrophoresed on a SDS-6% polyacrylamide gel, the gel was transferred and then Western blotted for SV40 LT.

A


200
CUT
97
SV40 LT
97
SV40 LT
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 SVT2 SVT2
IP:
SVLT CT
SVLT NT

CUT A/W3
CUT CT
CUT NT

B


97
SV40 LT
1 2 3 4 5 6 7 8 9 10 11 12 13
Balb SVT2 Balb SVT2 Balb SVT2 Balb SVT2 SVT2 Balb SVT2 Balb SVT2
IP:
CUT NT
CUT CT

SVLT CT

CUT CT
Washes:
150 mM NaCl
150 mM Nacl
150 mM Nacl
Washes:
150 mM NaCl
500 mM Nacl
150 mM Nacl

antibodies more efficiently than in previously mentioned experiments (lanes 9-12 in the upper panel of A) and a possible explanation could be that protease inhibitors were used in the washing steps. It is important to note however, that these immunoprecipitations were performed with a lysis buffer that contained 0.5% NP40 and 150 mM of NaCl. This lysis buffer was used because it is similar to the TNT buffer usually used in our laboratory to immunoprecipitate Cut (Chapter 2). The results shown in Figure 3.10 suggest that Cut can be immunoprecipitated even more efficiently when a lysis buffer with 1% NP40 and 200 mM of NaCl is used.

Attempts at reproducing the result shown in Panel A of Figure 3.11 were hampered by the presence of protein bands that co-migrated with SV40 LT in control lanes where antibodies were not used to immunoprecipitate proteins (Panel B of Figure 3.11, lane 6) suggesting that SV40 LT stuck to the protein A Sepharose beads. This was also seen when more stringent washes were performed (lane 11). In summary, at this point in time it is not possible to conclude whether or not Cut interacts with SV40 LT *in vivo*.

II. THE EFFECT OF THE CUT:SV40 LT INTERACTION ON THE FUNCTION OF CUT

The effect of SV40 LT on transcriptional repression by Cut proteins.

The p53 and pRb tumor suppressor proteins can be inactivated by the DNA tumor virus proteins (Chapter 3 Introduction). Binding of SV40 LT to p53 and pRb interferes with the ability of both proteins to regulate transcription (83, 85, 86, 87, 88), so it was similarly hypothesized that binding of SV40 LT to Cut may affect transcriptional repression mediated

by Cut. Figure 3.12 shows a CAT assay investigating the effect of SV40 LT on transcriptional repression mediated by Cut and Cux. The human Cut and murine Cux proteins have been shown to repress the *thymidine kinase* gene promoter (74). As expected, Cut and Cux repressed the *tkCAT* reporter gene (lanes 3 and 5 respectively) and SV40 LT activated the *tkCAT* reporter gene (lane 2). Although Cut and Cux no longer repressed *tkCAT* when transfected with SV40 LT (lanes 4 and 6 respectively), it is not possible to determine whether this was a direct effect on Cut and Cux or a cancelling out effect between Cut/Cux mediated repression and SV40 transactivation.

The effect of SV40 LT on DNA binding by Cut proteins.

In addition to inhibiting the transcriptional activation and repression abilities of p53, binding of SV40 LT also interferes with the ability of p53 to bind DNA (87). Therefore, the effect that SV40 LT has on DNA binding by Cut was investigated. Experiments were first performed to determine the effect that an interaction with SV40 LT would have on DNA binding by the CR3-HD and HD GST/Cut fusion proteins. As shown in Figure 3.13, the DNA binding complexes were supershifted slightly when SV40 LT was included in the reaction. However, pre-incubation with SV40 LT antibodies did not produce any further shifts. In light of the finding that the interaction between the CR3-HD and HD fusion proteins was stabilized by DNA, it is possible that SV40 LT bound to the DNA probe used in the reaction. However this did not seem to be the case since a protein-DNA complex was not detected when SV40 LT

Figure 3.12 CAT assay with extracts from NIH 3T3 cells transfected with *cut*, *cux*, and *SV40 LT*. NIH 3T3 cells were transfected with 6 μg of *cut*, *cux*, and *SV40 LT* plasmids with 4 μg of the tk*CAT* reporter plasmid. Extracts were prepared as described in Materials and Methods. 30 μg of each cell extract was assayed for CAT activity as described in Materials and Methods.

