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Regulation of HIV-1 TAT pre-mRNA splicing

Martha A. E. Klosevych

Thesis submitted to
the Department of Biochemistry, Microbiology, and Immunology in partial fulfilment of the
requirements for the degree of Masters of Science

University of Ottawa
Ottawa, Ontario, Canada
2000

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ABSTRACT

Pre-mRNA splicing has been shown to be regulated in a cell- and tissue-specific as well as a developmentally specific manner. A substantial number of proteins have been identified that play a specific role in pre-mRNA processing. Included in these, are serine/arginine-rich (SR) proteins, which are thought to be involved in the regulation of splicing. SR protein activity is believed to be modulated by their state of phosphorylation. A family of autophosphorylating dual-specific nuclear kinases termed Clk (CDC2/CDC28-like kinases) have been shown to interact with, phosphorylate, and cause the nuclear redistribution of, SR proteins. Previous studies in our laboratory have shown that catalytically active Clks influence their own pre-mRNA splicing and that active Clks also influence the alternative splicing of an E1A adenovirus expression vector *in vivo*.

Here, we describe the development of a triple primer RT-PCR assay to measure the spliced and unspliced RNA transcripts produced from the HIV-1 TAT expression vector pgTAT. The splicing of this vector has previously shown to be influenced by the SR protein ASF/SF2 in both *in vitro* and *in vivo* studies. Using the RT-PCR approach, we show that the over expression of any of the four known human full-length Clks (hClk1 to 4) does not alter the RNA ratio of spliced to unspliced TAT transiently or stably expressed in 293T cells. As well, by this approach, we show that the splicing of this same vector, stably transfected in P19 embryonal carcinoma cells, is altered upon neuronal and endodermal differentiation with retinoic acid or dimethyl sulfoxide respectively, whereby differentiation caused an increase in the unspliced transcript.

DEDICATION

I would like to dedicate this thesis to my parents and my brother Michael for their continuous support and dedication to my life.

Portrait of a Life

A portrait of my life
Hangs before me now.
It was painted by the Master –
Each stroke made with love.
To date it remains unfinished,
Yet worked on day by day,
Continually unfolding,
Revealing my divine destiny.
Only the Master will complete
The portrait of my life,
For only the Master knows
The stokes yet to be made.

Elaine Meyer

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To my best friend John Holt for his words of encouragement during my ups and my downs, for his patience and understanding, and for his inspirational whispers.

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LIST OF ABBREVIATIONS

α -MEM	alpha-minimal essential media
aa	amino acid
ASF	alternative splicing factor
bp	base pair
cDNA	complementary DNA
Clk	CDC2/cdc28-like kinase
Clk-GFP	green fluorescent protein tagged Clk
CMV	Cytomegalo virus
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DOA	<u>D</u> arkener of <u>A</u> pricot
DSK	dual specific kinase
DTT	dithiothreitol
EB	exon B of Clk
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
GFP	green fluorescent protein

GITC	guanidinium isothiocyanate
hClk	human Clk
HIV-1	human immunodeficiency virus-1
kb	kilobase pair
LTR	long terminal repeat
min	minute
mRNA	messenger RNA
NLS	nuclear localization signal
nt	nucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pEGFP	plasmid enhanced green fluorescent protein
PP1/PP2	protein phosphatase
pre-mRNA	precursor mRNA
RNA	ribonucleic acid
RS domain	arginine/serine-rich domain
RT-PCR	reverse transcriptase coupled with PCR
S	sedimentation constant
SF2	splicing factor 2
snRNP	small nuclear ribonucleoprotein particle
SR protein	serine/arginine-rich protein

SRPK	SR protein kinase
TAT	transactivator of transcription
U2AF U2	snRNP auxiliary factor

1. INTRODUCTION

1.1 MESSENGER RNA FORMATION

In eukaryotic cells, the primary messenger RNA transcript (pre-mRNA) of protein-coding genes, synthesized by RNA polymerase II (pol II), must undergo a number of extensive modifications in order to become mature, functional messenger RNA (mRNA). These modifications include capping at the 5' end, cleaving and polyadenylation of the 3' end, and the removal of intervening non-coding (intronic) sequences between exons (mRNA splicing). The 5' cap is important in protecting the RNA transcript from degradation and is necessary for protein synthesis. Basically, the 5' end is covalently linked by a triphosphate bond to a methyl guanosine residue. The polyadenylation is believed to protect the transcript from RNase degradation and aid in its nuclear export. These modifications must be timely and precise. The processed transcripts are then rapidly transported to the cytoplasm by RNA export factors where they can be used as template for protein translation by the ribosomal machinery (reviewed in Blencowe *et al.*, 1999).

1.2 mRNA SPLICING

The process by which introns are excised from the pre-mRNA transcripts is known as RNA splicing and occurs in the nucleus, before the transcript is transported to the cytoplasm. The number of introns varies considerably from one pre-mRNA to another. The schematic in figure 1 depicts the requirements within the pre-mRNA transcript for splicing as well as the general events leading to RNA splicing. The critical transcript sequences involved in pre-mRNA processing include the 5' splice site (5'ss) or donor site at the beginning of the intron, the branch site adenosine (A) located

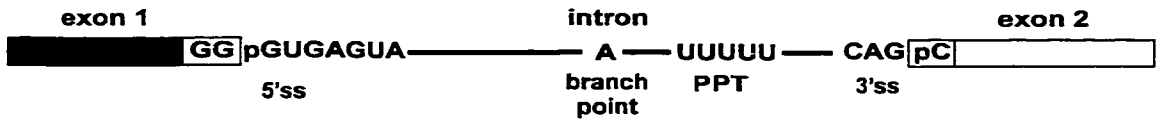
18 - 40 nucleotides (nt) upstream of the 3' splice site (3'ss), the polypyrimidine tract (depicted as "Us"), and the 3' ss or acceptor site of the intron. RNA splicing is critical for the proper expression of eukaryotic genes. Without correct 5' and 3' splice site pairing, there could be a loss of the correct reading frame and production of nonfunctional proteins (Black, 1995).

RNA splicing is a successive two-step enzymatic transesterification reaction (reviewed in Lewin, 1994). In the first step, the 2'-hydroxyl (-OH) group of the branch point adenosine initiates cleavage at the 5' donor splice site thus producing a phosphodiester bond between the adenosine and the 5' end of the intron and a branched lariat-exon 2 intermediate. In the second step, the free 3'-OH group of exon 1 initiates the cleavage at the 3' acceptor splice site thus ligating the two exons and releasing the branched lariat intron.

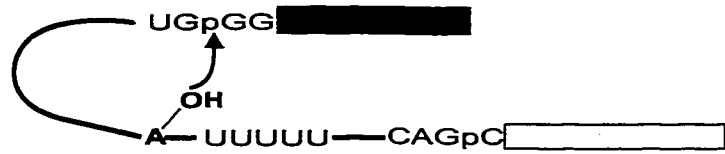
Figure 1 Mechanism of pre-mRNA splicing.

Precursor messenger RNA splicing is a successive two-step enzymatic transesterification reaction. The critical transcript sequences involved include the 5' splice site (5'ss), the branch site adenosine (A), the polypyrimidine tract (depicted as "Us"), and the 3' splice site (3'ss). In the first step, cleavage at the 5'ss is initiated by the 2'-hydroxyl (-OH) group of the branch point adenosine producing a phosphodiester bond between the adenosine and the 5' end of the intron as well as a branched lariat-exon 2 intermediate. In the second step, the free 3'-OH group of exon 1 initiates the cleavage at the 3'-ss thus ligating the two exons and releasing the branched lariat intron. (Modified from Moore et al. (Moore *et al.*, 1993))

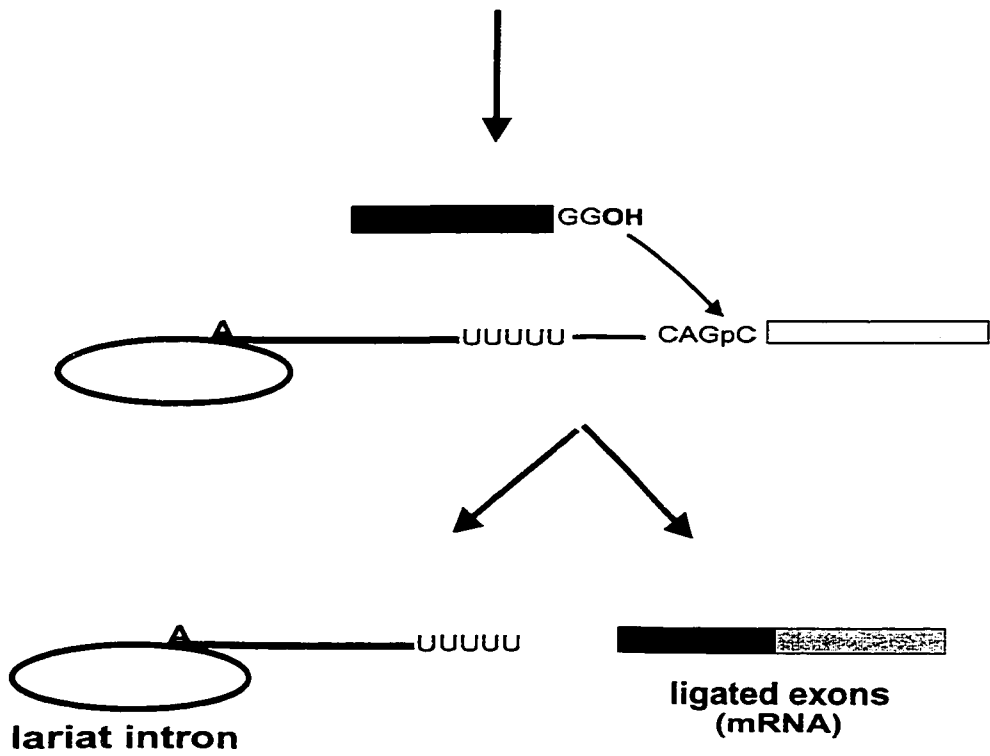
pre-mRNA



Step 1



Step 2



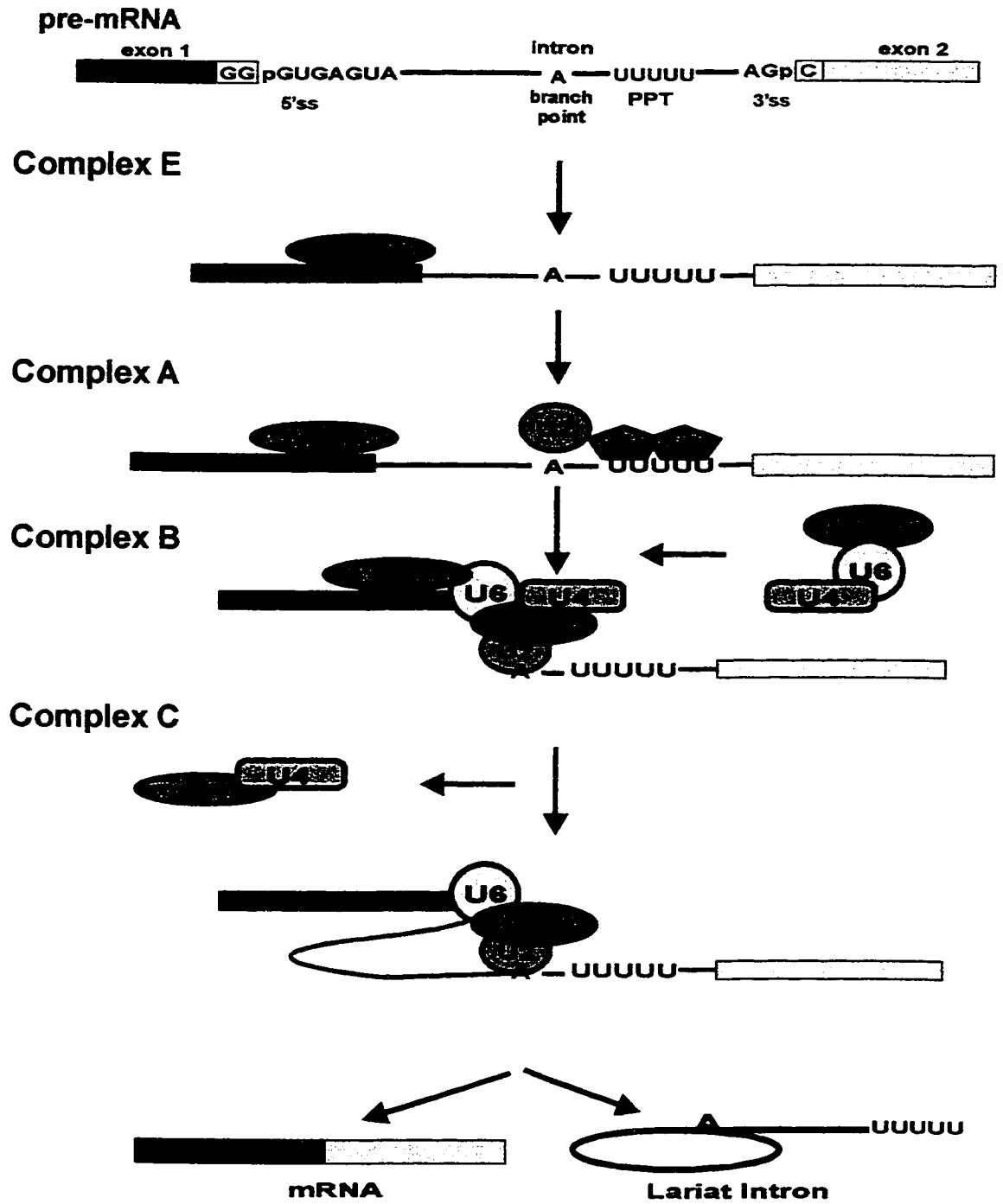
1.3 SPLICING WITHIN THE SPLICEOSOME

Our understanding of RNA splicing has made significant progress in recent years. This complex process requires that a number of cellular components come together in the right sequence and at just the right time in order to catalyse the reaction. Numerous RNA-RNA, protein-RNA, and protein-protein interactions determine the precision and efficiency of the splicing reactions. Over 50 proteins are essential for constitutive splicing (Mayeda *et al.*, 1999). The cellular machinery involved in this reaction forms a large 50-60S macromolecular enzymatic complex commonly referred to as the spliceosome. The major components of the spliceosome are the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6, and non-snRNP protein factors (Staley and Guthrie, 1998). These molecules interact with each other and the intron and are believed to allow the correct structure to form thus enabling the splicing reaction to occur (Lewin, 1994).

