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Protein kinases and the regulation of mRNA splicing and translation

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Acknowledgments

To the following people, I raise a glass and toast my good fortune for having them in my life:

Thank you John for your mentorship these past years. I've benefited greatly from your "lead by example" approach and system of "subtle manipulations". By allowing me to "muddle through" and find my own way, you've given me a chance to grow. It is for this, I am the most grateful.

To Susan, who has endured with me the deepest depths and shared in the triumphs these past years, making each more memorable and special. If ever they gave out honorary degrees for the "spouses", Susan would surely deserve one.

To all those that I've served along side throughout this whole experience. I've learned so much from these people, and some of it even had something to do with science. Peter and Ninan, are two of those people the likes of which will rarely be seen again. I wish you all success and hope our paths cross again.

Finally to Frank, Jana, and Marek, from whom I've learned almost everything and continue to learn from, long after I thought I'd learned it all. Your "Joie de Vie" inspires me, and renews my excitement for all that life has in store.

Abstract

Regulation of genetic information occurs through many intricate and varied mechanisms. We have explored two such mechanism, splicing and translation initiation, by studying two kinase families involved in these processes. The work in this thesis represents our ongoing efforts to understand the role of these proteins and the mechanisms by which they work. In the first section of this thesis, we present evidence that the Clk family of kinases are able to influence the regulatory process known as alternative splicing. In the second portion, we discuss PKR, a regulator of protein translation, and demonstrate it to be an essential mediator of antiviral defence. In the final chapter, we consider a possible application of the data from our PKR studies, and propose an oncolytic therapy based on putative defects in regulatory pathways controlling PKR and other members of the IFN pathway.

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List of Abbreviations

2'5'OAS	2'5' oligoadenylate sythetase
BFP	blue fluorescent protein
Bp	base pair
cpe	cytopathic effect
CTD	carboxy terminal domain (RNA polymerase II large subunit)
DNA	deoxyribonucleic acid
dsRBDs	double stranded RNA binding domains
DsRNA	double stranded RNA
EIF2α	eukaryotic initiation factor 2 alpha
eIF2B	eukaryotic initiation factor 2B
EMCV	Encephalomyocarditis Virus
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
GAS	gamma activated sequence
GFP	green fluorescent protein
GTFs	general transcription factors
HCV	Hepatitis C Virus
HHF	human foreskin fibroblast
HIV	human Immunodeficiency Virus
IFN	Interferon
IFNAR1	interferon alpha receptor subunit 1
IFNAR2	interferon alpha receptor subunit 2
IFNGR1	interferon gamma receptor subunit 1
IFNGR2	interferon gamma receptor subunit 2
IN	intranasal
IRS	insulin receptor substrate
ISRE	interferon stimulated response element
IV	intravenous
JAK	Janus kinase
kb	kilobase
kDa	kilodalton
LD_{50}	lethal dose for 50% of effected animals
MEF	mouse embryo fibroblast
MOI	multiplicity of infection
mRNA	messenger RNA
PAP	Poly A polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction

pfu	plaque forming units
pi	post-infection
RNA	ribonucleic acid
RRM	RNA recognition motif
RT-PCR	reverse transcription coupled Polymerase Chain Reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS – polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH-2	src homology domain 2
SH-3	src homology domain 3
STAT	signal transducers and activators of transcription
VSV	Vesicular Stomatitis Virus