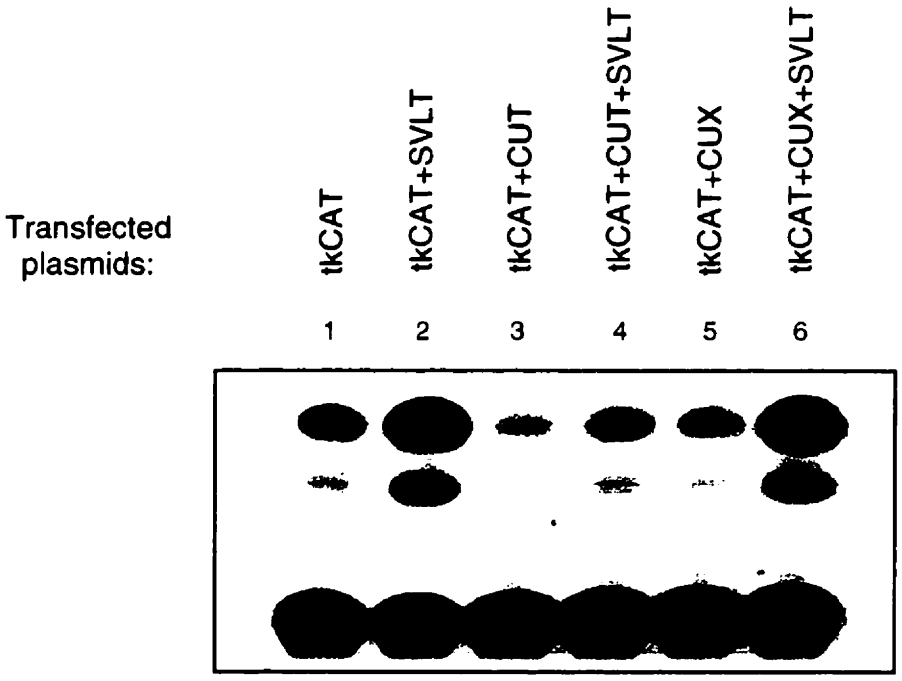
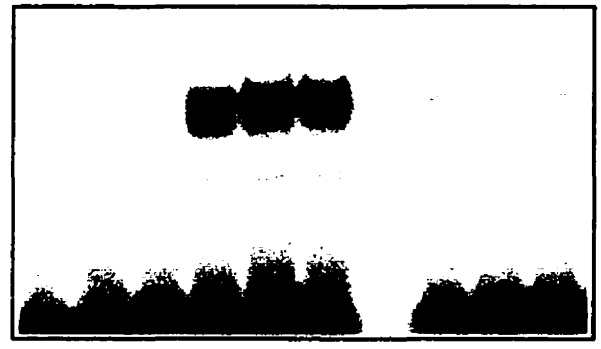

Transfected plasmids:
tkCAT
tkCAT+SVLT
tkCAT+CUT
tkCAT+CUT+SVLT
tkCAT+CUX
tkCAT+CUX+SVLT
1
2
3
4
5
6

Figure 3.13 Gel-shift assay for Cut with CR3-HD and HD Cut fusion proteins with or without incubation with SV40 LT. GST fusion proteins were prepared, bound to GST beads and then eluted as described in Materials and Methods. These proteins are described in Figures 3.1 and 3.2. One hundred nanograms of each fusion protein was incubated with or without 100 ng of purified SV40 LT as well as 100 ng of dI-dC and 1 µl of the radiolabeled FP probe. Some protein samples were pre-incubated with 1 µl of the carboxy-terminal SV40 LT antibody before addition of dI-dC and probe as indicated in the figure. The probe sample was included as a negative control and did not contain any protein. Samples were electrophoresed on a 5% polyacrylamide gel at 100 V for 3 hours. The gel was dried and exposed to film.



1	2	3	4	5	6		7	8	9
Probe	GST	GST	CR3-HD	CR3-HD	CR3-HD		HD	HD	HD
SV40LT		+		+	+			+	+
SV40 LT Ab					+				+

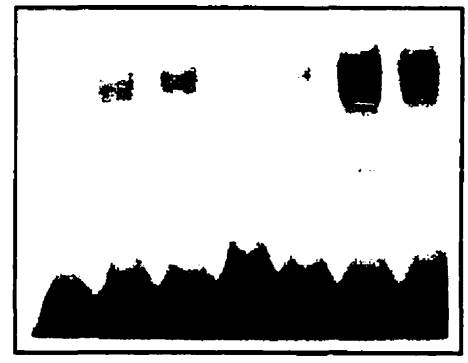
was incubated with the GST protein and the DNA probe (lane 3). It is important to note that cold competitor probe was not used as a control for the specificity of Cut DNA binding so these results should be interpreted with this in mind.

Next, DNA binding by Cut was investigated in extracts from cells transiently transfected with human *cut*, *cux*, and *SV40 LT*. Figure 3.14 shows a gel shift assay with protein extracts corresponding to the Western blots with NIH 3T3 extracts from cells transfected with *cut*, *cux* and *SV40 LT* shown in Figure 3.8. There was an increase in DNA binding in extracts from cells transfected with *cut* and *SV40 LT* (Panel A lanes 6 and 7), however the level of DNA binding by transfected Cut was low (Panel A lane 5) which makes these results difficult to interpret. Similar increases in binding were also observed in extracts from cells transfected with cux and *SV40 LT* (Panel B lanes 4 and 5), however the level of DNA binding by transfected Cux was low (lane 3) which also makes these results difficult to interpret. Unfortunately, these results could not be reproducibly repeated.

One of the potential problems with these initial experiments in cells transfected with human *cut* and murine *cux* was that high expression of Cut may have been saturating and therefore could have masked the effects of SV40 LT. Therefore, further experiments were performed to investigate the effect that transfected *SV40 LT* would have on DNA binding by endogenous murine Cut (mCut) in established cell lines. Since DNA tumor viruses force cells into S phase, it was hypothesized that SV40 LT may stimulate DNA binding by Cut (which usually only occurs in G1/S) in phases of the cell cycle when Cut is usually inactive (G0/G1). Since Cut is inactive in G0/G1, cells were serum starved to synchronize them in G0.