As their name implies, snRNPs are composed of small nuclear RNA (snRNA) as well as protein components (Guthrie and Patterson, 1988). Figure 2 depicts the splicing reaction with the components that recognize the consensus sequences. The U1 snRNP recognizes the 5' splice site and binds to it through its RNA component thus forming the first commitment complex, complex "E". Then, the U2 snRNP auxiliary factor (U2AF) binds to the polypyrimidine tract which in turn stimulates the binding of the U2 snRNP to the branch point and forms the "A" complex. Next, the U4/U6 and U5 snRNPs associate to form a tri-snRNP complex and together bind to the pre-mRNA and form the "B" complex. A number of conformational changes follow, resulting in the binding of U5 to the

Figure 2 Schematic diagram summarizing the step-wise assembly of the spliceosome.

Sequence elements crucial for splicing are as described in figure 1 with various splicing factors added . The splicing reaction proceeds through discrete stages in which numerous protein components recognize the crucial sequence elements, interact with the pre-mRNA, and form the spliceosome. The U1 snRNP recognizes the 5' splice site and binds to it through its RNA component and forms the first commitment complex (complex E). Then, the U2AF binds to the polypyrimidine tract (UUUUUU), which in turn stimulates the binding of the U2 snRNP to the branch point (A) and forms complex A. Next, the U4/U6 and U5 snRNPs form a tri-snRNP complex which then binds to the pre-mRNA to form complex B. A number of conformational changes follow resulting in the binding of U5 to the exon at the 5' splice site, the subsequent release of U1 along with U4, the binding of U6 to U2, and the formation of complex C. At this point the spliceosome is catalytically active and the transesterification reactions can occur. (Modified from (Lewin, 1994)



exon at the 5'ss, the subsequent release of U1 along with U4, the binding of U6 to U2, and the formation of complex "C". At this point, the spliceosome is catalytically active and the transesterification reactions can occur. Once the ligated exons are released, remaining snRNPs bound to the lariat-intron intermediate dissociate and individual snRNPs are free to participate in other splicing reactions (reviewed in Stojdl and Bell, 1999 and Blencowe *et al.*, 1999).

1.4 ALTERNATIVE SPLICING

As well as constitutive splicing, where one 5'ss is paired with one 3'ss, there is alternative splicing where either one splice site (either 3' or 5') is used with multiple complementary splice sites or entire exons are removed or introns retained. Alternative splicing allows various mRNA transcripts to be produced from one single gene. Higher eukaryotes utilize alternative splicing to regulate gene expression and generate isoform diversity within individual genes. A single primary transcript can generate multiple proteins with distinct functions in a tissue- and/or developmental-specific manner. For example, the chicken cardiac troponin T (cTnT) gene has an alternatively spliced exon (exon 5) which is included in mRNAs in embryonic skeletal and cardiac muscle, and excluded in adult mRNAs (Stark *et al.*, 1999). In some cases, the multiple products are formed in the same cell, in other cases, the process is regulated so that particular splicing patterns occur only under particular conditions (Lewin, 1994). Patterns of alternative splicing can be simple or very complex. A simple model is the *bcl-x* gene, which produces two distinct mRNAs, *Bcl-xL* and *Bcl-xS*, coding for two isoforms (Boise *et al.*, 1993). One of the best described examples of a complex model of alternative splicing is that of the gene for the lymphocyte homing receptor CD44. This gene contains

20 exons of which 10 can be included or removed to produce over 1000 potential isoforms (Bell *et al.*, 1998). It has been shown that alternative splicing regulates a number of developmental processes. The hallmark example and most well understood is the sex determination pathway in *Drosophila melanogaster*. This pathway involves a number of interactions between genes in which alternative splicing events distinguish males from females (Hedley and Maniatis, 1991). The ratio of X chromosomes to autosomes is the first sex determining signal allowing for the activation of the early sex-lethal (*sxl*) promoter through the formation of transcriptional complexes. Exon 3 of the *sxl* gene contains a termination codon, which, when included, prevents the synthesis of functional protein. Only females produce the *sxl* protein, as this exon is retained in the mRNA produced in males and skipped in female produced mRNA. The *transformer* (*tra*) gene, the next in the sex-determining cascade, is spliced similarly in both males and females, however, the presence of *sxl* protein promotes female-specific splicing of *tra* pre-mRNA by blocking the utilization of the default *tra* 3' splice site, resulting in the exclusion of a complete exon and synthesis of a specific protein only in females. This *Tra* protein influences the splicing of *tra2*, which in turn affects the splicing of the *doublesex* (*dsx*) gene. *Tra* and *tra2* are required for female specific splicing of *dsx*. Basically, male products block female sexual differentiation and female products inhibit male-specific gene expression (Lewin, 1994 and Du *et al.* 1998).

1.5 ENHANCERS/SUPPRESSORS

Although the previously mentioned intronic sequences are necessary for the splicing reaction to occur, these are weakly conserved and not sufficient for splicing recognition and pairing. The

choice of 5' and 3' splice sites in both constitutive (Mayeda *et al.*, 1999, Schaal and Maniatis, 1999) and alternative splicing is believed to be regulated by various *cis*- and *trans*-acting factors. *Cis*-acting factors have been associated primarily to exonic sequences, however, recent discoveries have identified *cis*-acting factors within intronic sequences (Modafferi and Black, 1997). The exonic sequence factors can act either positively (exonic splicing enhancer (ESE)) or negatively (exonic splicing silencers (ESS)) in regulating splicing of the pre-mRNA. ESEs, the most characterized *cis*-acting sequences, are most often rich in purine residues, 6 to 13 nt in length, contain multiple repeats of the motif guanosine-adenosine-purine (GAR) (Xu *et al.*, 1993), and are located within 100 nt downstream of suboptimal 3' splice sites (suboptimal meaning not conforming to the consensus) thus favouring its usage (Bourgeois *et al.*, 1999, Lavigneur *et al.*, 1993, Sun *et al.*, 1993, Yue and Akusjarvi, 1999). ESSs on the other hand have little sequence homology (Zheng *et al.*, 1998) and suppress splicing by an unknown mechanism.

1.6 SR PROTEINS

As mentioned earlier the spliceosome is composed of snRNPs as well as non-snRNP proteins. Non-snRNPs assist in splice site selection both constitutive and alternative, organize the snRNPs, and regulate alternative splicing. The majority of non-snRNPs belong to a family known as SR proteins. These proteins take their name from the fact that they have a characteristic dipeptide serine/arginine repeat (RS domain) of variable length within their carboxyl-terminal sequence. As well, SR proteins involved in splicing have either one or two RNA binding domains (or RNA recognition motifs (RRM)) within their amino-terminus. The RS domain can be phosphorylated and

is involved in mediating protein-protein interactions, whereas, the RRM is involved in RNA sequence-specific binding (Wu and Maniatis, 1993, Xiao and Manley, 1997). There exists a number of "SR protein family members" (reviewed in Blencowe *et al.*, 1999) including; U2AF, the *Drosophila melanogaster* splicing regulators transformer tra-1 and tra-2, and the *Drosophila melanogaster* alternative splicing regulator suppressor of white apricot (SWAP) (Fu, 1995), however, the term SR protein is more commonly used to describe proteins that are immunoreactive with the monoclonal antibody mAb 104 and can activate splicing in a splicing deficient cytoplasmic extract (termed S-100 extract). ASF/SF2 (alternative splicing factor/splicing factor 2), the first identified SR protein, was discovered by two different groups. Ge and Manley (1990) discovered ASF as a factor being able to influence alternative 5'ss selection in viral pre-mRNA. SF2 was isolated through fractionation experiments (Krainer *et al.*, 1990b) and was identified by complementation assays as a factor essential for splicing. Subsequently, it was revealed that ASF was in fact SF2 (Ge *et al.*, 1991).

Nine human SR proteins are known, to date, and these include ASF/SF2, SRp20, SC35, SRp30c, 9G8, SRp40, SRp46, SRp55, and SRp75 (summarized in Blencowe *et al.*, 1999). SR proteins are highly homologous and may fulfill identical functions in some assays such as restoring splicing in S-100 extracts. Nevertheless, a number of groups have demonstrated that SR proteins are not functionally redundant. Individual SR proteins have different effects on the selections of alternative splice sites and recognize distinct RNA sequences (Cavaloc *et al.*, 1999). As well, Wang *et al.* (1996) have shown that the inactivation of ASF/SF2 in the chicken cell line DT40 was lethal

(Wang *et al.*, 1996) and Jumaa *et al.* (1999), using Cre-loxP-mediated recombination to inactivate the SRp20 gene, have shown that this gene was essential for mouse development.

1.7 SR PROTEINS WITHIN THE SPLICEOSOME

It has been demonstrated by others that SR proteins play a role in both constitutive splicing as well as alternative splicing. In constitutive splicing, SR proteins act early in the formation of the spliceosomal “E” complex by attracting the U1 snRNP to the 5'ss. SR proteins are also involved in forming bridges across introns and exons and thus bring spliceosomal factors into close proximity. As well, *in vitro* splicing assays (Fu *et al.*, 1992, Krainer *et al.*, 1990a) and *in vivo* transient transfections in cultured cells (Caceres *et al.*, 1994, Wang and Manley, 1995) have revealed that individual SR proteins can influence alternative splicing.

Figure 3 shows the splicing reaction with the addition of some SR protein interactions.

It has been proposed that SR proteins such as ASF/SF2 and SC35 bridge the interaction between the 5' and 3' splice sites, allow for the recruitment of additional snRNPs and non-snRNPs to the spliceosomal complex, and stabilize snRNP binding to the pre-mRNA transcript (Blencowe *et al.*, 1999). For example the SR protein ASF/SF2 binds to the same 5' splice site as the U1 snRNP and thus promotes U1 binding.

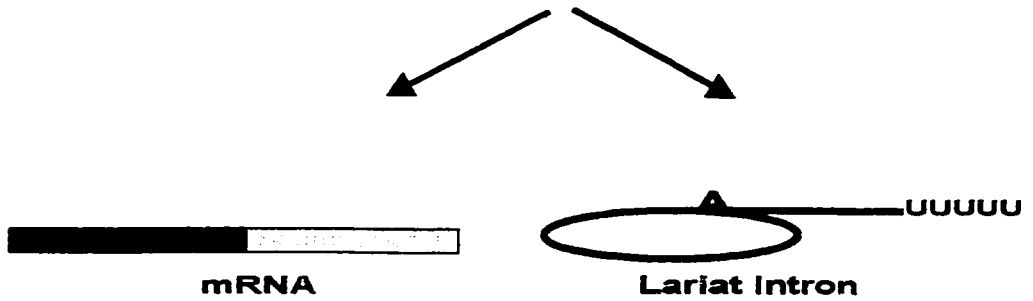
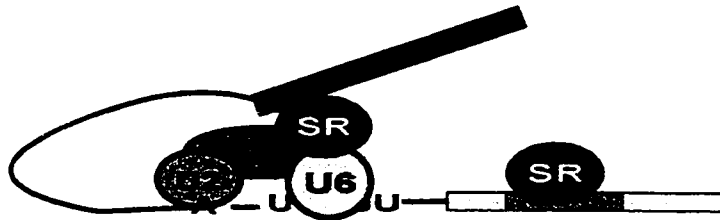
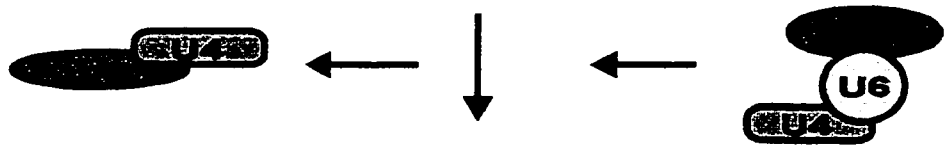
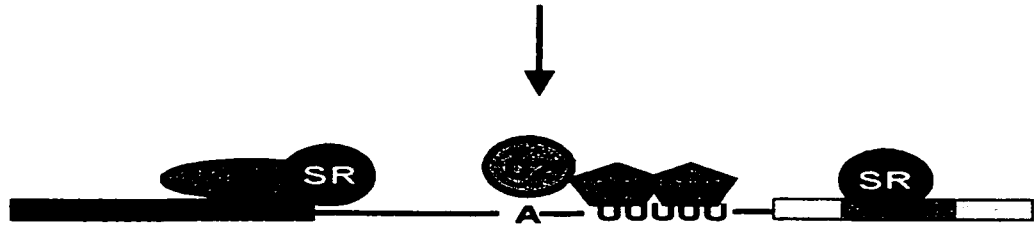
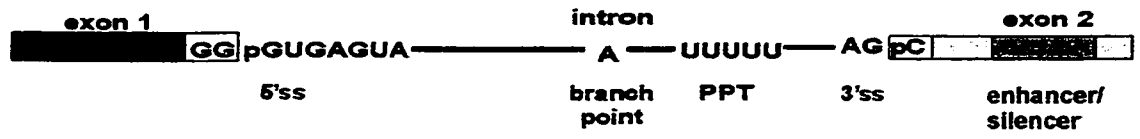
Through their RNA binding abilities and protein-protein interactions, it has been suggested that SR proteins allow the 5' ss, 3' ss, and branch points to come in proximity of each other, allowing for

the cleavage and ligation reactions to occur. A number of groups have identified binding sites for SR and SR-related proteins within intronic and exonic enhancer/silencer sequences. These sequences influence splicing through their binding of SR proteins. If an SR protein binds to enhancer sequences, it can facilitate splicing by recruiting other spliceosomal factors. Why the binding of SR proteins to silencers alters splicing still remains to be elucidated. It has been proposed that an SR protein-bound silencer represses splicing by limiting the amount of SR proteins available for splice site interaction or by preventing subsequent binding of appropriate snRNPs to the pre-mRNA transcript through steric hindrance (Zheng *et al.*, 1998).