Chapter 1 General introduction

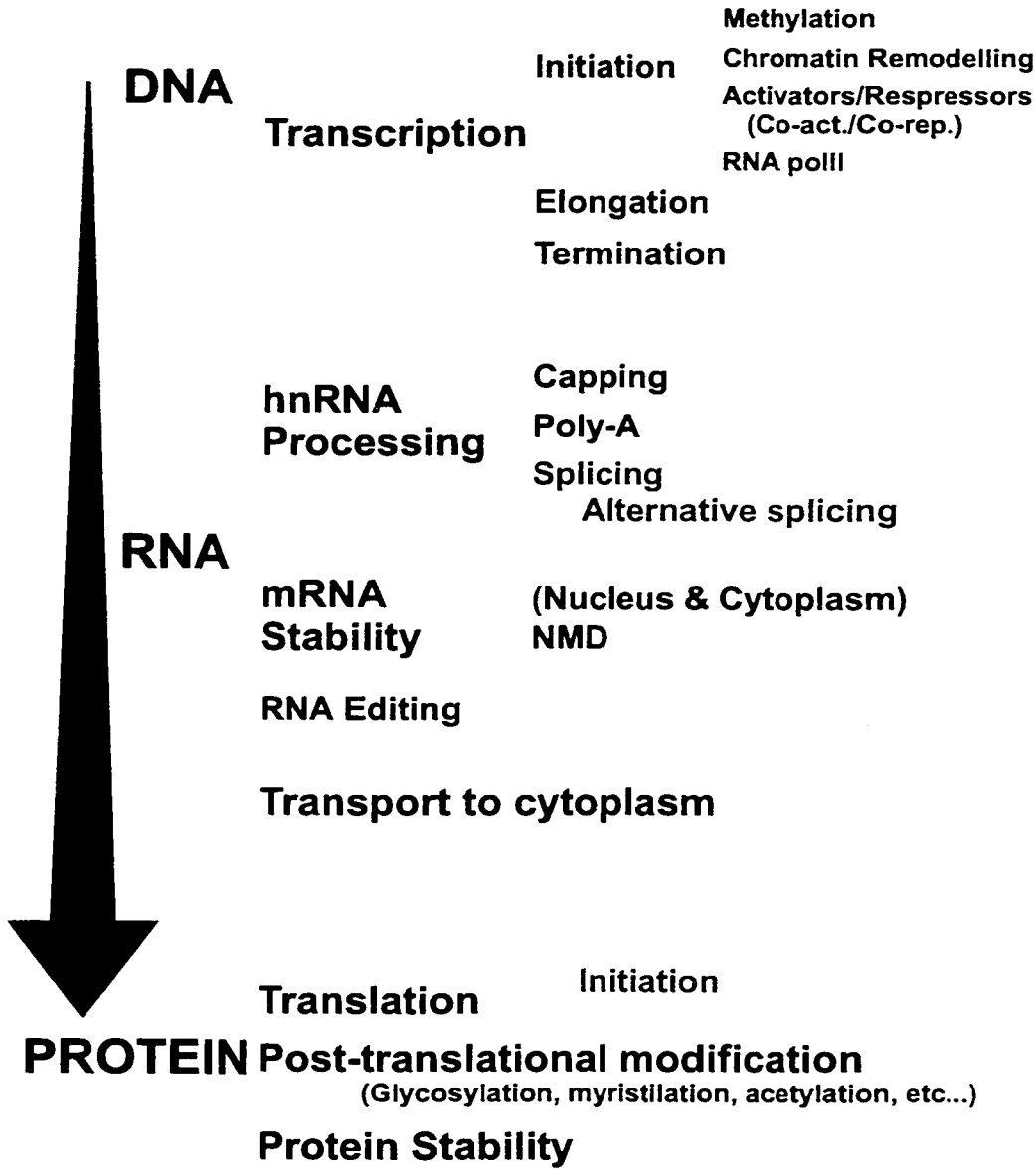
DNA to RNA to Protein

Humans as well as other animals and plants arise from a single cell, into a complex organism made up of billions of cells arranged into organs and tissues. Cells which make up each of the diverse tissues in our body are themselves very different and specialized for their role in that particular tissue. How can a single cell give rise to such precisely controlled diversity? The answer lies in how the flow of genetic information is controlled. Each cell contains the same library of information in the form of DNA; but what makes a lung cell different from a brain cell is the complement of proteins expressed in each of these types of cells. Humans possess some 100 000 genes which code for proteins that carryout all the various tasks by each cell type. The decision to become a particular type of cell (ie kidney, brain, muscle cell, etc) depends on the subset of proteins it expresses. There are a great variety of mechanisms which have evolved to control the flow of information from DNA to RNA and ultimately to proteins. In the following text and accompanying figure (Figure 1), I will briefly introduce several of these processes and highlight the mechanism(s) which relate to the original work in this thesis.

Figure 1. Flow of Genetic information.

A schematic representation of the various regulatory checkpoints encountered as a gene is transcribed and translated. Transcriptional initiation is dependent on a variety of factors including activator/repressor and co-activator/co-repressor complexes facilitating chromatin remodeling and transcriptional transactivation. The RNA polII complex initiates transcription, but must be phosphorylated on its CTD to continue elongation. The nascent RNA must be heavily processed (capped, poly adenylated and spliced) before being exported to the cytoplasm to be translated into a polypeptide by the ribosome complex. See text for more detailed descriptions of these regulatory mechanisms. Indicated in red are the processes most relevant to the thesis.

Figure 1


DNA
Transcription
Initiation
Methylation
Chromatin Remodelling
Activators/Respressors
(Co-act./Co-rep.)
RNA polII
Elongation
Termination
hnRNA
Processing
Capping
Poly-A
Splicing
Alternative splicing
RNA
mRNA
Stability
(Nucleus & Cytoplasm)
NMD
RNA Editing
Transport to cytoplasm
Translation
Initiation
PROTEIN
Post-translational modification
(Glycosylation, myristilation, acetylation, etc...)
Protein Stability

Transcription

DNA contains the primary sequence to all our genes, which in turn encode all our various proteins and RNAs. Along with these coding sequences, the DNA also contains almost all of the necessary regulatory elements required for proper expression of these molecules. Transcription of messenger RNA (mRNA) from the template DNA is a complex process requiring hundreds of proteins and multiple levels of regulation. In its basest form, transcription of mRNA requires that the RNA polymerase II (RNA polII) enzyme complex be brought to the site of a gene which is to be expressed and initiate transcription from the gene's promotor. The RNA polII complex is thought to recognize the proper site of transcription initiation through cis elements in the promoter region of the gene such as the TATA box and CAT box sequences. The proper site of initiation is specified by an intricate network of proteins which bind to cis acting DNA elements in and around the promoter. These complexes can be roughly divided into three groups: the basal factors or general transcription factors (GTFs), the activator/suppressors, and the co-activator/co-suppressors. Activation of transcriptional initiation begins with the binding of activator complexes to their cognate enhancer elements up to tens of thousands of bases away from the actual site of initiation. These activators recruit chromatin remodeling complexes in the form of co-activators with histone acetylating activities, which "loosen" the DNA coiled around histones, exposing cis-elements in the promoter. The activation domains of the activators then attract the first members of the GTFs, TFIIA, TFIID and TBP, which serve to precisely mark the transcriptional initiation site. RNA polII is attracted to this site by the interactions between the mediator/SRB co-activator complex associated with its carboxy terminal tail

(CTD) and the activation domains of the transcriptional activators (reviewed in (12, 69)).