Figure 3.14 Gel-shift assay for Cut DNA binding with extracts from NIH 3T3 cells transfected with *cut* and *cux*, with or without transfection with *SV40 LT*. NIH 3T3 cells were transfected with 6 μg of *cut*, *cux*, and *SV40 LT* plasmids as described in Materials and Methods. Twenty micrograms of each protein extract was incubated with 1 μg dI-dC and 1 μl of the radiolabeled FP probe. Some protein samples were pre-incubated with 1 μl of a non-specific antibody (NS), a SV40 LT carboxy-terminal monoclonal antibody, or a carboxy-terminal monoclonal Cut antibody (W3) before addition of dI-dC and probe. The probe samples were included as negative controls and did not contain any protein. Samples were electrophoresed on a 5% polyacrylamide gel at 100 V for 3 hours. The gel was dried and exposed to film. Panel **(A)** shows the results with Cut and panel **(B)** shows the results with Cux.

A



	1	2	3	4	5	6	7
Transfected plasmids:	Probe	---	CUT	CUT	CUT	CUT+SV40LT	CUT+SV40LT
NS Ab			+				
CUT W3 Ab				+			
SV40 LT Ab							+

B

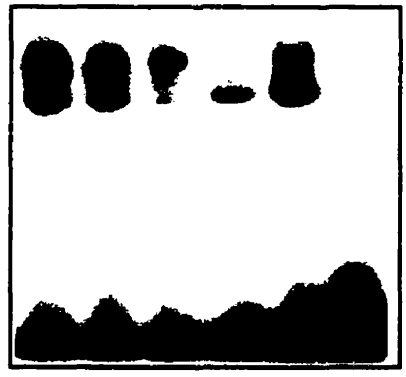
	1	2	3	4	5	6
Transfected plasmids:	CUX	CUX	CUX	CUX+SV40LT	CUX+SV40LT	Probe
NS Ab	+					
CUT W3 Ab		+				
SV40 LT Ab					+	

The gel-shift depicted in panel A of Figure 3.15 illustrates that there was an increase in DNA binding by endogenous murine Cut (mCut) in extracts from NIH 3T3 cells that were transfected with *SV40 LT* and then serum starved for one day (lanes 1 and 2). Since endogenous Cut still bound to DNA in extracts from these cells (thereby suggesting that most of the cells were not synchronized in G0/G1), the same experiment was performed but this time the cells were serum starved for two days. DNA binding by endogenous murine Cut was not detected in extracts from untransfected cells or from cells transfected with *SV40 LT* (panel B, Figure 3.15). Since the dark bands present in all lanes were not diminished by addition of a Cut antibody, these bands can likely be attributed to non-specific binding. Two bands were also seen in panel A of Figure 3.15 and the lower band was not diminished by addition of a Cut antibody (lane 4). Although further experiments must be performed, these results suggest that transfected SV40 LT may have a stimulatory effect on DNA binding by endogenous murine Cut in cycling cells, but not in cells synchronized in G0/G1.

Finally, DNA binding by endogenous murine Cut was examined in the SV40-transformed cell line SV-T2. Panel A of Figure 3.16 shows a gel-shift with SV-T2 and Balb/c/3T3 extracts derived from two different plates of cells. There was an increase in DNA binding by endogenous murine Cut (mCut) in SV-T2 cell extracts (lanes 2 and 3) in comparison to the control Balb/c/3T3 cell extracts that do not contain SV40 LT (lanes 5 and 6). In order to determine if these increases were attributed to higher levels of mCut protein in the SV-T2 cell extracts, Western blotting was performed. Panel B of Figure 3.16 is a Western blot for mCut in the extracts shown from panel A. The amount of mCut in the SVT2-2 and Balb-2 extracts was similar thereby suggesting that the increase in DNA binding by mCut in the

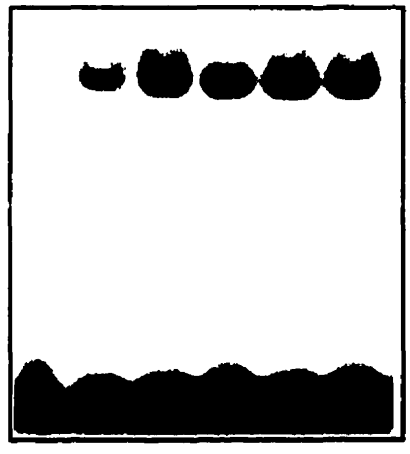
Figure 3.15 Gel-shift assay for Cut DNA binding with extracts from NIH 3T3 cells transfected with SV40 LT and serum-starved. NIH 3T3 cells were transfected with 6 μg of the *SV40 LT* plasmid (SVLT) as described in Materials and Methods. Then, cells were serum-starved in 0.4% serum for one day **(A)** or for two days **(B)**. Twenty micrograms of each protein extract was incubated with 1 μl of the radiolabeled FP probe. Some protein samples were pre-incubated with 1 μl of a non-specific antibody (NS) or a carboxy-terminal monoclonal Cut antibody (W3) before addition of dI-dC and probe. The probe samples were included as negative controls and did not contain any protein. SV40LT-1 and SV40LT-2 in **(A)** represent two different extracts from two different plates of cells transfected with SV40 LT. Samples were electrophoresed on a 5% polyacrylamide gel at 100 V for 3 hours. The gel was dried and exposed to film.