Figure 3 Schematic representation of possible SR protein interactions within the spliceosome.

Sequence elements crucial for splicing are as described in figure 1 with a hypothetical enhancer/silencer element within exon 2 (darker rectangle). Enhancer/silencer elements may also be located within introns or other exons and have binding sites for SR and SR-related proteins. Through both their RNA and protein binding properties, it has been suggested that SR proteins allow the 5' splice site, 3' splice site, and branch points to come in proximity of each other. This favours the recruitment of additional snRNPs and non-snRNPs to the spliceosome, therefore allowing the splicing reaction to occur. (Adapted from Lewin, 1994 and Stojdl and Bell, 1999)

pre-mRNA



1.8 SR PROTEIN PHOSPHORYLATION AND ACTIVATION

SR proteins as well as most other splicing factors are unevenly distributed within the nucleus and concentrated in nuclear structures referred to as speckles. On average, the nucleus has between 20 and 40 speckles (Spector, 1993). Splicing occurs cotranscriptionally in the nucleoplasm and not in nuclear speckles. Speckles are thus considered to be sites of storage and/or assembly of SR proteins and other splicing factors. Misteli et al. (1997) have noticed that when transcription of a gene is activated, various splicing factors, including ASF/SF2, leave the speckles in peripheral extensions and accumulate at what appears to be the new site of transcription. It was later concluded that splicing factors need to be recruited from their sites of storage to sites of active transcription and splicing (Misteli *et al.*, 1998).

SR protein distribution and activity is believed to be regulated by cycles of phosphorylation and dephosphorylation. Overexpression of kinases that alter the phosphorylation state of SR proteins leads to the dispersal of SR proteins from speckles (Colwill *et al.*, 1996b). As well, phosphorylating SR proteins enhances their RNA-independent protein-protein interactions and prevents them from binding non-specifically to RNA (Xiao and Manley, 1997). A number of studies suggest that changes in the phosphorylation state of SR proteins plays an important role in both constitutive and alternative RNA splicing. In fact, a number of groups have shown that phosphorylation and dephosphorylation of SR proteins is required for splicing to occur *in vitro*, however, the mechanistic importance of SR protein phosphorylation remains unknown. When treated with phosphatase 1 (PP1), expressed in *Escherichia coli* (*E. coli*) or purified from rabbit

skeletal muscle, pre-spliceosomal complex “E” could not form in HeLa nuclear splicing extracts and the U2 and U4/U6/U5 snRNPs could not stably bind to the pre-mRNA transcript. The addition of purified phosphorylated SR splicing factors to the PP1-inhibited extracts restored spliceosome formation. As well, it was observed that when treated with phosphatase inhibitors, splicing complexes could form, but splicing of pre-mRNA would be blocked and only restored upon addition of PP1 or PP2A (Mermoud *et al.*, 1994).

1.9 SR PROTEIN KINASES

A number of SR protein kinases that phosphorylate RS domains, predominantly on serine residues, have been isolated from mammalian cells and subsequently identified. The first kinase to be reported was the U1 snRNP-associated kinase (Woppmann *et al.*, 1993). However, the cDNA encoding this kinase has not yet been isolated. Other kinases able to phosphorylate RS domains include DNA topoisomerase I (Rossi *et al.*, 1996), an RS domain kinase that is associated with a nuclear envelope subcomplex containing the lamin B receptor (Nikolakaki *et al.*, 1996), the SR protein kinase family (SRPK1 and SRPK2), and the CDC2/CDC28-like kinase (Clks) family (for a review see Misteli, 1999), the latter two being the best characterized. The physiological relevance of the lamin B receptor kinase and DNA topoisomerase I is not yet known. Although SRPK 1 and Clk1 share 32% homology at the amino acid level within their kinase domains, these two families are structurally distinct, exhibit different substrate specificities, and are differentially expressed (Colwill *et al.*, 1996a, Colwill *et al.*, 1996b). SRPK1, the first member of its family to be identified, shares great homology at the amino acid level and is closely related to SRPK2. Both

these kinases are expressed during interphase and upregulated during mitosis suggesting an important role during the cell cycle (Blencowe *et al.*, 1999). As well, both have similar enzymatic activity and substrate specificity, as overexpression of either leads to the redistribution of splicing factors from speckles.

The Clk family of kinases belongs to a group of proteins known as dual-specific kinases (DSK) and, as this name implies, are able to phosphorylate substrates on both tyrosine and serine/threonine residues both *in vitro* and *in vivo* (Duncan *et al.*, 1998). Murine Clk1 (also referred to as STY (serine-threonine-tyrosine) kinase) was the first DSK identified and the first of its family to be discovered. It was initially identified during an anti-phosphotyrosine antibody screen of P19 embryonal carcinoma (P19EC) cell and mouse erythroleukemia cDNA expression libraries in an attempt to discover new protein tyrosine kinases that may be involved in the differentiation process (Ben-David, 1991 *et al.*, Howell *et al.*, 1991). Subsequently, four mammalian Clk kinases (Clks 1 to 4) in mouse and human have been identified as well as counterparts in *Drosophila*, plants, and other species. Besides possessing dual specific kinase activity, most DSK family members identified to date are characterized by the peptide motif "EHLLAMMERILG" within their catalytic subdomain X (Yun *et al.*, 1994), and are therefore also referred to as LAMMER protein kinases (Lee *et al.*, 1996).

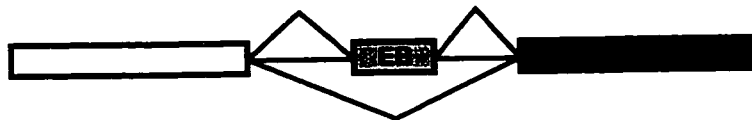
1.10 CLK KINASE FAMILY

All four Clk family members contain two major domains: a C-terminal kinase domain, for catalytic activity, and a N-terminal regulatory domain of about 156 aa with a putative nuclear localization signal (NLS) and a arginine/serine (RS)-rich non-catalytic region, the latter having been shown to be important for its association with SR proteins (or protein-protein interactions in general). The scheme in figure 4 represents the Clk pre-mRNA splicing and translated protein products. The Clk pre-mRNA possesses an alternatively spliced exon (EB). When this exon is included, full length, catalytically active Clk protein is produced, when it is excluded there is a frame shift and subsequent introduction of a premature stop codon upstream of the catalytic domain resulting in the production of a truncated catalytically inactive protein (Duncan *et al.*, 1997, Duncan *et al.*, 1998). It is believed that Clk can autoregulate itself, whereby increased expression of Clk protein influences splicing to generate transcripts encoding truncated Clk protein and increased expression of truncated Clk protein influences splicing to generate transcripts encoding catalytically active Clk protein (Duncan *et al.* 1997).

Figure 4 Schematic representation of the alternative splicing of Clk pre-mRNA and protein isoforms.

For simplicity, Clk pre-mRNA is represented by 3 exons (boxes) separated by introns (lines). The alternatively spliced exon is indicated by EB. Upon inclusion of this exon, full length, catalytically active Clk protein is produced, when it is excluded there is a frame shift and subsequent introduction of a premature stop codon resulting in the production of a truncated catalytically inactive protein. Full length Clk protein contains two major domains: a C-terminal catalytic domain and a N-terminal regulatory domain with a putative nuclear localization signal (NLS). (Adapted from Duncan *et al.*, 1997)

Clk pre-mRNA



Clk mRNA



Catalytically active protein



Truncated inactive protein

Natural substrates and biological functions for the Clk family of kinases remain unknown. Work performed on the only known LAMMER protein kinase in *Drosophila melanogaster* and Clk homologue, DOA (Darkener of Apricot) has suggested that Clk plays a critical role in development. The DOA kinase is required at many stages of development and influences sexual differentiation. As well, a loss of the DOA allele is embryonically lethal (Yun *et al.*, 1994). Recent work on murine Clk1 has implicated Clk in regulating mRNA splicing. Colwill *et al.* (1996) have demonstrated that mClk1 can phosphorylate SR proteins (specifically ASF/SF2) *in vitro*, at the same sites phosphorylated on these proteins *in vivo*. A yeast-two-hybrid screen has shown that Clk1 can interact with at least 5 RNA-binding proteins including three proteins belonging to the SR family of splicing factors; ASF/SF2 (SRp30a), X16 (SRp20), and SRp75 (Colwill *et al.*, 1996b). As well, other studies on DOA have also associated Clk to splicing. A mutation in the DOA protein alters the splicing of a pre-mRNA transcript encoding an important regulator of sex determination. Finally, transfection experiments, using Clk expression vectors, have shown that the Clk protein itself is also located in nuclear speckles and that just like SRPK family proteins, over-expressing any of the Clk proteins leads to a redistribution of SR proteins within the nucleus (Colwill *et al.*, 1996b, Duncan *et al.*, 1997, Duncan *et al.*, 1998). Caceres *et al.* (1998) noticed that upon overexpression of murine Clk1 there was an accumulation of the SR protein ASF/SF2 in the cytoplasm, suggesting that Clk's role in splicing may be through its regulation of SR protein activity and compartmentalization.

1.11 THESIS WORK

A number of model pre-mRNAs are commonly used in *in vitro* and *in vivo* experiments to study splicing and the effects of various splicing components on this process. We have chosen the human immunodeficiency virus-1 transactivator of transcription (HIV-1 TAT) expression vector pgTAT, a vector for which the effects of the SR proteins such as ASF/SF2 have been well documented. In the following experiments we proceeded to study the regulation of the TAT pre-mRNA splicing within two contexts. In the first part, we will examine the *in vivo* effects of the human Clk kinases (hClk1 to 4) on the expression of TAT mRNA in cells transiently and stably expressing this gene. Previous results observed with Clk in splicing assays with other pre-mRNAs suggest that Clk would have an influence on the splicing of HIV-1 TAT pre-mRNA. The second part will look at alterations in TAT splicing upon cellular differentiation of P19 embryonal carcinoma cells (P19EC) stably expressing TAT. To this end, we developed a triple primer semi-quantitative RT-PCR based approach and proceeded to measure the production TAT specific transcripts.

1.12 HIV-1 TAT PROTEIN FUNCTIONS

HIV-1 is a retrovirus that transcribes its RNA from an integrated proviral genome and utilizes the host cell splicing machinery to produce multiple mRNAs (Swanson and Stoltzfus, 1998). The single 9.2-kb primary transcript undergoes splicing by complex pathways producing singly and multiply spliced mRNAs. HIV-1's multiply spliced mRNAs encode a number of regulatory proteins including TAT (Purcell and Martin, 1993). TAT is a small 86 aa nuclear protein with RNA-binding

properties, required for viral replication *in vivo*. Although TAT has been shown to fulfill some functions unrelated to transcription, its primary role is in transactivating viral genes by regulating productive and processive transcription from the HIV-1 long terminal repeat (LTR) (reviewed in Jeang *et al.*, 1999). The TAT expression vector pgTAT, will be used in the following studies as a splicing reporter.

2. MATERIALS AND METHODS

2.1 PLASMID CONSTRUCTS USED

The pgTAT expression vector, a generous gift from Michael H. Malim (University of Pennsylvania), is a 2686bp genomic HIV-1 DNA fragment from the replication competent HXB-3 proviral HIV-1 clone ((Malim *et al.*, 1988)) inserted into the pBC12/CMV vector. The insert spans the Tat gene as well as part of the Rev and Env coding regions. A schematic of pgTAT is seen in figure 5A. The GFP-tagged human Clk vectors and the CMV/CR-1 Clk1 minigene were made in our laboratory. The pEGFP and pgk-Puro plasmids used were kindly provide by Drs Douglas Gray and Michael McBurney (University of Ottawa) respectively.

2.2 293T AND 293T TAT CELL CULTURE AND TRANSIENT TRANSFECTION

293T cells (human kidney, Pear *et al.*, 1993) and 293T TAT clones were maintained in α -minimal essential medium (α -MEM) supplemented with 10% serum (2.5% fetal calf serum and 7.5% new born calf serum) at 37°C and 5% CO₂. As well, 293T TAT clone media was supplemented with 0.25 μ g/ml puromycin to maintain positive selection. At 60-75% confluency, cells plated in 6-well dishes were transfected with 15 μ l of the cationic polymer transfection reagent, ExGen500 (MBI Fermentas) and 4 μ g of plasmid DNA according to manufacturer's instructions. For the splicing experiments with the pgTAT and Clk vectors, 0.36 μ g of pgTAT plasmid was cotransfected with 3.6 μ g of either hClk1-GFP, hClk2-GFP, hClk3-GFP, or hClk4-GFP. pEGFP was used as filler DNA. Dishes were then spun at 1500 rpms for 5 mins at room temperature and returned to the incubator. CMV/CR-1 and Clk co-transfections were done with 18 μ l of Lipofectamine reagent

(Gibco BRL) and 6.5 μ g of plasmid DNA according to the manufacturer's instructions. Basically 2.5 μ g of CMV/CR-1 plasmid was cotransfected with either 4 μ g of hClk plasmids (1 to 4), or the pEGFP (control) vector.

For all transient transfections, cells were harvested 24 hours post transfection with trypsin-EDTA and collected in 15ml centrifuge (Falcon) tubes. Cells were washed once with phosphate buffered saline (PBS) and resuspended in 100 μ l PBS. The guanidinium isothiocyanate (GITC)-containing buffer, RLT (Qiagen), was used to lyse the cells and total RNA was extracted using the QiaShredder columns (Qiagen) and the RNeasy purification kit (Qiagen) as per manufacturer's instructions. After extraction, the RNA yield was quantified using the Beckman spectrophotometer in a 50 μ l microcuvette. The average yield of RNA was 60 μ g with minimal DNA contamination (optical density^{260/280} >1.8). RNA was stored at -80°C.