Elongation

Shortly after the initiation of transcription, the RNA pol II transcription complex pauses on the DNA template. This pausing by RNA pol II requires the presence of nucleosomes on the DNA template(18). Alleviation of nucleosome-mediated inhibition of elongation by pol II has been implicated as a major component of activator-dependent stimulation of elongation (18). Phosphorylation of the CTD has been proposed as a molecular switch to re-activate paused transcription (198). An example of this pathway as a regulatory mechanism has been described for the von Hippel-Lindau (VHL) gene product which has been suggested to act as a tumour suppressor protein by inhibiting transcriptional elongation(6).

Post Transcriptional Processing

5' mRNA Capping

All eukaryotic mRNAs are modified at their 5' terminus with the addition of a 7'methylguanylate structure called a cap. Capping of mRNA occurs shortly after transcription initiation, preceding other mRNA processing events such as mRNA splicing and polyadenylation, and is mediated by a capping enzyme recruited to the transcription complex via phosphorylation of the RNA polymerase CTD(117). In the yeast Saccharomyces cerevisiae, capping enzyme is composed of two subunits, the mRNA 5'-triphosphatase (Cet1) and the mRNA guanyltransferase (Ceg1)(29). Caps contribute to mRNA stability as well as enhance mRNA splicing and translation initiation(105). Many links have been demonstrated between the processes of capping, poly-adenylation and splicing, leading some to speculate

that this represents a form of quality control, whereby exon definition is coupled to proper capping and poly-adenylation(125).

Polyadenylation

Following transcription, most eukaryotic mRNAs are cleaved at the 3' terminus and polyadenylated. The constituents of this system have been well characterized and include five important components: the cleavage and polyadenylation specificity factor (CPSF) which interacts with the highly conserved AAUAAA sequence upstream of the cleavage site; cleavage stimulation factor (CstF) which binds to down-stream elements in the 3'-untranslated regions of pre-mRNAs; cleavage factors (CF) I m and CF II m which act in the cleavage step; and poly(A) polymerase (PAP) which is required for efficient cleavage of most pre-mRNAs(125). Poly-adenylation is known to be essential for mRNA stability as well as efficient mRNA export(125). An example of regulated polyadenylation has been described for the IgM heavy chain. In this case, alternative polyadenylation gives rise to either secreted or membrane bound forms of IgM depending on the differentiation state of the B cell(125).

Splicing

Transcribed heteronuclear RNA (hnRNA) contains introns which must be removed during the course of mRNA maturation to create a contiguous coding sequence which can be later translated by the ribosome. Alternative splicing is a process whereby two or more proteins can be coded from a single gene by regulating the selection of the exons to be spliced together. It is well established that splicing, and alternative splicing are important regulatory steps in gene expression(73, 137, 189). A detailed description of the splicing

process is included later in the text.

RNA editing

RNA editing is a post-transcriptional/co-transcriptional modification that results in the generation of nucleotides within an RNA transcript that do not match the bases present within the genome. In mammals, adenosine deaminases ADAR I and II are believed to meditate these events by catalyzing cytidine-to-uridine and adenosine-to-inosine conversions, predominantly by base deamination(16). It occurs in the nucleus, as well as in mitochondria and has been observed in tRNA, rRNA and mRNA molecules in eukaryotes(16). RNA editing within mammalian substrates can have profound consequences in protein function, including the regulation of the serotonin receptor(53) and ADARII itself(167), implicating this post-transcriptional modification as an important step in the production of molecular diversity.

mRNA Stability

Once the mRNA has been processed it can be subject to regulation by degradation. From yeast we know there are 3 steps to mRNA degradation: 1) the poly A tail is progressively degraded from the 3' end towards the 5' end. Pab1p is a poly A binding protein resident on the stable mRNA(200). As the tail gets shorter, more and more pab1p's are displaced until some critical threshold is reached and the final pabp1 is released. This triggers the second event is the cascade, decapping. In this step, the m7GpppG cap is removed through the hydrolysis of the triphosphate linkage(200). DCP1 is an essential gene involved in decapping and is believed to be responsible for this hydrolysis(200). Finally, the cap-less and tail-less mRNA is degraded 5' to 3' by the XRN1 exonuclease gene

product(200). Other 3'-5' exonuclease pathways have been reported but are believed to represent minor constituents in the stability of mRNA.

Nonsense mediated decay

Another mechanism of RNA regulation comes in the form of the nonsense mediated decay pathway (NMD). This process is an mRNA surveillance mechanism akin to DNA surveillance (involved in repair). The NMD system removes two broad categories of aberrant mRNAs including transcriptional mistakes, aberrant splicing including exon skipping and use of cryptic splice sites, and erroneous transport of intron-containing mRNAs to the cytoplasm(38). Where NMD occurs is still contentious, however, many believe it occurs during the first round of translation as the mRNA enters the cytoplasm cap first. Others believe that it may occur by a ribosome scanning-like mechanism in the nucleoplasm, while still others postulate a direct codon recognition mechanism taking place in the nucleoplasm, that is distinct from scanning(38).

mRNA export

Translation occurs in the cytoplasm yet transcription and mRNA maturation occurs in the nucleus. This necessitates the export of processed mRNA across the nuclear membrane, through the nuclear pore complex to the cytoplasm. The protein complexes required for this process are still poorly characterized but appear to be distinct from the well characterized Ran-importin system which is responsible for protein transport across the nuclear pore(130). To date, viral mechanisms of gene regulation by RNA export have been the best characterized, including human immunodeficiency virus (HIV)(50), vesicular stomatitis virus (VSV) and hepatitis B virus (HBV)(212).