A



	1	2	3	4	5	6
Transfected plasmids:	SV40LT1	SV40LT2	---	---	---	Probe
NS Ab					+	
CUT W3 Ab				+		

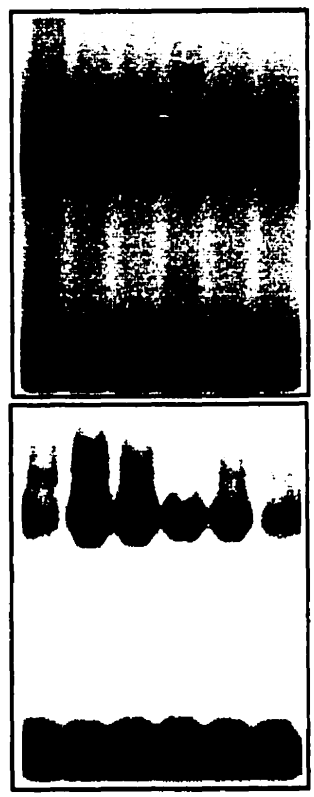
B



	1	2	3	4	5	6
Transfected plasmids:	Probe	SV40LT	---	---	---	---
NS Ab					+	
CUT W3 Ab				+		

Figure 3.16 (A) Gel-shift assay for Cut DNA binding with extracts from Balb/c/3T3 and SV-T2 cells. Protein extracts were prepared from two 100 mm plates of SV-T2 and Balb/c/3T3 cells. SVT2-1 and SVT2-2 represent the two different SV-T2 protein extracts and Balb-2 and Balb-3 represent the two different Balb/c/3T3 protein extracts. Balb/c/3T3 cell extracts were included as negative controls for extracts from SV-T2 cells. Twenty micrograms of each protein extract was incubated with 1 µl of a diluted (1:10) radiolabeled FP probe. Some samples were pre-incubated with 1 µl of a Cut monoclonal carboxy-terminal antibody (W3) before addition of the probe. Samples were electrophoresed on a 5% polyacrylamide gel at 100 V for 3 hours. The gel was dried and exposed to film. Two different exposures are shown. **(B) Western blot for Cut in Balb/c/3T3 and SV-T2 cell extracts.** Fifty micrograms of each protein extract described in **(A)** was electrophoresed on a SDS-6% polyacrylamide gel, transferred to a membrane and Western blotted with an amino-terminal antibody for Cut.

A

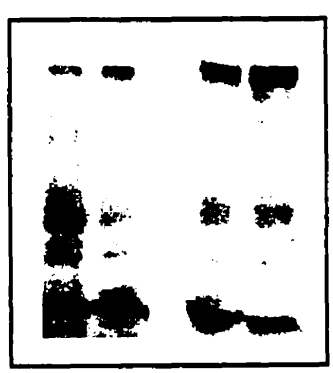


1 2 3 4 5 6

SVT2-2 SVT2-2 SVT2-1 Balb-3 Balb-3 Balb-2

CUT W3 Ab + + +

B



1 2 3 4

SVT2-2 Balb-2 Balb-3 SVT2-1

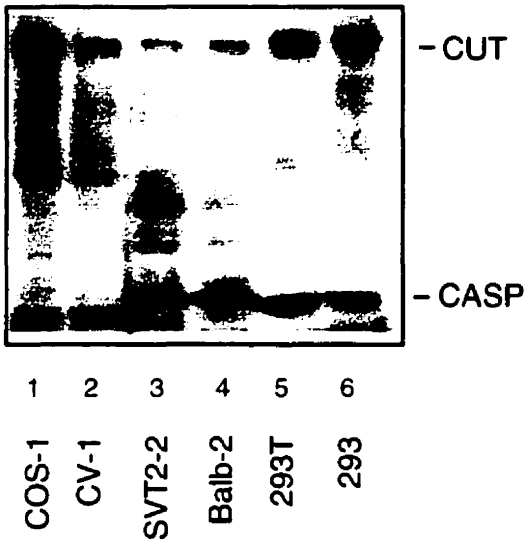
SVT2-2 extract was not attributed to an increase in mCut protein. However, there was an increase in the amount of mCut protein present in the SVT2-1 extract when compared to Balb-2 and Balb-3 extracts thereby suggesting that the increase in DNA binding by mCut in the SVT2-1 extract was attributed to an increase in mCut protein. Although further experiments must be performed, these results suggest that there may be an increase in DNA binding by endogenous mCut in some SV40 transformed mouse cells and this increase may be attributed to an increase in the amount of mCut protein.

The effect of SV40 LT on the levels of Cut protein in cells.

Increases in the level of and stability of the p53 protein have been observed in cells transformed with SV40 (140). In this study, there was an increase in endogenous murine Cut in NIH 3T3 cells transfected with *SV40 LT* as compared to untransfected cells (panel A in Figure 3.8). In addition, in some SV-T2 (Figure 3.16) and in some COS-1 (Figure 3.17) cell extracts, there was an increase in the levels of endogenous murine Cut and simian Cut respectively as compared to the levels in the comparable untransformed cells.