2.3 DNase TREATMENT

For pgTAT and E1A splicing analysis, 2 μ g of total RNA was DNase treated prior to RT-PCR. DNase treatment was performed in a final volume of 16 μ l (3 μ l 5X First Strand Buffer (Gibco BRL), 1 μ l dithiothreitol (DTT) (0.1M), 1 μ l (20 units) RNase-Inhibitor (MBI Fermentas), and 1 μ l (10 units) of RNase free DNase I (Roche)) at 37°C for 1 hour. Following DNase treatment, samples were heat inactivated at 70°C for 15 mins and immediately cooled on ice. RT-PCR was then performed on DNase treated samples.

2.4 RT-PCR ANALYSIS OF pgTAT TRANSCRIPTS

First strand cDNA synthesis occurred in a 21 μ l total reaction volume. DNase treated RNA (16 μ l) was reverse transcribed using 200 units of SuperScript ITM RNase H⁻ reverse transcriptase (Gibco BRL) and a mixture of 1 μ l OligodT₁₅ primers (50pmols/ μ l), 2 μ l of deoxynucleotide 5'-triphosphate (dNTP) (200 μ M), 1 μ l 5X First Strand Buffer (Gibco BRL), and 1 μ l DTT (0.1M). Following a 1 hour incubation at 37°C, samples were heat inactivated at 95°C for 3 mins.

The first strand cDNA product was used as template in 50 μ l PCR reactions. 4 μ l of cDNA was amplified with 1.25 units *Taq* DNA Polymerase (Gibco BRL) in a buffer consisting of: 5 μ l 10X PCR Reaction Buffer (Gibco), 2.5 μ l MgCl₂ (50nM), 1 μ l dNTPs (10mM), and 0.5 μ l of each primer (25 μ l). The following primers were used to amplify TAT specific transcripts: one forward primer, TAT-F (5'atggagccagtagatcctag³), within the first coding exon at position 87, and two reverse primers, TAT-RI (3'gggtgcttcctccacac³) in the adjacent intron and TAT-R (3'tattccttcgggcctgtc³) in the second coding exon. 32 cycles of amplification were performed with 30 sec denaturation at 94°C, 30 sec annealing at 60°C, and 1 min elongation at 72°C followed by a final elongation at 72°C for 10 mins. All RNAs were tested to be free of DNA contamination by RT-PCR without reverse transcriptase.

2.5 PCR OPTIMIZATION

PCR conditions, including number of cycles and template concentrations, were optimized to maintain linearity during the amplification of both the spliced and unspliced TAT transcripts.

Linearity was verified by diluting the RNA or cDNA templates and measuring a corresponding decrease in signal, as well as consistent ratios between the spliced and unspliced bands. All PCR products were separated on a 2% agarose gel, poststained with SYBR Green II (Molecular Probes), visualized, and quantified with a Storm Fluorescence Imager and ImageQuant software (Molecular Dynamics).

2.6 RT-PCR ANALYSIS OF CR-1 TRANSCRIPTS

For the CMV/CR-1 transcripts, 1 µg of total RNA, isolated using RNeasy total RNA isolation kit (Qiagen), was used to synthesize oligo (dT) primed first strand cDNA with Superscript I™ RNase H⁻ reverse transcriptase (Gibco BRL). RT was performed in a final volume of 21 µl: 4 µl 5X First Strand Buffer (Gibco), 2 µl DTT (0.1 M), 2 µl dNTPs (200 µM), and 1 µl Oligo dT₁₅ primer (50 pmol/µl). Samples were heated at 65°C for 5 mins and cooled to ambient temperature, after which 1 µl (200 units) of RT enzyme was added. Samples were then incubated at 37°C for 50 min and heat inactivated for 3 min at 95°C. The first strand cDNA product (2 µl) was used as template in 25 µl PCR reactions with final volumes of 2.5 µl 10x PCR Reaction Buffer (Qiagen), 0.5 µl of MgCl₂ (50 mM), 0.5 µl dNTPs (10 mM), 0.5 µl of each primer (25 pmol/µl), and 0.25 µl (1.25 units) Taq DNA Polymerase (Gibco BRL). The following primers were used to amplify CMV/CR-1 specific transcripts: 5' *tggtaggagtggaagaag* 3' as forward primer and 5' *gatggctggcaactagaa* 3' as reverse primer. 22 cycles of amplification were performed with 30 sec denaturation at 94°C, 30 sec annealing at 58°C, and 30 sec elongation at 72°C followed by a final elongation at 72°C for 10 mins.

2.7 ESTABLISHMENT OF STABLE CELL LINES

293T (Pear *et al.*, 1993) and COS-1 (monkey kidney cells, Gluzman, 1981) cells were transfected using the Lipofectamine transfection reagent (Gibco BRL). P19 mouse embryonal carcinoma (EC) cells (McBurney and Rogers, 1982) and HeLa (human cervical carcinoma (Gey *et al.* 1952) cells were transfected using the Fugene transfection reagent (Roche). All transfections were performed according to respective manufacturer's instructions. Cells were co-transfected with pgk-Puro and pgTAT at a 1 to 10 ratio. DNA:transfection ratios were as follows: 6 μ g:18 μ l Lipofectamine for 293T and COS-1 cells, 6 μ g:12 μ l Fugene for HeLa cells, and 4 μ g:12 μ l Fugene for P19EC cells.

The DNA:Lipofectamine or DNA:Fugene complexes were applied over a semi-confluent layer of cells plated the night before at a density of 1×10^6 cells per 60mm dish for 293T, COS-1, and HeLa cells, and 5×10^5 cells for P19EC cells. The cells were cultured for 24 h and then dispersed with trypsin-EDTA and plated in a 100mm dish with α -MEM supplemented with 10% new born calf serum and the antibiotic puromycin (0.25 μ g/ml for 293T and COS-1 cells, 0.20 μ g/ml for HeLa cells, and 2 μ g/ml for P19EC cells) to generate stable clones. Cells were cultured in the presence of antibiotic for 7 to 10 days. The surviving colonies were picked with the aid of a pipette man and expanded into cell lines or pooled and cultured en masse. Puromycin was kept in the culture media to maintain positive selection. Positive clones expressing pgTAT were identified by RT-PCR.

2.8 RT-PCR ANALYSIS OF E1A TRANSCRIPTS

Following DNase treatment and first strand cDNA synthesis, described above for pgTAT transcripts, 2 μ l of cDNA was used as template for the amplification of E1A specific transcripts in a 25 μ l PCR reaction. The final volumes of PCR components were as follows: 2.5 μ l 10x PCR Reaction Buffer, 0.5 μ l of MgCl₂(50mM), 0.5 μ l dNTPs (10mM), 0.5 μ l of each primer (25pmol/ μ l), and 0.25 μ l (1.25units) *Taq* DNA Polymerase (Gibco BRL). The following primers were used to amplify E1A specific transcripts ((Yang *et al.*, 1994)): 5'attatctgccacggaggtg^{3'} as forward primer and 5'ggatagcaggcgccatttta^{3'} as reverse primer. One round of PCR was performed with 22 cycles consisting of a 30 sec denaturation at 94°C, 30 sec annealing at 58°C, and 30 sec elongation at 72°C, followed by a final elongation at 72°C for 10 mins.

2.9 P19 TAT CLONE CULTURE AND DIFFERENTIATION

P19 TAT expressing clones were cultured and differentiated as described in Rudnicki and McBurney (1987). To initiate differentiation, cells were plated into bacterial grade Petri dishes at 10⁵ cells/ml in medium containing either 0.5% dimethyl sulfoxide (DMSO) or 0.3 μ M retinoic acid (RA). After 48hrs the medium was changed and aggregates of cells were plated in 100mm bacterial grade Petri dishes with media containing either DMSO or RA. Cultures were expanded for an additional 48hrs for RA differentiation and 72hrs for DMSO differentiation after which about 100 cell aggregates were plated onto 100mm tissue culture grade plastic dishes and gelatin coated (0.2% gelatin in PBS) coverslips for immunofluorescence. At this point DMSO and RA were omitted from the media. The media was replenished every 2 days and puromycin was maintained

throughout the experiment in order to retain positive selection for TAT expressing cells. Cells in culture dishes were harvested on day 7. RNA was isolated and RT-PCR performed as described above for pgTAT transcripts.

2.10 INDIRECT IMMUNOFLUORESCENCE MICROSCOPY OF DIFFERENTIATED CELLS

For cultures to be studied by indirect immunofluorescence, cells grown on gelatin coated coverslips were washed with PBS and fixed in methanol at -20°C for 5min. After fixation, the cells were rehydrated with PBS. The following monoclonal antibodies were used as markers for differentiated cells: TROMA-1, which reacts with the mouse endoderm marker keratin (Kemler *et al.*, 1981); A60, a neuronal differentiation marker which recognizes the neuron-specific nuclear protein Neuronal Nuclei (NeuN) (Mullen *et al.*, 1992); and MF-20 which reacts with the myosin heavy chain present in muscle (Bader *et al.*, 1982). The fixed cells were incubated with the primary antibodies, diluted 1:100 in 0.3% Triton X-100 in PBS, in a humid chamber at 37°C for 45min. Then, the cells were washed 3 times with PBS and incubated with secondary antibody (anti-mouse CY3 conjugated), diluted 1:100 in 0.3% Triton X-100 in PBS, in a humid chamber at 37°C for 45 mins followed by three washes in PBS. DAPI (4',6-diamidino-2-phenylindole, Sigma) was used at a 1:10000 dilution as a counterstain to detect nuclei. Coverslips were then mounted on slides with Vectashield

mounting medium for fluorescence (Vector) and sealed to avoid dehydration. Phase contrast and immunofluorescence were examined and captured using an Axiophot microscope and the Intelcam computer software program.

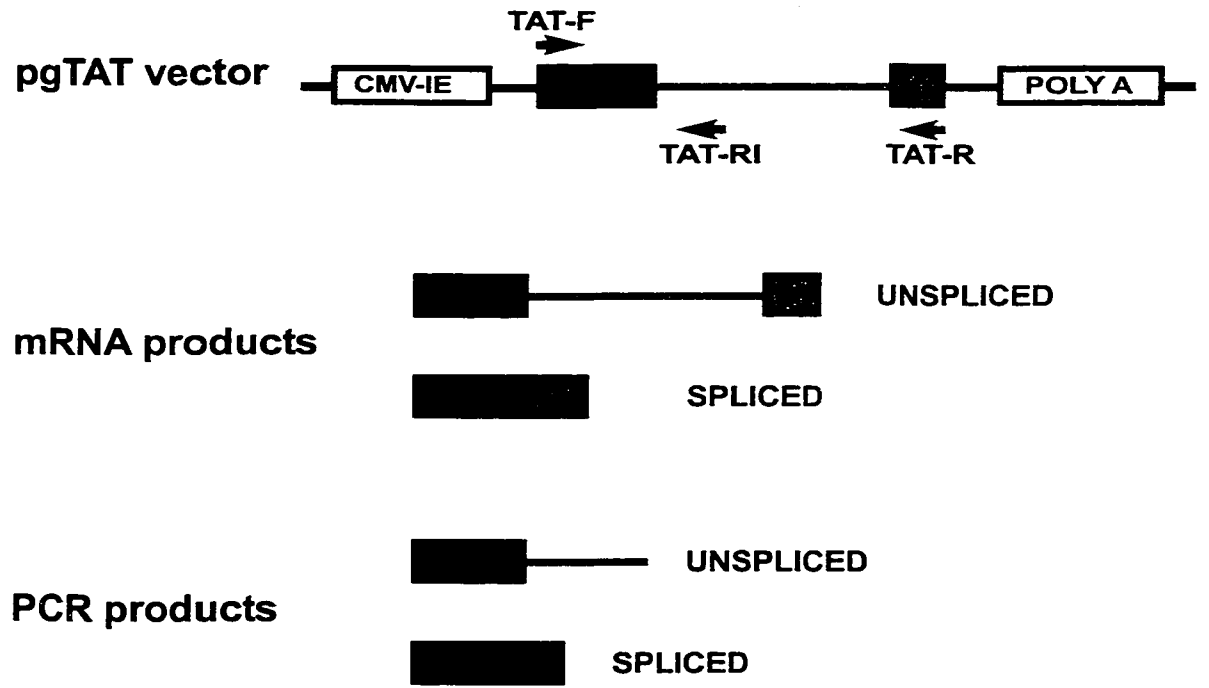
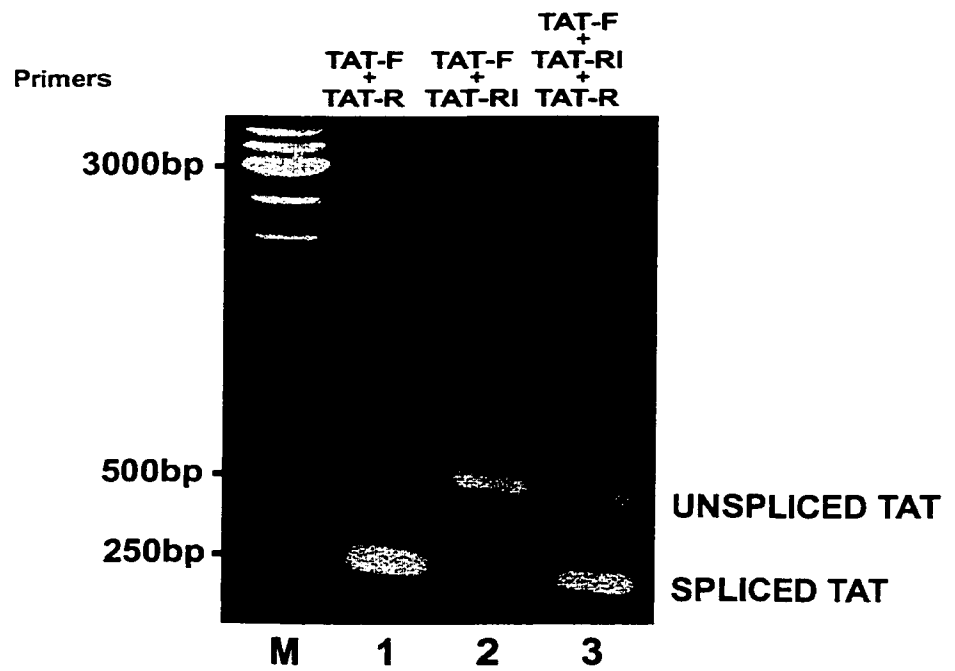
3. RESULTS