Translation

Translation is the process whereby the 4 base code of mRNA is converted to the 20 amino acid code of a polypeptide. It is mediated by a vast number of multi-protein complexes making up the ribosome and its associated initiation and elongation factors, which act in a tightly choreographed, step-wise progression. Translational initiation is generally regarded as the rate limiting step in protein translation, and represents the point at which the majority of regulation takes place(64, 152). Many examples have been reported of translational regulation in response to stress(17), growth factors and the cell cycle (180). A more detailed treatment of translational initiation is included later in the text.

All of the above processes have been implicated in the regulation of genetic information as it proceeds from the template DNA to intermediary RNA molecules, and finally to proteins. The work in this thesis is divided into two sections focusing on two of these regulatory checkpoints: Firstly, splicing or more specifically, alternative splicing as we discuss the Clk family of protein kinases; and secondly, translational regulation involved in the antiviral response mediated by the protein kinase, PKR. Included in this second portion is a final subsection discussing a possible technology derived from our studies of PKR.

Splicing

The genome of higher eukaryotics codes for most of its proteins though discontinuous coding sequences called exons, which are separated by non-coding sequences known as introns. Following transcription of a gene, these exons must be spliced precisely, removing the intervening introns, to form meaningful mature messenger RNAs (mRNA) that are

transported to the cytoplasm and translated by the ribosomal machinery. To add yet another level of complexity, a process known as alternative splicing exists, whereby a single pre-mRNA can give rise to two or more mature mRNAs depending on the combination of exons spliced together. Alternative splicing of pre-mRNAs is emerging as an important mechanism for gene regulation in many organisms. The classic example of splicing as a regulator of genetic information during a developmental process is sex determination in *Drosophila*. The now well characterized cascade of sex specific alternative splicing events demonstrates nicely how the control of splice site selection during pre-mRNA processing can have a profound effect on the development of an organism. The factors involved in pre-mRNA splicing and alternative splice site selection have been the subject of active study in recent years. Emerging from these studies is a picture of regulation based on protein-protein, protein-RNA and RNA-RNA interactions. How the interaction of the various splicing constituents is controlled however, is still poorly understood. One of the mechanisms of regulation that has received attention recently is that of post-translational phosphorylation. The following is a summary of the evidence for a role of phosphorylation in constitutive and alternative splicing and a discussion of some of the recent information on the biochemistry and biology of the enzymes involved.

The "E"-A-B-Cs of the Spliceosome

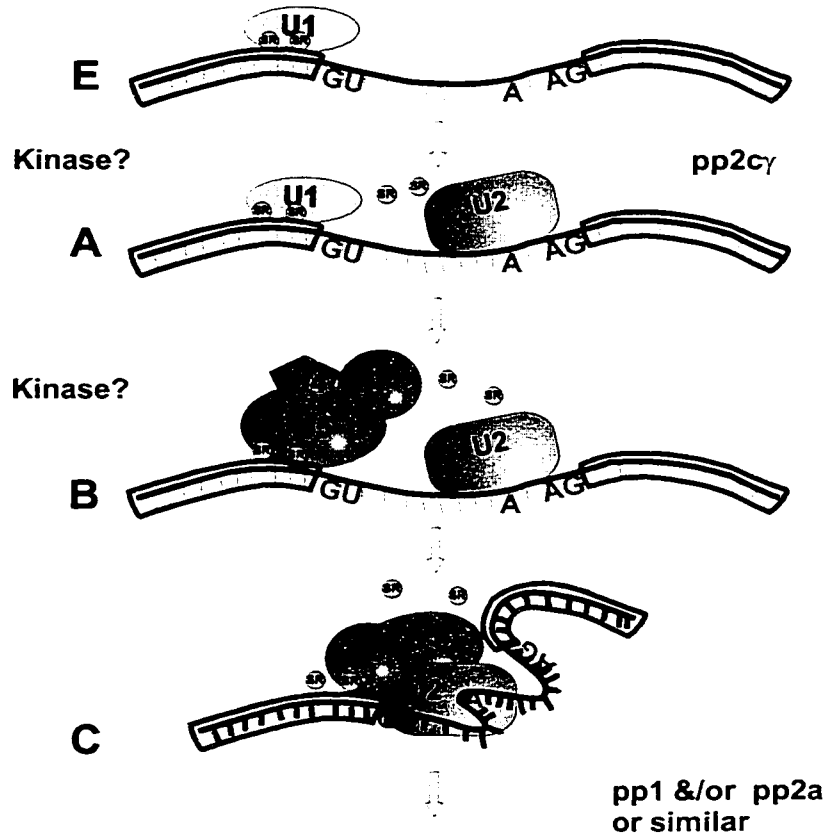
As is becoming the rule for many metabolic events in the cell, splicing of pre-mRNAs is mediated by a macro-molecular machine; in this case, the spliceosome. This mega-complex is assembled in a coordinated manner from 5 subunits termed the U small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4, U5 and U6); each composed of a small nuclear RNA (snRNA) and several or more proteins. The formation of the spliceosome begins by the recognition and subsequent base-pairing of the U1 snRNP to the conserved 5' splice donor sequence on the pre-mRNA (Figure 2). This complex has been referred to as the "E" complex. Next, the U2 snRNP binds to the branch point region of the pre-mRNA and in doing so, forms the "A" complex. The "B" complex is formed with the addition of the U4/U6·U5 tri snRNP complex. A number of rearrangements in the components of the snRNPs results in the base pairing of U6 to U2 and the displacement of U1 at the 5' splice donor, by U6. This leads to the release of U1 and U4 and ultimately to the mature, catalytically active spliceosome known as the "C" complex (157, 184).