It was originally proposed that binding of SV40 LT to p53 was responsible for the increase in level of and stability of p53, however now it has been suggested that this effect is a consequence of the SV40 transformed phenotype (162). Therefore, it is possible that transformation by SV40 could have a similar effect on Cut. The effect that SV40-transformation (or infection) may have on Cut is important to consider when interpreting these

Figure 3.17 Western blot for Cut in extracts from cells transformed with SV40 LT and in untransformed control cells. Extracts were prepared from COS-1, CV-1, 293T and 293 cells as described in Materials and Methods. The SVT2-2 and Balb-2 extracts were previously described in Figure 3.16. Fifty micrograms of each protein extract was electrophoresed on a SDS-6% polyacrylamide gel. The gel was transferred to a membrane and Western blotted with an amino terminal antibody for Cut as described in Materials and Methods.


- CUT
- CASP
1
2
3
4
5
6
COS-1
CV-1
SVT2-2
Balb-2
293T
293

results and the results in the previous sections on DNA binding since the presence of an interaction between Cut proteins and SV40 LT was not confirmed in any of these studies. In other words it is possible that Cut and SV40 LT do not interact *in vivo*, however, Cut function may be altered as a consequence of the SV40 transformed phenotype.

3.4 DISCUSSION

The results presented in this study demonstrate that the SV40 LT antigen interacts with the homeodomain region of the human Cut protein *in vitro* (Figure 3.2, panel B). However, at this time there is not any convincing evidence suggesting that Cut and SV40 LT interact *in vivo*. The Cut homeodomain interacted with baculovirus-produced SV40 LT suggesting that there is a direct interaction between Cut and SV40 LT and that other cellular factors are not needed for this interaction (Figures 3.2-3.4). Since it was recently reported that Cut may form DNA binding protein complexes with members of the pRb family (61), it was necessary to prove that the SV40 LT preparation was pure. Examination of the purified protein preparation of SV40 LT by silver staining confirmed that pRb was not present and therefore suggests that the interaction between Cut and SV40 LT is not mediated by the Rb protein (Figure 3.5). In addition, since SV40 LT and Cut are both DNA binding proteins it was also necessary to rule out the possibility that the interaction between SV40 LT and the Cut homeodomain depended on contaminating DNA (21, 171). The interaction between the homeodomain and SV40 LT was stabilized in the presence of DNA since the amount of SV40 LT bound decreased as the volume of DNAse increased (Figure 3.4). This observation is similar to previous results reported on the Bcd protein since Bcd molecules interact less efficiently in the presence of ethidium bromide (23, 24).

The homeodomain also interacted with SV40 LT from SV-T2 and COS-1 cell extracts (Figure 3.6) confirming that the interaction between Cut and SV40 LT was not dependent on one source of SV40 LT. These findings are more physiologically relevant since the Cut fusion proteins were required to interact with SV40 LT in the presence of many other proteins.

However, this interaction should be confirmed by performing experiments with both full length proteins. This study is the first reported account of an interaction between a homeodomain and a DNA tumor virus oncoprotein. Interaction with a DNA tumor virus oncoprotein can be interpreted to mean that the interacting protein has a role in regulating cellular proliferation (89) since DNA tumor virus oncoproteins target and inactivate such proteins in order to force cells into S phase.

The consequences of an interaction between Cut and SV40 LT on DNA binding and transcriptional repression by Cut were investigated. It has been shown that binding of SV40 LT to wild-type p53 inhibits sequence specific DNA binding and transcriptional activation by p53 (87, 88) and binding of SV40 LT to pRb blocks transcriptional repression by pRb (83, 86). It was similarly hypothesized that SV40 LT may influence DNA binding and transcriptional repression by Cut. More specifically, it was proposed that SV40 LT may stimulate DNA binding (and therefore, transcriptional repression) by Cut since Cut is only active in S phase and DNA tumor virus oncoproteins attempt to force cells into S phase. There is some evidence suggesting that SV40 LT may increase DNA binding by Cut, however, further experiments are needed before any conclusions can be drawn from these results. At least in some cases, DNA binding by exogenous Cut was elevated in proliferating cells transfected with either *cut* or *cux* and *SV40 LT* (Figure 3.14) and DNA binding by endogenous murine Cut was elevated in cells transfected with *SV40 LT* (Figure 3.15, panel A). In addition, DNA binding

by endogenous murine Cut (mCut or Cux) in some cells transformed with SV40 (SV-T2) was elevated in comparison to untransformed Balb/c/3T3 cells (Figure 3.16). However, transfected SV40 LT did not stimulate DNA binding by Cut in cells synchronized in G0 (Figure 3.15, panel B).

Although there is some evidence suggesting that SV40 LT can influence the levels of DNA binding by Cut, it is possible that SV40 LT could affect DNA binding in other ways. For example, it has been proposed that interactions mediated by homeodomains may confer DNA binding specificity *in vivo* (17, 18). If the Cut homeodomain has a similar role in determining DNA binding specificity, it would make sense that the DNA tumor virus proteins would target this region of Cut in an attempt to inactivate the protein. If this were the case, levels of DNA binding would not necessarily be altered by an interaction with SV40 LT but the ability of Cut to bind to specific DNA sequences would be affected.