3.1 SEMI-QUANTITATIVE COAMPLIFICATION OF SPLICED AND UNSPLICED HIV-1 TAT TRANSCRIPTS USING RT-PCR

The pgTAT expression vector (depicted in figure 5A), transfected into eukaryotic cells, forms two mRNA species, an ~2600bp unspliced product and an ~260bp product resulting from the ligation of exons 2 and 3 and removal of the intervening intron. Before measuring changes in the levels of the two mRNA products under specific conditions it was necessary to optimize and set various RT and PCR conditions in order to make the measurements semi-quantitative. When one forward (TAT-F) and reverse (TAT-R) primer combination was used (figure 5A), the expected size of the products was about 250bp for the spliced product and 2500bp for the unspliced product. Upon expression of this vector in eukaryotic cells, isolation of total RNA, DNase treatment, and subsequent RT-PCR, it was noticed that only the much smaller spliced product would amplify (figure 5B lane 1). Since PCR reactions favour smaller products over larger ones, and that the two products to be compared differed by over 2000bp, a second reverse primer (TAT-RI) was designed, within the intron (figure 5A). This additional primer allowed the detection of both the spliced and unspliced products in the same RT-PCR reaction. Using TAT-F and TAT-R primers, a band of about 250bp, corresponding to the spliced transcript was obtained (figure 5B lane 1), and using the TAT-F and TAT-RI primers, a band of ~500bp, corresponding to the unspliced transcript (figure 5B lane 2) was produced. When the three primers were used in the same PCR reaction both bands were obtained (figure 5B lane 3).

Figure 5 Amplification of HIV-1 TAT cDNA using two or three primers in PCR.

(A) Schematic representation of part of the pgTAT vector, mRNA transcripts and PCR products. Exons are indicated as boxes and the intron as a line. Arrows represent PCR primers and their sites of hybridization (NOTE: primers are not to scale). The forward primer (TAT-F) is able to recognize both the spliced and unspliced products. The reverse primers (TAT-R and TAT-RI) are able to recognize either the cDNA from spliced and unspliced products respectively. (B) Products formed using the different TAT specific primers indicated (lanes 1 to 3). RT-PCR was performed on total RNA isolated from 293T cells transiently transfected with the pgTAT vector. Positions of the cDNAs representing spliced and unspliced TAT RNA are indicated on the right side of the panel. The positions of the molecular size standards (lane M) are indicated to the left of the panel. (Schematic modified from Malim et al., 1988)

A**B**

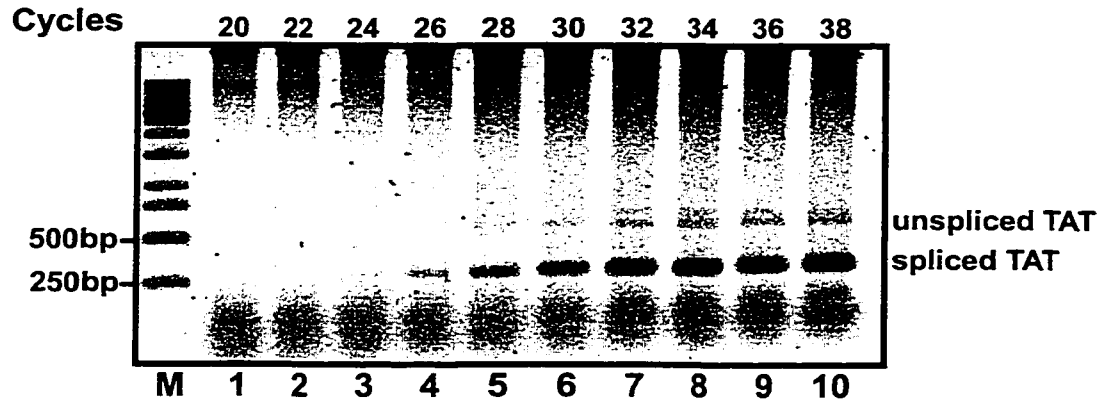
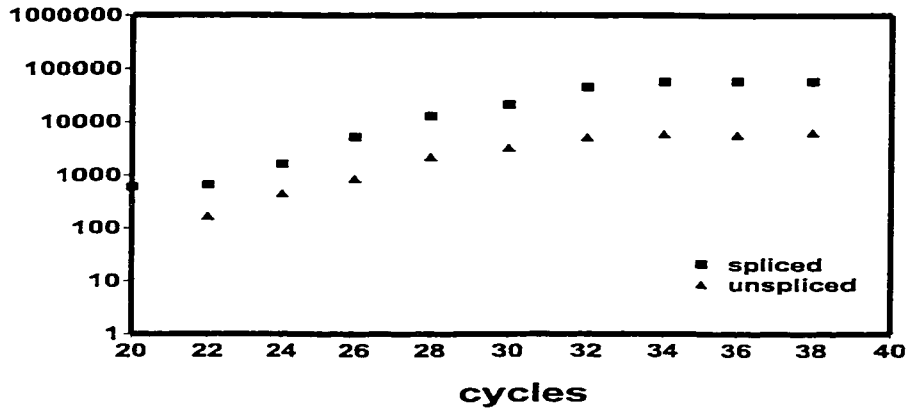
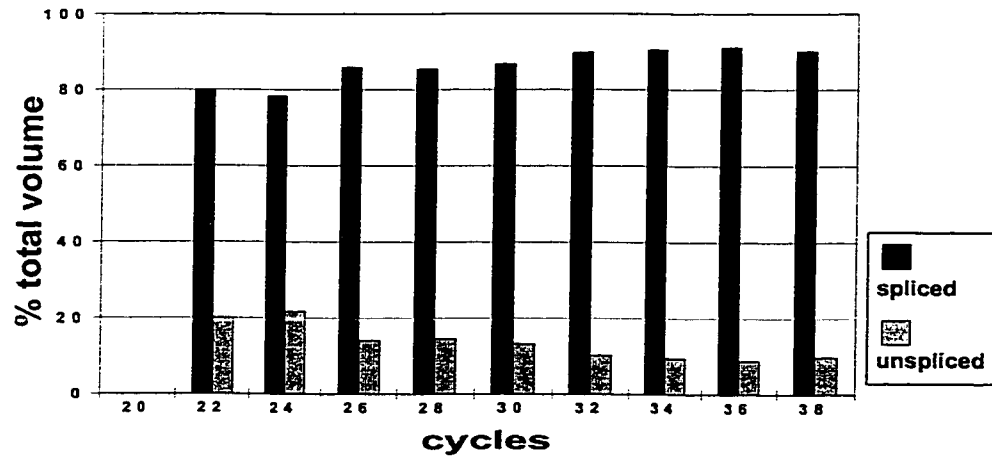
In order to assure the maintenance of a constant ratio of spliced and unspliced signals after RT-PCR, it was necessary to examine the products under various RT and PCR conditions. RNA from cells expressing TAT both transiently and stably, was reverse transcribed and amplified using the three primers. cDNA was sampled every two cycles from 22-38 cycles. With increasing number of cycles both the spliced and unspliced products co-amplified linearly within cycles 26 and 32 (figure 6A and 6B) This figure depicts one representative experiment. Thirty-two cycles was chosen for all subsequent reactions. Quantification of signals within the linear range showed that the ratio of spliced to unspliced signals did not vary significantly (figure 6C). Likewise using a constant number of PCR cycles, 32 cycles, the quantity of either total RNA or template cDNA was varied and the ratio did not change significantly.

3.2 TESTING THE GFP-TAGGED CLK PLASMIDS

Previous studies in our laboratory, looking at effect of Clk protein on splicing, have utilized a myc-tagged form of the protein. More recently, new GFP-tagged constructs for all four human Clk members known to date (Stojdl and Bell, 1999) were made in our laboratory. In order to assure that these new constructs functioned in the same fashion as the myc-tagged clones, their ability to splice a murine Clk1 splicing mini-gene, CMV/CR-1 (figure 7A), commonly used for splicing assays in our laboratory (Duncan *et al.*, 1997), was tested. The CMV/CR-1 minigene contains an alternatively spliced exon, EB, and can produce two transcripts, EB+ (about 550bp) and EB- (about 400bp) (Figure 7). The CMV/CR-1 minigene was cotransfected into the human cell line 293T along with either the vectors encoding hClk2-GFP, hClk3-GFP, hClk4-GFP, or the pEGFP

Figure 6 PCR linearity.

Total RNA was isolated from a P19 clone stably transfected with the HIV-1 TAT expression vector pgTAT. TAT specific RNAs were amplified by triple primer RT-PCR as described in Materials and Methods. (A) PCR samples taken every two cycles between 20 and 38 and separated on a 2% agarose gel (lanes 1 to 10). Positions of the cDNAs representing spliced and unspliced TAT RNAs are indicated on the right side of the panel. The positions of the molecular size standards (lane M) are indicated to the left of the panel. (B) A standard PCR curve was generated from quantification of TAT unspliced and spliced RNAs shown in A. (C) The percentage of each isoform (quantitated in B) relative to the total signal is represented for each PCR cycle as shown in A.

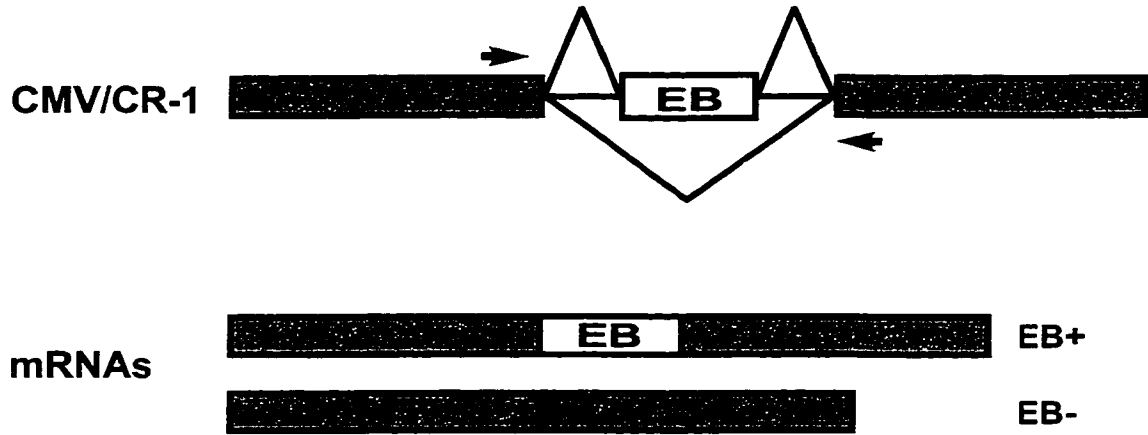
A**B****C**

vector as control. The CR-1 spliced mRNA products were then assessed by RT-PCR analysis as described in Materials and Methods. As previously shown in both COS-1 and 293T cells (Duncan *et al.*, 1997, Duncan *et al.*, 1998), the CMV/CR-1 minigene, when transiently transfected, gives rise to both forms of transcripts (figure 7B lane 2). Upon overexpression of cotransfected hClk2, hClk3, or hClk4, the splicing of the alternative exon is favoured (figure 7B lanes 3-5). These results on the splicing of CMV/CR-1, as well as those for hClk1-GFP (results not shown), are similar to those seen with the myc-tagged Clk proteins and therefore these proteins function in the same manner as their myc-tagged counterparts (Duncan *et al.*, 1997, Duncan *et al.*, 1998).

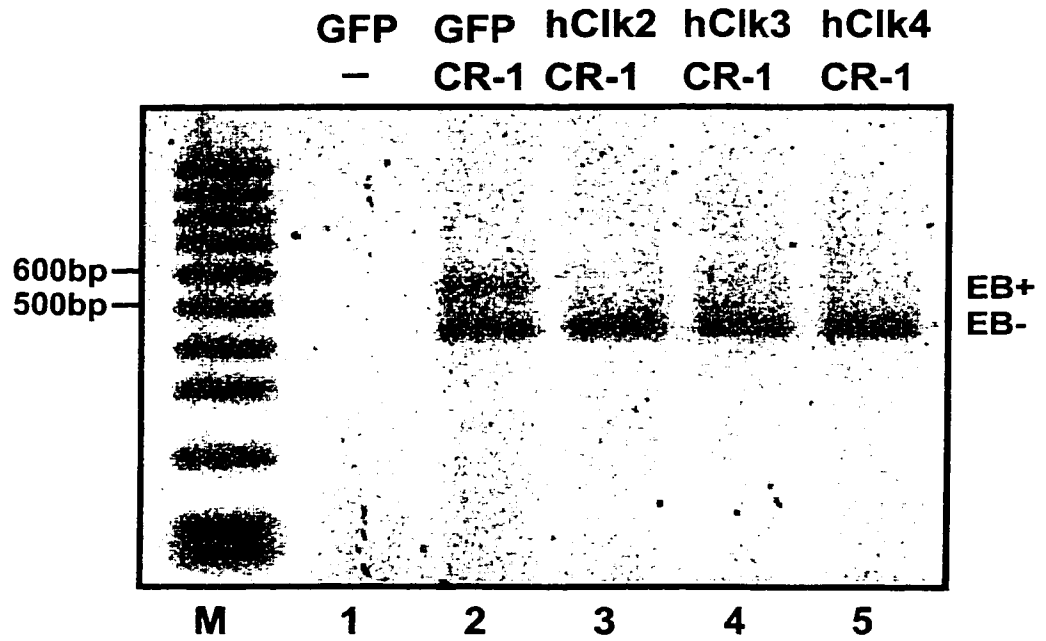
Figure 7 Effects of GFP-tagged Clks transient overexpression on the splicing of Clk1 pre-mRNA.