Several lines of evidence have recently emerged to suggest that phosphorylation status may play a role in spliceosome maturation. For example, general inhibitors of pp1 and pp2a phosphatase activities, block the catalytic function of the spliceosome, and not the assembly of "A", "B" or "C" complexes (120, 135, 194). Furthermore, thiophosphorylation of a constituent of the U1 snRNP, U1 70K, as well as an SR protein (discussed below) were each shown to block the catalytic steps of splicing, without disturbing spliceosome assembly. In contrast, the phosphatase pp2cγ has recently

Figure 2. Summary of the step-wise assembly of the spliceosome.

The events which are effected by dephosphorylation are indicated by the name of the phosphatase, while the label "kinase?" designates events thought to be dependent on phosphorylation, by an as yet unknown kinase(s) (see text). The U1 , U2 , U4, U5 & U6 small nuclear ribonuclear proteins are shown sequentially binding to a pre-mRNA with two exons (red outline) separated by an intron (see text). The small spheres represent SR proteins. The GU...A...AG lettering in red denotes portions of the consensus sequences at the 5', branch point and 3' extremities of the unspliced intron, respectively.

Figure 2


E
U1
GU
A
AG
Kinase?
pp2cγ
A
U1
U2
GU
A
AG
Kinase?
B
U2
GU
A
AG
C
pp1 &/or pp2a
or similar
Splicing Reactions

been shown to affect spliceosome assembly at the "A" complex. This protein is a nuclear serine/threonine (S/T) phosphatase reportedly associated with the spliceosome throughout the splicing reaction. Therefore, dephosphorylation of the as yet poorly defined targets substrates of the spliceosome, seems to be a prerequisite in formation and activity of the macro-molecular machine. A recent report has demonstrated that phosphorylation of SR proteins (essential factors found associated with the splicing complexes; discussed below) is required for the recruitment of U4/U6·U5 tri-snRNP complex during the formation of the "B" complex(161). Taken together these data support the widely held theory that multiple rounds of phosphorylation and dephosphorylation of components of the splicesome and its accessory factors may be required for assembly and function of the mature spliceosome.

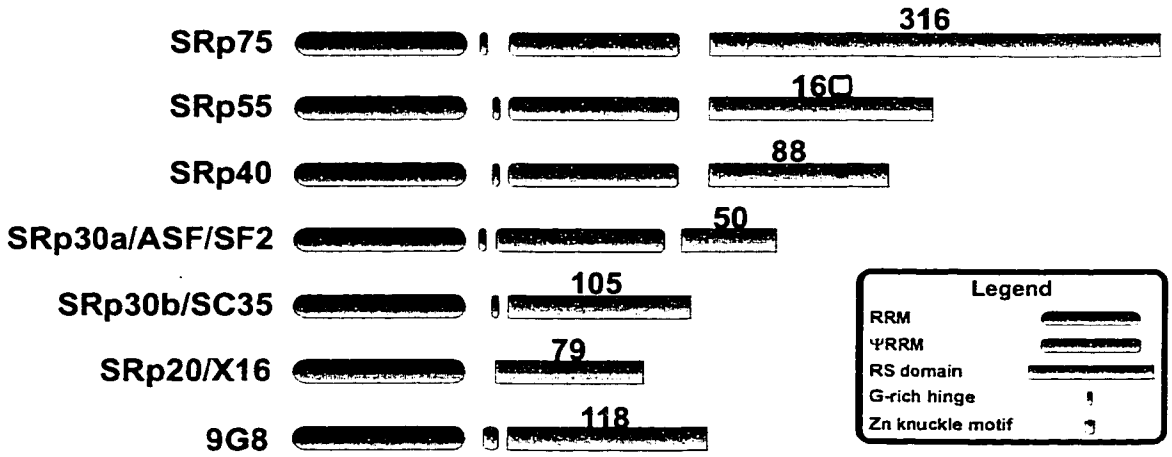
Phosphorylation and SR protein function

In addition to the U snRNPs, many non-snRNP proteins have been identified which play important, indeed crucial, roles during pre-mRNA splicing. One such family of splicing accessory factors is the SR proteins (Figure 3). These proteins are characterized by their common primary amino acid structures and sequences; namely the possession of a COOH-terminal domain rich in arginine/serine dipeptides called the RS domain, and either 1 or 2 amino-terminal RNA recognition motifs (RRMs). SR proteins are required for splice site selection in both constitutive and alternative splicing events. SR proteins bind via their RRM domain(s), to discrete RNA sequences within the exonic and intronic regions of pre-mRNAs. Once positioned by their RRM(s) on the pre-mRNA, SR proteins interact with other components of the splicing machinery through RS domain mediated protein-protein

Figure 3 Schematic representation of the domain structures of human SR proteins.