The ability of the Cut homeodomain to mediate protein interactions also suggests that Cut may bind other proteins via its homeodomain and that these interactions may be important to the function of the Cut protein. Similar to the case with pRb and E2F (E2F and the viral proteins bind to the same region of pRb), it is possible that SV40 LT blocks interactions between Cut and other proteins by occupying protein binding sites in the homeodomain. This could interfere with the function of Cut, or it could interfere with the normal activity of proteins that complex with and regulate Cut. For example, it has been suggested that Cut may interact with pRb and regulate pRb in an E2F-independent manner (61). Binding of SV40 LT to Cut could prevent Cut from regulating pRb and this would not be reflected in changes in Cut DNA binding or transcriptional repression.

The effect of the interaction between SV40 LT and Cut on transcriptional repression by Cut was also investigated. In this study, SV40 LT activated the *tkCAT* reporter gene, Cut and Cux repressed the *tkCAT* reporter gene as expected, but Cux and Cut no longer repressed *tkCAT* when transfected with *SV40 LT* (Figure 3.12). However, it was not possible to conclude if this was a direct effect on Cut and Cux, or a cancelling out effect between Cut/Cux mediated repression and SV40 transactivation. SV40 LT has been shown to activate transcription from virtually all promoters studied (176) which makes these types of experiments difficult to interpret. The study that investigated the effect of SV40 LT on pRb repression also utilized the *tkCAT* promoter (83). Cells were transfected with 1.5 μg of GAL4- *tkCAT* and 2 μg of *Rb* plasmids with increasing amounts (0.1-0.9 μg) of the *SV40 LT* plasmid. When as little as 0.9 μg of the *SV40 LT* plasmid was transfected, transcription of the *tkCAT* reporter gene was activated. In the present study, 6 μg of *SV40 LT* plasmid was transfected. Future experiments may produce results that are easier to interpret if the amount of SV40 LT transfected is decreased. In addition, *SV40 LT* plasmids encoding proteins that are defective in transactivation that have been developed in other laboratories can also be used (176).

The inability to show a convincing *in vivo* interaction between Cut and SV40 LT raises the issue of the biological significance of the *in vitro* results. There is some evidence suggestive of an interaction *in vivo*, however further experiments must be performed before any conclusions are drawn from these results. For example, SV40 LT antibodies immuno-precipitated a band that co-migrated with Cut from ^{35}S-labelled 293T cells that had been

transfected with Cut and SV40 LT (Figure 3.7) and from SV-T2 cells (Figure 3.10). In addition, a band that co-migrated with SV40 LT was immunoprecipitated with Cut antibodies from SV-T2 cells, however, it is likely that SV40 LT stuck to the protein A beads (Figure 3.11).

There are many credible explanations that may account for the inability to show that Cut and SV40 LT interact *in vivo*. For example, it is possible that other protein factors present in certain cells may stabilize or destabilize this interaction in different cell types. Some evidence supporting this hypothesis is that the interaction between Polyoma Large T (LT) and Cux was only detected in leiomyomas and breast tumors but not in testes tumors (67, 125). The finding that Cut bound to baculovirus-produced SV40 LT suggests that other protein factors may destabilize or prevent this interaction in some cases. In fact, it has been shown that an unidentified 193 kDa protein was immunoprecipitated with SV40 LT antibodies from AT-2 cardiomyocytes which do not express pRb (169). Therefore, it is possible that pRb binds to SV40 LT in the same region that Cut binds and that pRb must be absent in order for Cut and SV40 LT to interact. On the other hand, since an interaction between Cut and pRb has been detected (61) it is possible that pRb binds Cut via the homeodomain and prevents Cut from interacting with SV40 LT. These hypotheses can be tested by determining the binding site of Cut on the SV40 LT antigen, and the pRb binding site on the Cut protein.

It is also possible that the interaction between Cut and SV40 LT is dependent on the phosphorylation state of Cut and SV40 LT. At least in the case of pRb, it has been shown that only hypophosphorylated pRb binds SV40 LT. If this is also the case with Cut, this suggests that only the unphosphorylated Cut protein from cells in G0/G1 may be able to bind to

SV40 LT. Finally, some more general explanations that may account for the inability to identify an *in vivo* interaction between Cut and SV40 LT may be that: a) endogenous Cut expression is quite low; b) the Cut protein is large (which makes it highly susceptible to degradation); and c) the lysis buffers used were either too stringent to detect interactions or too weak to extract enough Cut protein from the nucleus.

In the absence of an interaction between Cut and SV40 LT *in vivo*, it is possible that the process of infection and/or transformation of cells by SV40 will have an effect on Cut. Although the presence of an interaction between Cut and SV40 LT was not confirmed in the studies investigating the effect of SV40 LT on DNA binding by Cut, there is some evidence that SV40 LT stimulated Cut DNA binding. In addition, there was an apparent increase in the level of endogenous Cut protein in cells transiently transfected with *SV40 LT* (Figure 3.8) and in SV40 transformed cell lines (Figures 3.16, 3.17). Conversely, murine Cut (mCut or Cux) may affect the level or stability of SV40 LT since noticeable expression of transiently transfected SV40 LT was only observed in cells also transfected with *cux* (Figures 3.7, 3.8). With this in mind, it is worthwhile to infect cells with SV40 and to investigate the effects that infection and/or transformation could have on the function of Cut.