293T cells were transiently co-transfected with the Clk1 minigene expression vector (CMV/CR-1) and either the GFP or one of three human Clk (hClk 2 to 4) vectors. Total RNA was extracted 24 hours post transfection and reverse transcribed as described in Materials and Methods. CR-1 specific RNAs were amplified by RT-PCR. Samples were separated on a 2% agarose gel, stained with SYBR Green I, and visualized by fluorimager. (A) Schematic representation of part of the Clk1 pre-mRNA minigene (CMV/CR-1) along with its spliced mRNA transcripts. Exons are indicated as boxes and the intron as a line. CR-1 specific primers are indicated by the arrows. (B) Pattern of CR-1 splicing upon co-transfection with either GFP (lane 1) or one of hClks 2 to 4 (lanes 2 to 5). The position of EB included and excluded transcripts are indicated on the right side of the panel. The positions of the molecular size standards (lane M) are indicated to the left of the panel. (Schematic modified from Duncan *et al*, 1997)

A



B



3.3 ESTABLISHMENT OF TAT STABLE CELL LINES

To test the effects of the various Clks on the splicing of TAT mRNA, various eukaryotic cells lines stably expressing this vector were established. The pgTAT expression vector was co-transfected with the pkgPuro plasmid, allowing resistance to the antibiotic puromycin, in 293T (human kidney), P19 mouse embryonal carcinoma (EC), COS-1 (monkey kidney cells), and HeLa (human cervical carcinoma) cells. Cells were incubated for 7 to 10 days in puromycin and 24 resistant clones for each cell line were picked. Only 293T, P19 EC, and COS-1 clones survived and were expanded to establish colonies. RNA was isolated from the various clones and RT-PCR analysis was then performed to determine those clones expressing TAT mRNA. As result of the technique used to pick the clones, none of the COS-1 clones expressed TAT. Twelve 293T clones were positive for TAT mRNA expression and these expressed both the 250bp and 500bp RT-PCR products representing the spliced and unspliced forms of TAT mRNA respectively (figure 8A shows some of these clones). Most P19 EC TAT positive clones also expressed both the 250bp and 500bp PCR products (figure 8B). Four clones from each cell line were maintained in culture and the remainder stored in aliquots at -80°C.

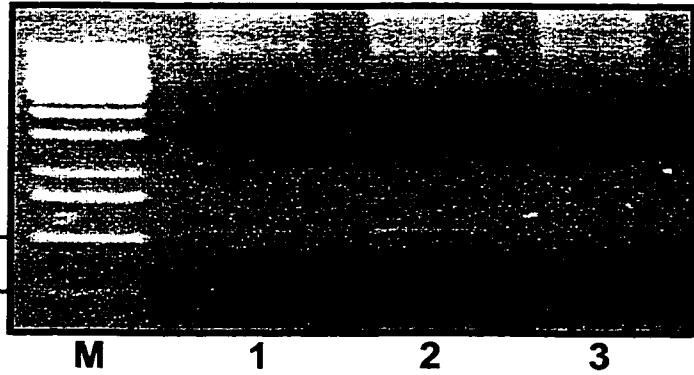
Figure 8 Stable expression of HIV-1 TAT in P19 EC and 293T cells.

P19 EC and 293T cells were co-transfected with the pgTAT and the pgk-Puro expression vectors. Drug resistant colonies were picked and expanded. Total RNA was isolated from expanded clones. Positive clones were identified by RT-PCR with TAT specific primers. Samples were separated by gel electrophoresis on a 2% gel. (A) 293T drug resistant clones (lanes 1 to 3), (B) P19 EC drug resistant clones (lanes 1 to 12). Positions of the cDNAs representing spliced and unspliced TAT RNA transcripts are indicated on the right side of the panels. The positions of the molecular size standards (lanes M) are indicated to the left of the panels.

A

293T

500bp
250bp



unspliced TAT
spliced TAT

M

1

2

3

B

P19EC

500bp
250bp



unspliced TAT
spliced TAT

M

1

2

3

4

5

6

7

8

9

10

11

12

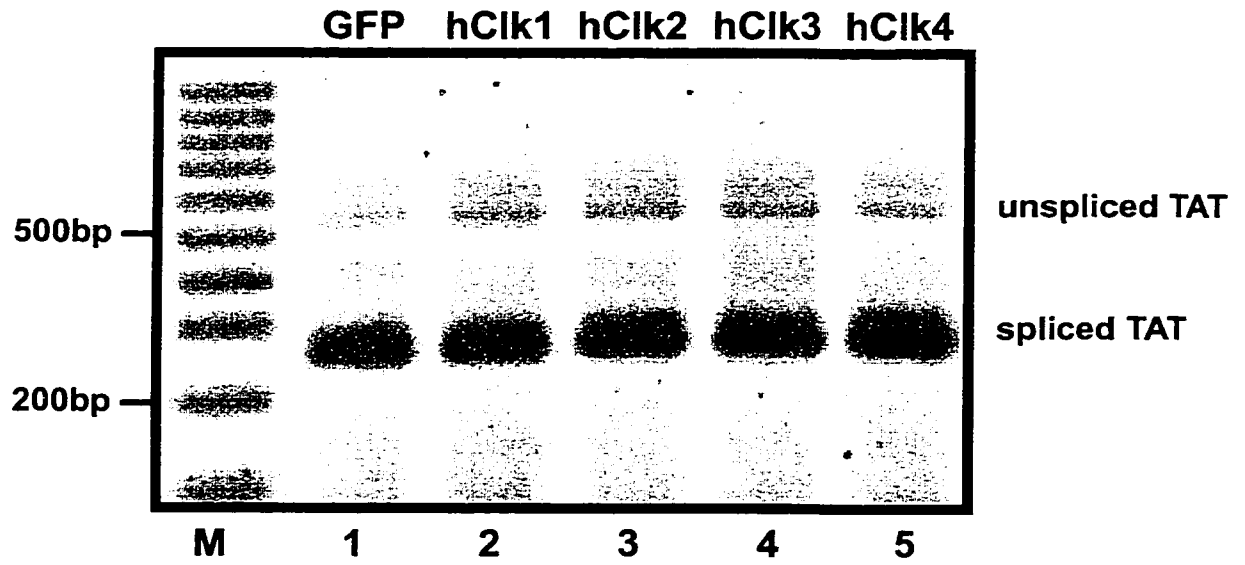
3.4 TAT mRNA SPLICING DOES NOT CHANGE UPON OVER-EXPRESSION OF THE SR PROTEIN KINASE CLK

Previous studies have suggested that Clk may be involved in mRNA splicing. *In vitro* splicing assays have shown that Clk can alter the splicing of a number of pre-mRNAs (Prasad *et al.*, 1999). As well, *in vivo* studies have shown that all 4 Clk kinases can regulate the splicing of both their own pre-mRNA as well as that of an adenovirus E1A reporter gene *in vivo* (Duncan *et al.*, 1997, Duncan *et al.*, 1998). To determine if the various Clks could regulate the splicing of other transcripts *in vivo*, we transfected the pgTAT plasmid (Malim *et al.*, 1988) into 293T cells in the absence and presence of vectors encoding one of the 4 human Clks (hClk1 to 4). RNA was harvested from these cells 24hrs post transfection and DNase treated and RT-PCR analysis was performed using the optimized conditions set with the triple primer combination. Analysis of the RNA isolated from cells transfected with the pgTAT vector alone showed both the 250bp and 500bp PCR products representing the spliced and unspliced forms of TAT mRNA respectively (figure 9A lane 1). The ratio of spliced to unspliced product was approximately 7 to 1 (figure 9B). In the presence of any of the hClk vectors there was no significant difference in the ratio of spliced to unspliced product formed (figure 9A lanes 2-5 and 9B). The same results were seen for three independent experiments.

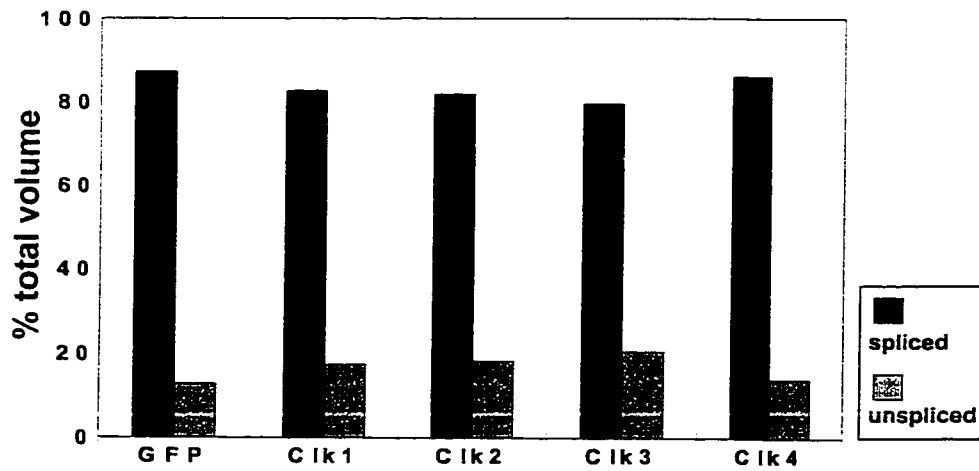
Figure 9 Transient overexpression of human Clk does not detectably affect the splicing of HIV-1 TAT pre-mRNA *in vivo*.

293T cells were transiently co-transfected with the pgTAT expression vector and either GFP or one of four human Clk (hClk1 to 4) vectors. Total RNA was extracted 24 hours post transfection and reverse transcribed as described in Materials and Methods. TAT specific transcripts were amplified using TAT specific primers described in figure 5. PCR samples were separated on a 2% agarose gel and stained with SYBR Green I. (A) Pattern of TAT splicing upon co-transfection with GFP (lane 1) or one of hClks 1 to 4 (lanes 2 to 5). The position of the unspliced and spliced TAT transcripts are indicated on the right side of the panel. The positions of the molecular size standards (lane 1) are indicated to the left of the panel. (B) The unspliced and spliced mRNA transcripts were quantitated and the percentage of each transcript, shown in A, relative to the total signal is shown for each co-transfection.

A



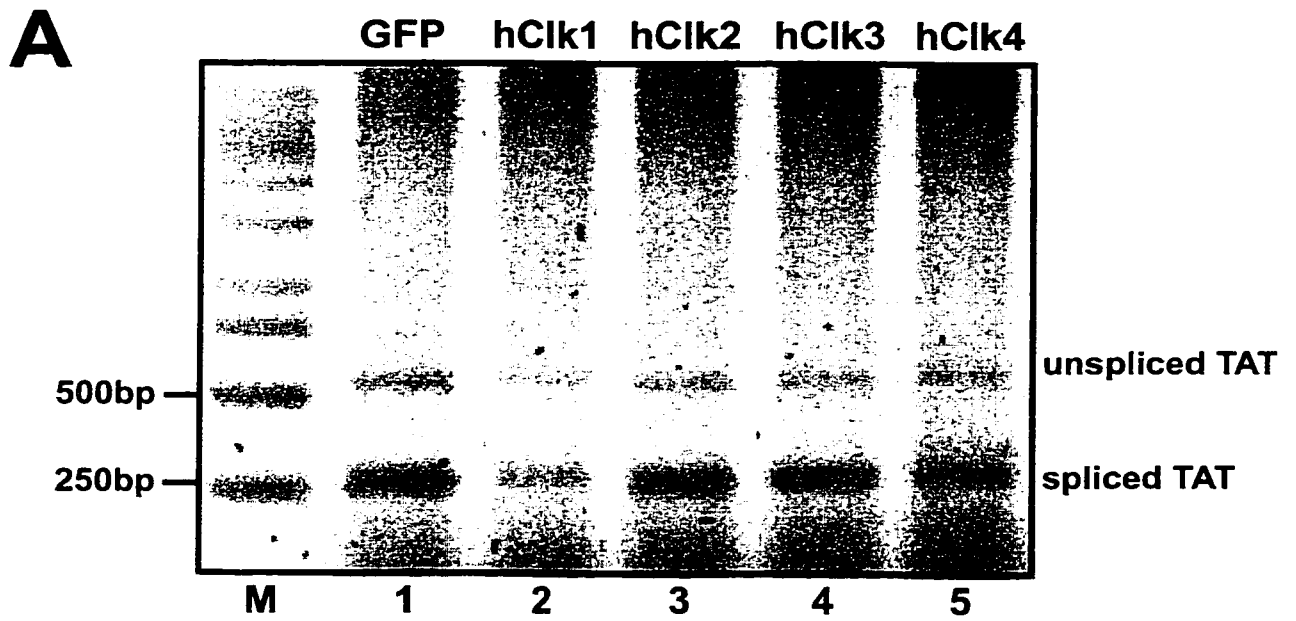
B



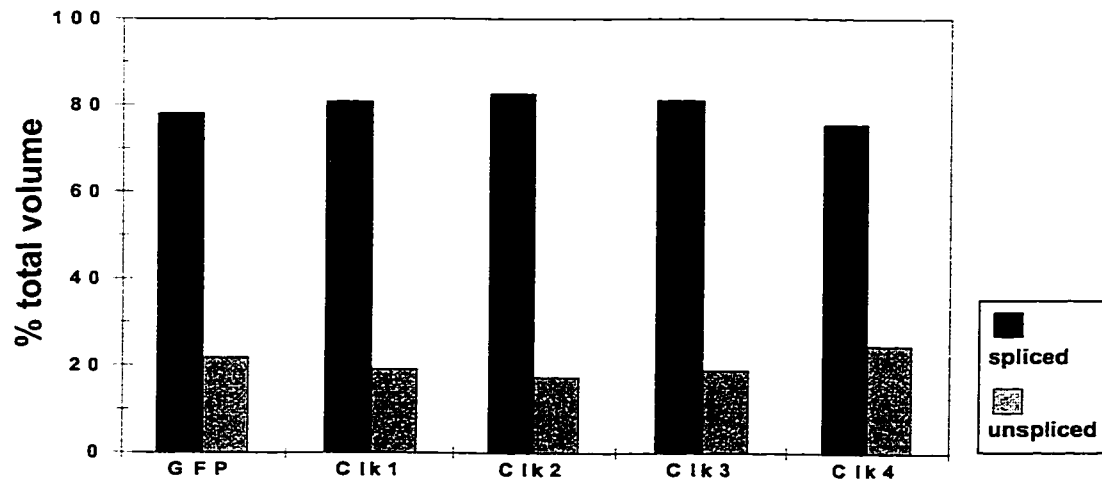
To examine the splicing effects of Clk on 293T cells in which the TAT vector was stably transfected, 293T TAT expressing clones, were transiently transfected with either the pEGFP vector (as control) or with one of the human Clk plasmids (hClk1 to 4). Twenty-four hours post-transfection, TAT mRNA was monitored by semi-quantitative RT-PCR with the triple primer conditions following DNase treatment. An aliquot of cells was viewed under the UV microscope and positive cells were counted in order to determine the transfection efficiencies. RT-PCR was only performed if transfection efficiencies were $\geq 50\%$. Figure 10A represents the results for one of the 293T clones (293TD4). Similar results were seen with two other 293T clones. Baseline levels of the TAT mRNAs show a ratio of about 4 to 1 spliced to unspliced (figure 10A lane 1). Upon transfection of any of the human Clks there was no significant change in the ratio of spliced to unspliced (figure 10A lanes 2 to 5 and 10B).