Each member of the family is composed of one N-terminal RRM-type RNA binding motif and a C-terminal arginine/serine repeat sequence called the RS domain. Several of the larger proteins also contain a RRM-related RNA binding motif, referred to here as a ψRRM, which is often separated from the RRM by a glycine-rich hinge region (also present in SC35). 9G8 possesses a Zn-knuckle motif between its RRM and RS domain which has been reported to contribute to RNA binding specificity (25). The numbers above the RS domains indicate their respective lengths in amino acids.

Figure 3


SRp75
316
SRp55
160
SRp40
88
SRp30a/ASF/SF2
50
SRp30b/SC35
105
SRp20/X16
79
9G8
118
Legend
RRM
ΨRRM
RS domain
G-rich hinge
Zn knuckle motif

interactions (37, 183, 218). It seems apparent from recent studies that different SR proteins bind to distinct splicing enhancer/silencer sequences, and that the combination of SR proteins bound at a particular splice site and the position of their binding relative to the splice site, determines its "strength", or probability that it will be used during the processing of that pre-mRNA (63, 171).As mentioned above, reversible protein phosphorylation is essential for both spliceosome assembly and the catalytic process of splicing (66, 119, 120, 195). While there are many phosphoproteins within the spliceosome it has been widely suggested that the SR protein splicing factors (114, 197)are one of the key targets of regulated protein kinase activity (32, 66, 114, 119, 120, 195, 197). Since the discovery that SR proteins were in fact phosphoproteins, many kinases have been identified which phosphorylate these factors either *in vitro*, *in vivo* or both. Clk/Sty1, 2, 3 and 4 (32, 132), SRPK1 and 2 (66, 96), Lamin B receptor (134), Topoisomerase I (162) as well as the fission yeast kinase DSK1(192) have all been shown to phosphoryate SR proteins *in vitro*. Overexpression of Clks 1-4 leads to the phosphorylation of co-transfected SF2/ASF (an SR protein) *in vivo* (132). As well, Prasad *et al* (150) have demonstrated that Clk mediated phosphorylation of SR proteins is critical for their activity in splicing assays. A recent study has also shown that SRPKs 1 &2 directly bind and phosphorylate SF2/ASF in Hela cells (96). However, the physiological consequences of SR protein phosphorylation remain unclear.

The Clk Kinase

The Clk kinase (also known as Sty) was originally isolated from a mouse embryocarcinoma cDNA library by virtue of its tyrosine-phosphorylating activity; but upon

sequencing the catalytic domain was found to be most similar to protein kinases of the serine/threonine family(1, 2). Subsequent biochemical data has shown that this kinase is able to autophosphoryate on tyrosine as well as serine and threonine(45, 78). These observations are what helped establish Clk as one of the founding members of a new class of kinases: the dual-specificity kinases. The Clk protein can be roughly divided into two domains; a carboxyl-terminal catalytic domain of approximately 320 amino acids, and an amino-terminal non-catalytic domain of approximately 160 amino acids. The amino-terminal region contains a putative nuclear localization signal (NLS), and a RS domain. No other characteristic domains (ie, SH2 domains, leucine zippers, myristylation signals, etc.) are apparent from the sequence thus far. Another relevant feature of this gene is depicted in figure 4. The *Clk* pre-mRNA is alternatively spliced such that inclusion of the alternatively spliced exon gives rise to an mRNA coding for the full-length catalytically active kinase, while exclusion of the exon results in a frame shift mutation leading to a truncated, catalytically inactive protein.

Preliminary studies demonstrate *Clk* mRNA to be ubiquitously expressed, albeit at various levels, in adult mouse tissues. However, expression of *Clk* RNA is developmentally regulated. Embryonic carcinoma cells express two mRNA species approximately 1.7 and 1.8-kb in size(78). Differentiation of embryonic cells *in vitro* leads to the expression of two additional *Clk* transcripts approximately 3.2 and 5.6-kb in size(78).

The Clk Family of Kinases and their Homologues

Since the initial cloning of Clk, several other protein kinases have been cloned that share considerable sequence similarity. Hanes *et al.* (70) independently cloned human Clk1 and two additional human Clk-related kinases, while Nayler *et al* (132)cloned the murine Clk

2, 3 and 4 genes. The genes for these kinases display a wide pattern of expression, similar to that of *Clk1*. Interesingly *Clk2, 3* and *4* show an RNA splicing pattern similar to that of *Clk1*(as in figure 4).

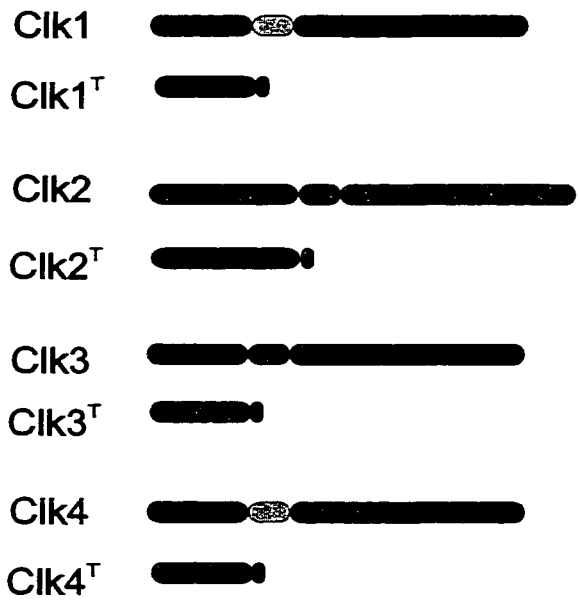
The *Doa* (*Darkener of apricot*) gene was identified in *Drosophila melanogaster* as a dosage-sensitive modifier of the *apricot* allele (w^a) of the *white eye* (*w*) locus(154). An insertion of the copia retrotransposon into the second intron of the *w* gene causes normally red eyes to appear "apricot-coloured". *Doa* mutations dominantly suppress the w^a phenotype and therefore flies expressing mutations in the *Doa* gene have eyes which are darker than "apricot-coloured". Most *Doa* mutations are recessive lethal suggesting that the *Doa* gene product is vital for fly development. The *Doa* gene product possesses intrinsic protein kinase activity although the amino acid specificity is currently unknown. Based on sequence homolgy, *Doa*'s most similar mammalian homologue identified to date is human Clk2.