CHAPTER 4

CONCLUSIONS, SUMMARY, AND FUTURE WORK

The *cut* gene has been shown to play an important role in determining cell fate in *Drosophila* (75, 76, 77). Although the biological function of *cut* genes in mammals has not been determined, it is expected that the human *cut* gene (CUTL1) also plays a role in determining cell type specificity. Over the past few years, indirect evidence suggesting that the CUTL1 gene is a tumor suppressor gene has been presented. In this study, functional assays were used to evaluate the abilities of human Cut to: a) suppress oncogene-mediated cell transformation, and b) interact with the SV40 Large T (LT) antigen.

Human Cut suppressed E1A/Ras and Myc/Ras cell transformation in the REF assay. These results could be confirmed by performing reversion assays. For example, transformed cells could be transfected with *cut* plasmids and reversion of these cells to a "normal" phenotype could be monitored. The mechanism by which human Cut suppressed transformation in the REF assay is unknown. Since Cut proteins repress transcription from a number of promoters, it is possible that human Cut suppressed transformation by repressing the promoters driving the oncogenes. Since two of the c-*myc* plasmids used in this study contain the endogenous c-*myc* promoter which is known to be repressed by hCut (49), it is possible that human Cut may have suppressed cell transformation by repressing the promoter in these

two cases. However, human Cut may have suppressed cell transformation by different mechanisms in the other cases. The ability of human Cut to repress the promoters driving the oncogenes used in this study can be investigated further by performing Northern or Western analyses.

All the transformed cell lines transfected with *E1A*, *ras*, and *cut*, expressed exogenous human Cut RNA and protein. Previous results from our laboratory indicate that high expression of Cut is incompatible with normal cell growth (67). However, in this study the transfected hCut proteins were not grossly mutated and they appeared to bind DNA in most cell lines tested thereby suggesting that high expression of Cut is compatible with the growth of transformed cells. It is important to note, however, that these experiments should be repeated in cells synchronized in G1/S because Cut is only able to bind to DNA and repress transcription during these phases of the cell cycle. It is possible, as has been suggested for the hsp70 protein (129), that very high threshold levels of Cut are needed to suppress transformation and that this threshold was not reached in surviving cells or that surviving cells may be derived from a small fraction of cells that are insensitive to suppression mediated by Cut.

On the other hand, the finding that transfected hCut proteins from some cell lines seemed to be unable to bind DNA suggests that the exogenous Cut protein was not functional in some cell lines. It is possible that the hCut proteins are complexed with E1A and inactivated by this interaction in these cells. This possibility can be addressed by performing co-immunoprecipitation experiments. Alternatively, exogenous Cut proteins may be mutated and

these mutations could either inactivate the proteins or, as is the case with p53, may cause them to act like oncoproteins. In the future it will be necessary to screen for mutations in the exogenous *cut* genes expressed in these cell lines and to design experiments to determine if normal cells can tolerate high levels of Cut.

Since tumor suppressor proteins interact with and can be inactivated by viral oncoproteins, an interaction with a DNA tumor virus protein is an indication that a protein plays a role regulating cell growth (89). The murine Cut protein (mCut or Cux) was previously shown to interact with the polyomavirus Large T antigen in uterine leiomyomas and breast tumors from mice transgenic for the MMTV/LT construct (67, 125). In this study, the human Cut homeodomain interacted *in vitro* with the SV40 Large T antigen. Although this interaction should be confirmed by performing experiments with both full length proteins, this is the first reported account of an interaction between a homeodomain and a viral oncoprotein. Furthermore, this is the first time that a Cut homeodomain has been shown to interact with other proteins. These results are consistent with other recent findings that the homeodomain is involved in protein-protein interactions *in vitro* and *in vivo* (18, 19, 21, 23, 24).

It was proposed that SV40 LT may stimulate DNA binding (and therefore transcriptional repression) by Cut since Cut is only active in the G1/S phases of the cell cycle and DNA tumor virus oncoproteins force cells into S phase. The effect of this interaction on transcriptional repression by Cut could not be determined in this study. Future experiments using decreased amounts of *SV40 LT* plasmid, or using plasmids encoding SV40 LT mutants that are unable to activate transcription should be performed. There is some evidence that DNA binding was elevated in proliferating cells transfected with *cut* or cux, and *SV40 LT* and

that endogenous murine Cut DNA binding was elevated in proliferating cells transfected with *SV40 LT*. In addition, DNA binding by endogenous murine Cut in some cells transformed with SV40 virus (SV-T2) was elevated. At least in some cases, this elevation could be attributed to an increase in the amount of Cut protein but this must be confirmed. However, DNA binding was not stimulated in cells transfected with *SV40 LT* and synchronized in G0. On the other hand, SV40 LT may affect the ability of Cut to bind to specific DNA sites since protein interactions mediated by residues in the homeodomain have been shown to be important to DNA binding specificity (17, 18).