Figure 10 HIV-1 TAT pre-mRNA splicing is not altered upon Clk transient overexpression in 293T cells stably expressing TAT.

293T cells stably expressing HIV-1 TAT were transiently transfected with one of four human Clk (hClk1 to 4) expression vectors. Total RNA was extracted 24 hours post transfection and reverse transcribed as described in Materials and Methods. TAT specific transcripts were amplified using TAT specific primers described in figure 5. PCR samples were separated on a 2% agarose gel and stained with SYBR Green I. (A) Pattern of TAT splicing upon transfection with GFP (lane 1) or one of hClks 1 to 4 (lanes 2 to 5). The position of the unspliced and spliced TAT transcripts are indicated on the right side of the panel. The positions of the molecular size standards (lane 1) are indicated to the left of the panel. (B) The unspliced and spliced mRNA transcripts were quantitated and the percentage of each transcript, shown in A, relative to the total signal is shown for each transfection.



B

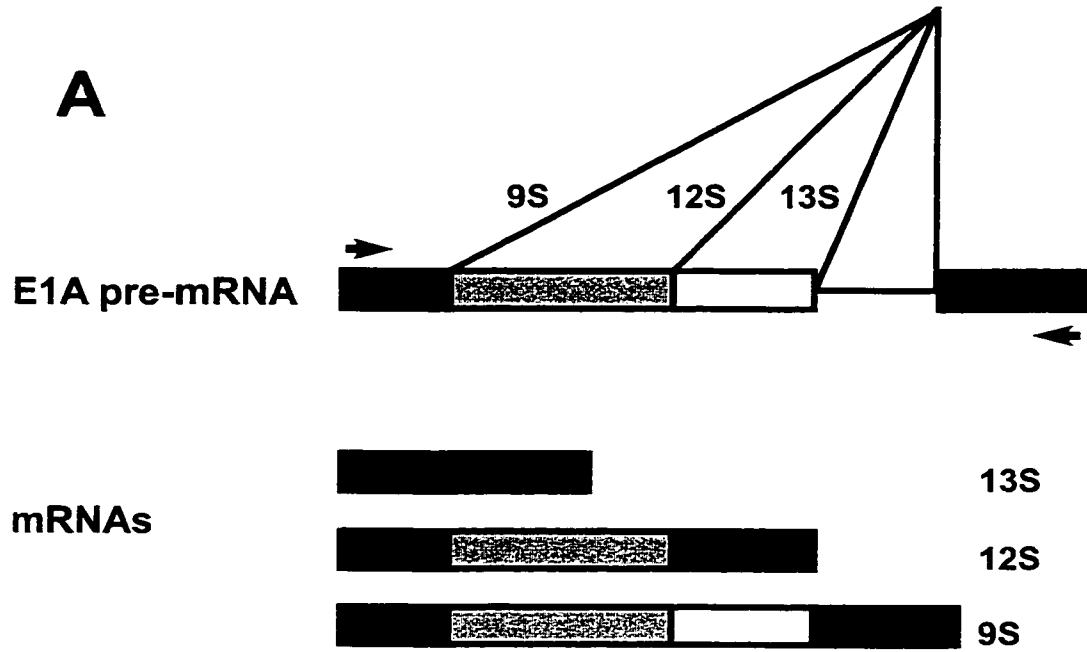


To verify that the Clk proteins expressed were functional in the preceding transfection experiments, and to rule out the possibility that transfection efficiencies were too low, a PCR reaction was performed using the cDNA from the transfected cells with primers specific to the endogenous E1A transcripts. 293T cells have endogenous E1A and express the various transcripts typical of this vector (schematized in figure 11A) (Berk and Sharp, 1978, Chow *et al.*, 1979). Previous experiments have shown that all 4 human Clks alter the splicing pattern of E1A mRNA transcripts (Duncan *et al.*, 1997, Duncan *et al.*, 1998, David Stojdl personal communication). Figure 11B illustrates the E1A specific PCR products from TAT expressing 293T cells transfected with and without hClks 1 to 4. When transfected with control DNA (pEGFP), the 293T clones express the classical expression of the three major alternatively spliced product, 13S, 12S, and 9S (Bourgeois *et al.*, 1999) (figure 11B lane 1). When either of the human Clks are transfected there is increase in the 9S transcript, consistent with previous experiments performed in our lab (figure 11B lanes 2 to 5).

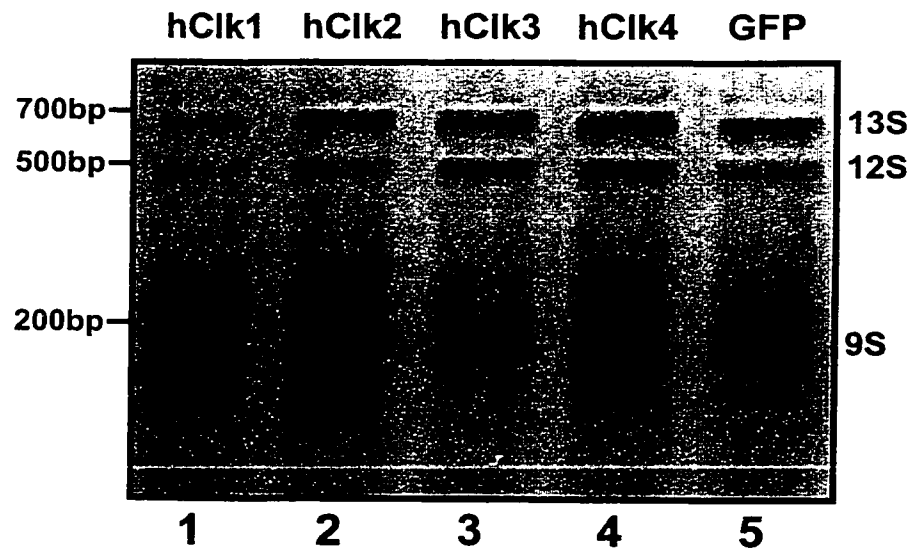
Figure 11 Effects of GFP-tagged hClks transient overexpression on the alternative splicing of endogenous E1A pre-mRNA.

293T cells, stably transfected with the pgTAT expression vector, were transiently transfected with either the GFP vector or one of four human Clk (hClk1 to 4) vectors. RNA was isolated and E1A specific transcripts were amplified by RT-PCR. (A) Representative diagram of the adenovirus E1A pre-mRNA with the major E1A mRNA transcripts generated by alternative 5' splice site selection. The primers used for RT-PCR analysis are indicated by the arrows. (B) Patterns of E1A alternative splicing in 293T TAT expressing cells, upon transfection with GFP (lane 1) or hClks 1 to 4 (lanes 2 to 5). (Diagram adapted from Caceres *et al*, 1994).

A



B



3.5 TAT mRNA SPLICING CHANGES DURING CELL DIFFERENTIATION OF P19EC CELLS STABLY EXPRESSING TAT

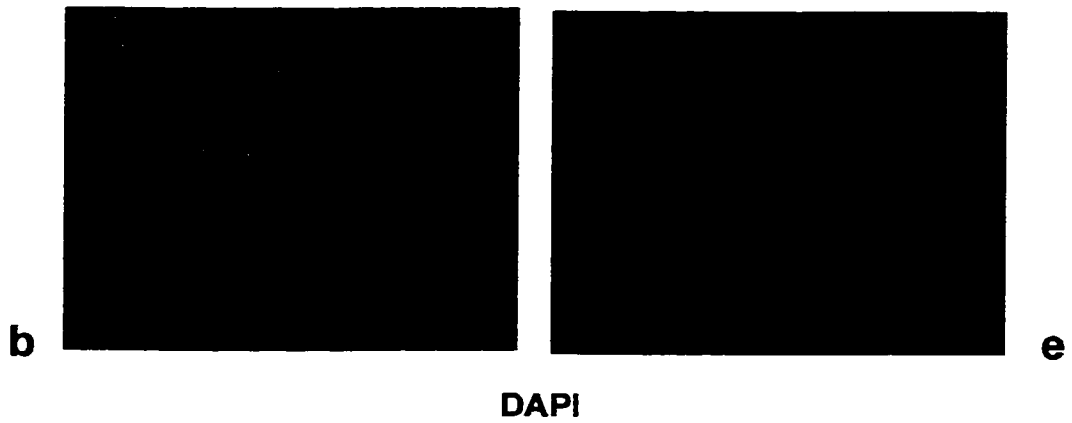
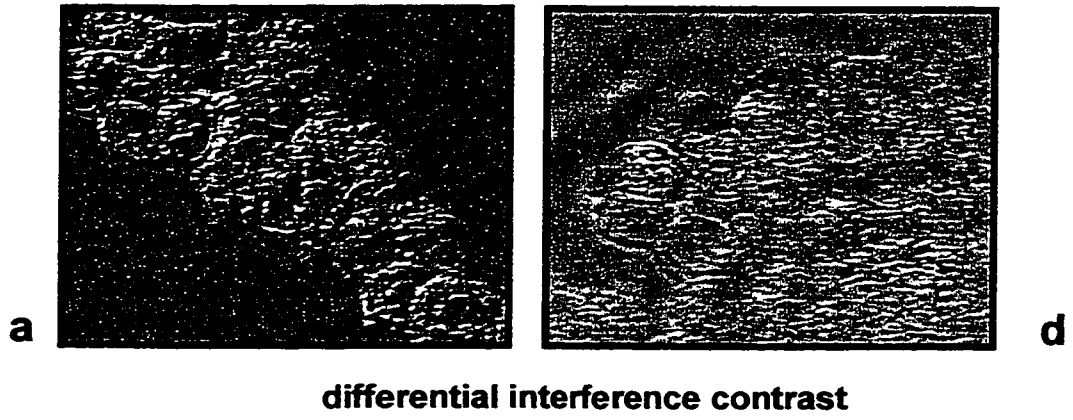
To examine the effects of differentiation on the splicing of HIV-1 TAT mRNA, one of the P19EC clones stably expressing TAT (P19D5) was induced to differentiate. Treatment of P19EC cells with dimethyl sulfoxide (DMSO) induces differentiation into cells with features of cardiac muscle, skeletal muscle, and epithelium (McBurney, 1993) whereas treatment with retinoic acid (RA) induces differentiation into a mixed population of cells consisting of neurons, astrocytes, and fibroblast-like cells (Rudnicki and McBurney, 1987, McBurney, 1993). Total RNA was isolated from P19D5 TAT cells on days 0 and 7 of differentiation, DNase treated, and subject to RT-PCR analysis using the triple primer conditions described earlier.

To verify the extent to which cells were differentiated, indirect immunofluorescence was performed on cells fixed on day 7 post differentiation (figure 12). DMSO treated cultures (figure 12A) stained positively with TROMA-1, a monoclonal antibody directed against a class of cytokeratin-like proteins (panel c), and showed little staining with MF-20 (panel f), an anti-myosin heavy chain antibody. RA treated cultures (figure 12B) presented a very different morphology to DMSO treated cells (compare 12B panel a to 12A panel a), and stained heavily with A60 (panel c), a neuronal differentiation marker antibody, with much less TROMA-1 staining (not shown).

Figure 12 P19 EC TAT expressing cells differentiate to neuronal and endodermal phenotype in response to RA and DMSO respectively.

Differentiation was stimulated by allowing the cells to aggregate in bacterial grade Petri dishes for 4 days in medium supplemented with 0.3 μ M RA for neuronal differentiation or 5 days in medium supplemented with 0.5% DMSO for muscle and endodermal differentiation. Then aggregates were dispersed and spread on tissue culture plates for an additional 2 or 3 days (total of seven days of differentiation). Cells were fixed in methanol at -20°C for 5min and subject to differential interference contrast (panels Aa, Ad, and Ba), DAPI staining (panels Ab, Ae, Bb), and indirect immunofluorescence microscopy (panels Ac, Af, Bc), as described in Materials and Methods. (A) DMSO treated cultures showed positive staining with the endodermal marker TROMA-1 (panel a) and very little staining with the muscle specific marker MF-20 (panel f). (B) RA treated cells stained positively with the neuronal specific marker A60 (panel c).