Bender and Fink (10)isolated *AFC1* (*Arabidopsis thaliana fus3-*

Figure 4. The Clk family of kinases

Schematic representation of the four members of the Clk family of protein kinases. The domain structures of all four are very similar with an N-terminal regulatory domain (containing an RS domain and nuclear localization sequence(s)) and a C-terminal catalytic domain. An alternatively spliced exon resides between these two domains. Inclusion of the exon leads to a full length catalytically active kinase, while exclusion of the exon results in a truncated catalytically inactive form. A matrix of the relative homologies (amino acid identity) of the murine kinases is shown.

Figure 4



	Homology to mClk1	mClk2	mClk3
Clk1	--	--	--
Clk1T			
Clk2	55	--	--
Clk2T			
Clk3	50	59	--
Clk3T			
Clk4	77	56	52
Clk4T			

complementing gene 1) in a screen to identify genes in higher plants that could complement the yeast *Saccharomyces cerevisiae* with mutations in their MAPK signalling pathways. Haploid yeast with mutations in both MAPK homologues, *fus3* and *kss1*, are sterile and cannot mate. *AFC1* is able to complement the *fus3/kss1* double mutant resulting in mating-specific gene expression in haploid yeast and mating of haploid yeast to yield diploids. AFC1 is not a MAPK homologue but most similar to Clk family members (41% identity to Clk kinase domain). Bender and Fink have also isolated two genes highly related to AFC1 called AFC2 (75% identity to AFC1) and AFC3 (68% identity)(10). The amino acid specificity of these kinases is unknown.

KNS1 (*Kinase Next to Spa2*) was cloned from *Saccharomyces cerevisiae* and has no identified function (139). When deleted or overexpressed in yeast *KNS1* displays no overt phenotype suggesting that it is a non-essential gene.

Thus, the Clk family of kinases appear to have been evolutionarily conserved from yeast to man arguing that these kinases probably perform critical function(s).

Translational regulation by eIF2-α phosphorylation

Eukaryotic translation initiation involves the association of an mRNA, an initiating methionine charged tRNA (Met-tRNA$_i$) and the 40S and 60S ribosomal subunits. The step-wise assembly of these components requires the help of a series of eukaryotic initiation factors (eIFs) (reviewed in64, 82). Of these, eIF-2 was determined to play a key role in regulating peptide chain initiation. eIF-2 is a member of the ternary complex consisting of eIF-2, Met-tRNAi and GTP which is required for the formation of the 43S preinitiation

complex (152). eIF-2 itself is a complex of three subunits, eIF-2α, eIF-2β and eIF-2γ (92). Subsequent binding of mRNA and the 60S ribosomal subunit is dependent on GTP hydrolysis to form a functional 80S initiation complex, and results in the release of eIF-2-GDP. For re-initiation to occur, eIF-2 must exchange the GDP for GTP to catalyze another cycle of initiation (92). This guanine nucleotide exchange is mediated by the eIF-2B. However, phosphorylation of eIF-2α inactivates eIF-2B, preventing GTP exchange and hence, ternary complex formation (177). In this way, phosphorylation of eIF-2α can block protein synthesis in response to a variety of triggers (ie. cellular stress (71), heme deprivation (28), amino acid starvation (77), heat shock (173), Ca2+ mobilization (151), and viral infection (168)).