It is important to note that the presence of an interaction between Cut proteins and SV40 LT was not confirmed in any of these experiments investigating the effect of SV40 LT on DNA binding. In fact, *in vivo* interactions between Cut proteins and SV40 LT were not detected in this study. It is possible that such an interaction will be detected in the future, for example, if: a) immunoprecipitation of Cut is improved; b) a better lysis buffer is found; c) results indicate that the interaction between Cut and SV40 LT is dependent on phosphorylation status or phases of the cell cycle; or d) it is shown that the presence of other proteins or the binding of other proteins to Cut (such as pRb) can inhibit or promote the interaction. In the event that SV40 LT does not interact with Cut proteins *in vivo*, it is possible that changes in DNA binding and transcriptional repression by Cut proteins could be a consequence of SV40 infection and/or transformation.

The results presented in this study are relevant even if interactions between Cut proteins and SV40 LT are not detected *in vivo* since they provide evidence for a role of the Cut homeodomain in mediating protein interactions that may have functional significance. As was the case with the retinoblastoma protein, identification of proteins that interact with Cut could give insight into the normal function of Cut as well as its potential role in the development of cancer.

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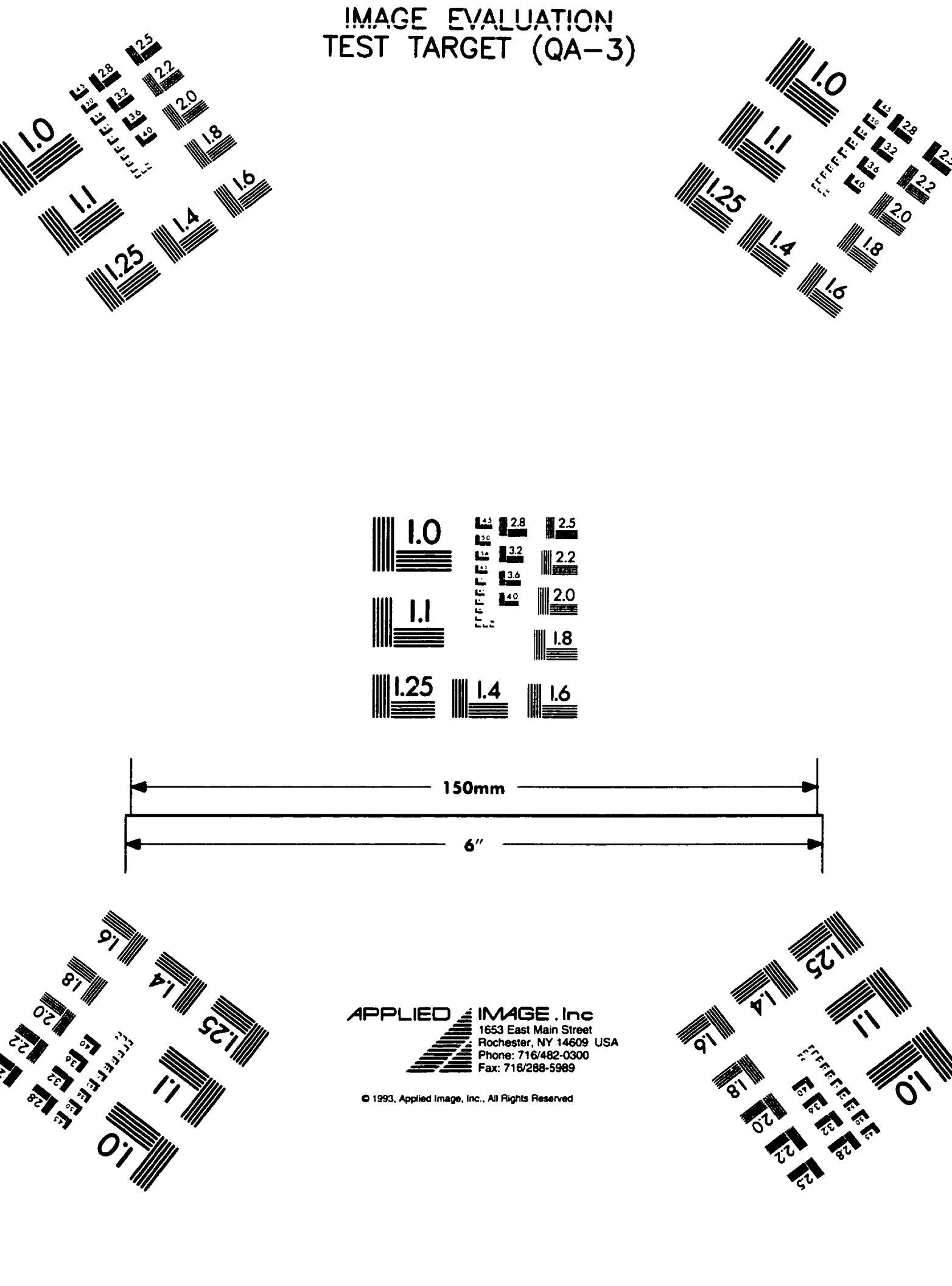
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