A 0.5% DMSO



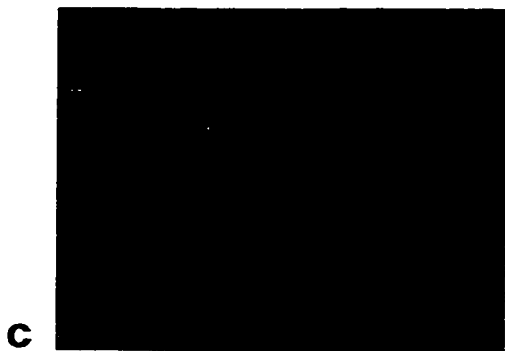
B 0.3 μ M RA



differential interference contrast



DAPI



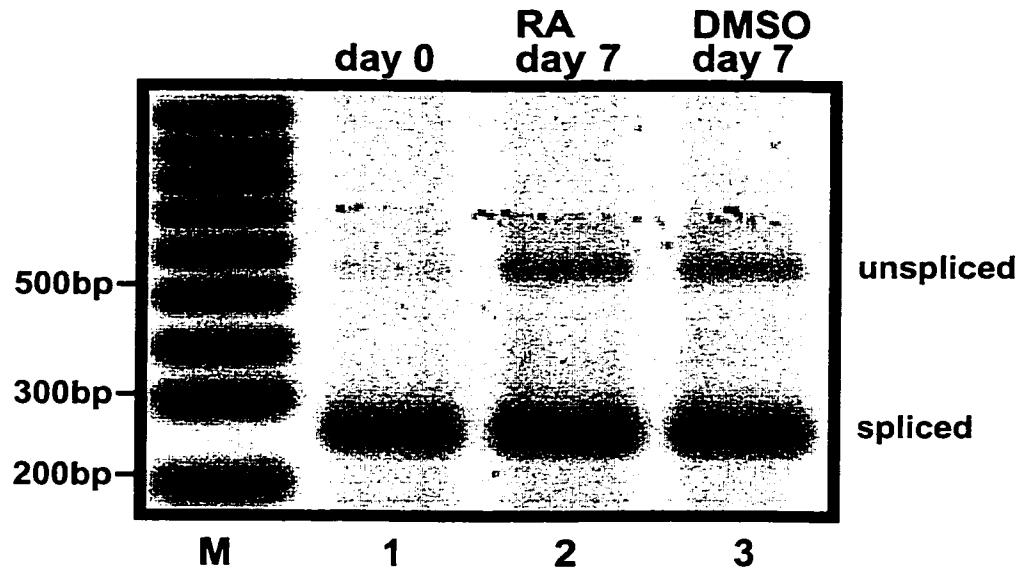
A60

Figure 13A shows the TAT RT-PCR products before and after 7 days of differentiation with RA or DMSO. Analysis of the RNA isolated prior to the differentiation of the P19D5 clone (day 0) primarily revealed the 250bp PCR product corresponding to the spliced form of TAT mRNA, with very little production of the 500bp PCR product, representing the unspliced pre-mRNA (figure 13A lane 1 and 13B). Upon differentiation with either RA or DMSO, the 500bp PCR product is increased ($p \leq 0.002$), thus significantly decreasing the spliced to unspliced ratio (figure 13A lanes 2 and 3 and figure 13B). The results represent the average \pm standard deviation of five independent experiments.

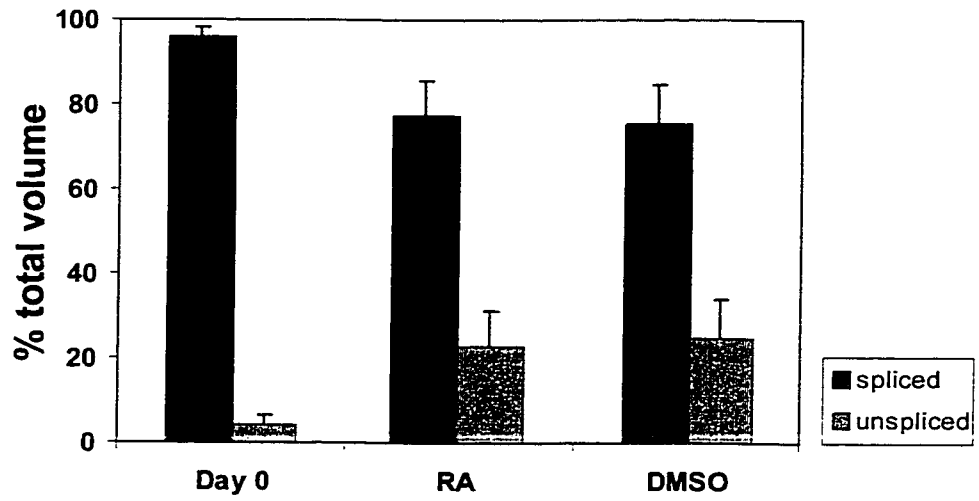
Figure 13 Expression of TAT mRNA before and after RA- or DMSO-induced differentiation of P19 EC TAT expression cells.

Total RNA was extracted from P19 EC cells stably transfected with the pgTAT expression vector on day 0 and day 7 after exposure to either RA or DMSO. The expression of TAT mRNA was detected by triple primer RT-PCR as described in Materials and Methods with the primers depicted in figure 5. Samples were separated on a 2% agarose gel and stained with SYBR Green I. (A) Pattern of TAT splicing on day 0 before differentiation (lane 2) and after RA (lane 3) or DMSO (lane 4) 7 day treatment. The position of the unspliced and spliced TAT transcripts are indicated on the right side of the panel. The positions of the molecular size standards (lane 1) are indicated to the left of the panel. (B) The unspliced and spliced mRNA transcripts were quantitated and the percentage of each transcript shown in A relative to the total signal is shown for each treatment condition. Bars represent the average \pm SD of 4 independent experiments.

A



B



4. DISCUSSION

The experiments described above demonstrate the development of a semi-quantitative reverse transcriptase (RT) triple primer polymerase chain reaction (PCR) assay which allows us to study the regulation of pre-mRNA splicing. mRNA splicing is a complicated process involving a large number of cellular components. Of the many factors involved in pre-mRNA splicing, serine-arginine (SR) rich proteins have been extensively studied and have been shown to play an essential role in the regulation of both constitutive and alternative splicing (reviewed in Blencowe *et al.*, 1999). A number of studies suggest that changes in the phosphorylation state of SR proteins, such as ASF/SF2, influences their activity and nuclear localization (Colwill *et al.*, 1996b, Duncan *et al.* 1997, Duncan *et al.*, 1998, Caceres *et al.*, 1998). Mammalian cells have evolved multiple protein kinases, including SRPK1 and 2 (SR protein kinase 1 and 2) and Clk (CDC2/CDC28-like) kinases, that phosphorylate the arginine-serine (RS) domain of SR proteins and thus regulate SR protein activity (Colwill *et al.*, 1996b) and influence pre-mRNA splicing.

To further address the role of Clk in the regulation of splicing *in vivo*, and determine whether Clk's influence on splicing could be extended to different types of mRNA precursors, the effects of the 4 human Clks on the splicing of the human immunodeficiency virus TAT expression vector (pgTAT) was analysed. We applied the triple primer RT-PCR approach to determine the relative expression of spliced and unspliced TAT mRNA transcripts in 293T cells co-transfected with one of four human GFP-tagged Clk constructs. *In vivo* splicing experiments using the Clk1 minigene indicated that the GFP-tagged constructs, used in these assays, functioned in a similar manner as

did the myc-tagged constructs used in previous splicing assays. Transient transfection of the TAT plasmid alone or cotransfected with the control plasmid, pEGFP, generated primarily the spliced isoform of TAT. This expression was similar to that seen in transfected COS-1 cells when detected by both S1 nuclease protection (Malim *et al.*, 1988) and RT-PCR (Powell *et al.*, 1997). Cotransfections with any one of the four hClk cDNAs (hClk1 to 4) had no effect on the splicing of this HIV-1 TAT vector, as the ratio of spliced to unspliced mRNA was not significantly altered (figure 9). Stable transfection of TAT in 293T cells also produced primarily the spliced isoform of this transcript, as measured by the triple primer RT-PCR, with no significant change in transcript splicing upon transient expression of any of the hClks, (figure 10). The increased usage of the 9S splice site seen with the endogenous E1A transcript in these transfected cells (figure 11), revealed that the lack of splicing seen with Clk is specific to the TAT pre-mRNA and not to the more general effects of poor transfection and/or expression of these Clk vectors.

Although Clk had no influence on the splicing of pgTAT, other studies have shown that all 4 Clks (Clk1 to 4), identified in mouse and human, are involved in mRNA splicing, specifically alternative splicing, possibly through their effects on SR proteins. First, it has been demonstrated that Clk1 can phosphorylate SR proteins, and that overexpression of any of the Clks leads to a redistribution of SR proteins within the nucleus (Colwill *et al.*, 1996b). Second, Clk1 has been shown to interact with splicing factors, including SR proteins, in the yeast-two-hybrid system (Colwill *et al.*, 1996b) and to specifically influence the *in vitro* splicing activity of various SR proteins in reconstituted splicing assays (Prasad *et al.*, 1999). Third, the various Clk pre-mRNAs are themselves

alternatively spliced and recent studies in our lab have shown that all four Clks can regulate the splicing of two pre-mRNA transcripts *in vivo*, that of a Clk1 minigene, and that of an E1A adenovirus expression vector (Duncan *et al.*, 1997, Duncan *et al.*, 1998). These *in vivo* results observed are consistent with results previously seen with a decrease in SR protein activity (Wang *et al.*, 1998).

Numerous studies have shown that TAT mRNA splicing is responsive to alterations in SR protein levels, specifically to ASF/SF2 levels. *In vitro* experiments using nuclear extracts, in which SR proteins are limiting, have shown that HIV-1 TAT pre-mRNA splicing requires the addition of an excess amount of ASF/SF2 (Krainer *et al.*, 1990b, Mayeda *et al.*, 1999) and that ASF/SF2, and not other SR proteins, such as SC35, could commit TAT pre-mRNA to splicing (Fu, 1993). In contrast, *in vivo* experiments have shown that genetic depletion of ASF/SF2 leads to an increase in the splicing of TAT pre-mRNA from a stable transfected minigene in a chicken B-lymphocyte cell line (Wang *et al.*, 1998). These previous experiments regarding TAT splicing, along with results seen with Clk in splicing assays and experiments suggesting Clk's association with and activation of SR proteins such as ASF/SF2, suggested that Clk would have an influence on the splicing of the HIV-1 TAT pre-mRNA.

The unchanged mRNA levels of the HIV-1 TAT transcript tested, following Clk overexpression in 293T cells, versus the positive effects of Clk on the splicing of the Clk1 minigene and E1A transcripts in these and previous *in vivo* studies, may simply reflect general differences in the

regulation of alternative splicing (E1A and ClkII minigene) versus constitutive splicing (TAT). On the other hand, these results may suggest that Clks effects on splicing may be through interactions with SR proteins or other splicing factors that do not influence TAT pre-mRNA splicing. This is supported by the fact that individual pre-mRNAs contain combinations of positive and/or negative cis-acting regulatory sequences (exon splicing enhancers (ESEs) and exon splicing silencers (ESSs)), all of which could interact with particular SR proteins (Amendt *et al.*, 1995). TAT exon 3 (the second exon in the pgTAT vector) has both an ESE and an ESS element as well as an ESS element within its second coding exon (the first exon in the pgTAT vector) (Amendt *et al.*, 1995, Staffa and Cochrane, 1995), all of which have been presumed to regulate its splicing, possibly through the binding of different SR proteins.

The expression of the HIV-1 TAT RNA transcripts, upon the differentiation of murine P19 embryonal carcinoma cells stably expressing the pgTAT vector, was analysed using the triple primer RT-PCR assay. P19 EC cells are pluripotent cells that can differentiate upon cellular aggregation in the presence of differentiating agents such as retinoic acid (RA) and dimethyl sulfoxide (DMSO). The present study shows that the spliced TAT mRNA is predominant in undifferentiated P19EC cells stably expressing TAT and that upon neuronal differentiation with RA and endodermal differentiation with DMSO, the unspliced transcript is expressed at higher levels (figure 13). The expression and splicing of a number of other endogenous transcripts, including the human AT motif binding factor 1 (ATBF1) gene (Miura *et al.*, 1995) and the cloned human cardiac actin (CH-actin) gene, has also been shown to be altered upon differentiation of P19 EC

cells. The molecular basis for these changes in splicing during differentiation is not yet fully understood. Studies looking at the neuronal-specific splicing of the neurotrophin receptor genes *trk* have revealed that the mRNA levels of many splicing factors, including ASF/SF2 decrease rapidly during differentiation of P19 cells with RA (Shinozaki *et al.*, 1999). Based on previous experiments, where ASF/SF2 expression lead to increased TAT splicing (Krainer *et al.*, 1990b) it is conceivable that the increase in HIV-TAT unspliced RNA seen in differentiated cells, is a result of a decrease in ASF/SF2 production.

In summary, we have shown through a semi-quantitative RT-PCR approach that the expression of full length catalytically active Clk does not affect the splicing of HIV-1 TAT mRNA transiently or stably expressed in 293T cells, suggesting that Clk does not influence the splicing of all transcripts. Clk pre-mRNA codes for both a full length (Clk) and a truncated (ClkT) protein upon exon inclusion or exclusion respectively. Whether ClkT alters the splicing of the HIV-1 TAT transcript remains to be seen. Earlier studies in our laboratory have shown that both Clk and ClkT influence the splicing of the Clk1 minigene but in opposite ways to each other (Duncan *et al.*, 1997). As well, previous *in vitro* complementation assays using nuclear extracts have shown that Clk and ClkT have different effects on different transcripts (Prasad *et al.*, 1999).

As well, we have shown that upon differentiation of P19EC cells expressing TAT mRNA there is an increase of unspliced transcript produced suggesting the splicing of this TAT pre-mRNA is repressed in neuronal and endodermal cells. The results presented here, along with other previous studies, support the idea that splicing events are regulated by the expression of a specific set of SR

proteins which may in turn be regulated by kinases with distinct activities and substrate specificities, all of which could be regulated in a cell-, a tissue-, and/or a developmentally specific manner.

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