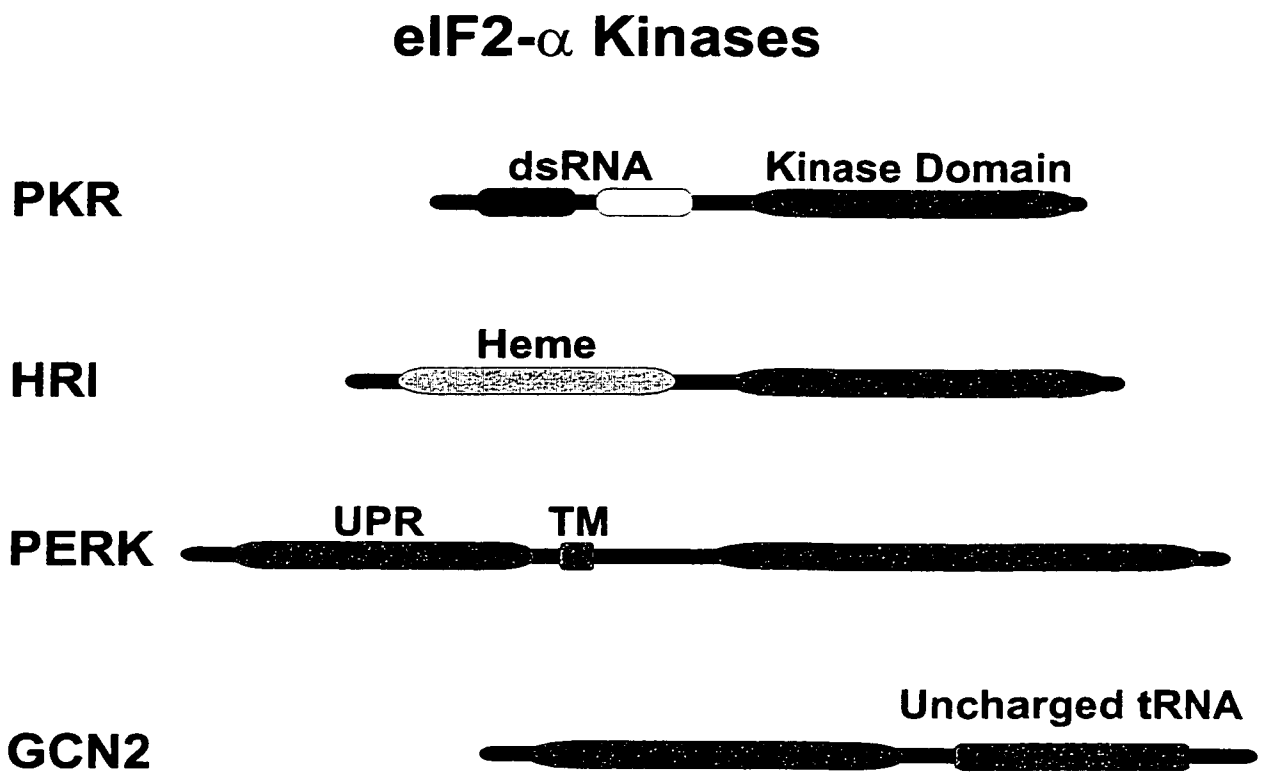
dsRNA dependent protein kinase, PKR

PKR is a member of the eIF2-α protein kinases, which all demonstrate the ability to phosphorylate eukaryotic initiation factor 2 α (eIF2-α) on serine 51. The consequences of this phosphorylation event are to block protein synthesis at the initiation step (discussed in detail below). Figure 5 shows a schematic of the eIF2-α kinases displaying the common kinase domain with variable regulatory domains responsive to divergent signals. As its name suggests, PKR is activated upon binding double stranded RNA, and as a dimer, autophosphorylates and then catalyses the phosphorylation of its substrate. The domain structure of PKR can be divided into a C-terminal catalytic domain with a N-terminal

Figure 5. The eIF2α kinases

Schematic representation of the members of the eukaryotic initiation factor α (eIF2α) kinase family and their domain structures. Each of these protein kinases has a similar kinase domain (orange), and all are able to phosphorylate eIF2α on serine 51. They are divergent in their regulatory domains and hence each responds to a unique stimulus. PKR has two dsRNA binding domains and is activated by dsRNA. Heme-regulated inhibitor (HRI) is activated by the absence of heme, and phosphorylates its substrate in response to low heme levels. PERK, an ER resident protein, is activated upon accumulation of unfolded proteins and hence blocks further protein synthesis by phosphorylating its substrate. GCN2 is a yeast kinase which halts protein synthesis in response to amino acid starvation. UPR is unfolded protein response. TM denotes a transmembrane domain.

Figure 5


eIF2-α Kinases
PKR
dsRNA
Kinase Domain
HRI
Heme
PERK
UPR
TM
GCN2
Uncharged tRNA

regulatory domain. The regulatory domain consists of two dsRNA binding domains (dsRBDs) which act co-operatively to bind RNA duplexes of 80bp or larger (113). The catalytic domain of PKR consists of the standard eleven subdomains common to all protein kinases with the addition of a 28 amino acid kinase insert domain of unknown function, between subdomains four and five (36).

PKR is transcriptionally induced subsequent to type I and type II interferon stimulation(98, 121). Many viruses display dsRNA genomes or intermediates during their life cycles, which could activate PKR. Biochemical studies of PKR have demonstrated the activation of PKR and the phosphorylation of eIF2α in response to viral infection and *in vitro* experiments have indicated a protective role for PKR against viral pathogens(13, 104, 122, 136, 158). Taken together, most researchers now agree that PKR plays a role in antiviral defense. In fact, it is clear now that many viruses such as influenza, adenovirus, vaccinia, hepatitis C, herpes simplex, polio and human immunodeficiency virus have devised various methods of counteracting this antiviral mediator(191). However, at the time we initiated this study, no clear role for PKR had been established on an organismal level.

Interferons

Discovered in 1957, interferons (IFNs) represent a class of multifunctional cytokines with potent antiviral properties as well as antiproliferative effects on target cells(81). Interferons have been characterized as the primary innate immune response mechanism against viruses and other pathogens. There are two classes of interferons: type I IFNs and type II IFNs. The family of type I IFNs includes IFNα, IFNβ and IFNω while IFNγ

represents the sole type II IFN (142). They exert their effects primarily by inducing the synthesis of many proteins required for the various antiviral and anti-proliferative pathways employed by the immune system to counter pathogens and tumour cells.

Type I IFNs

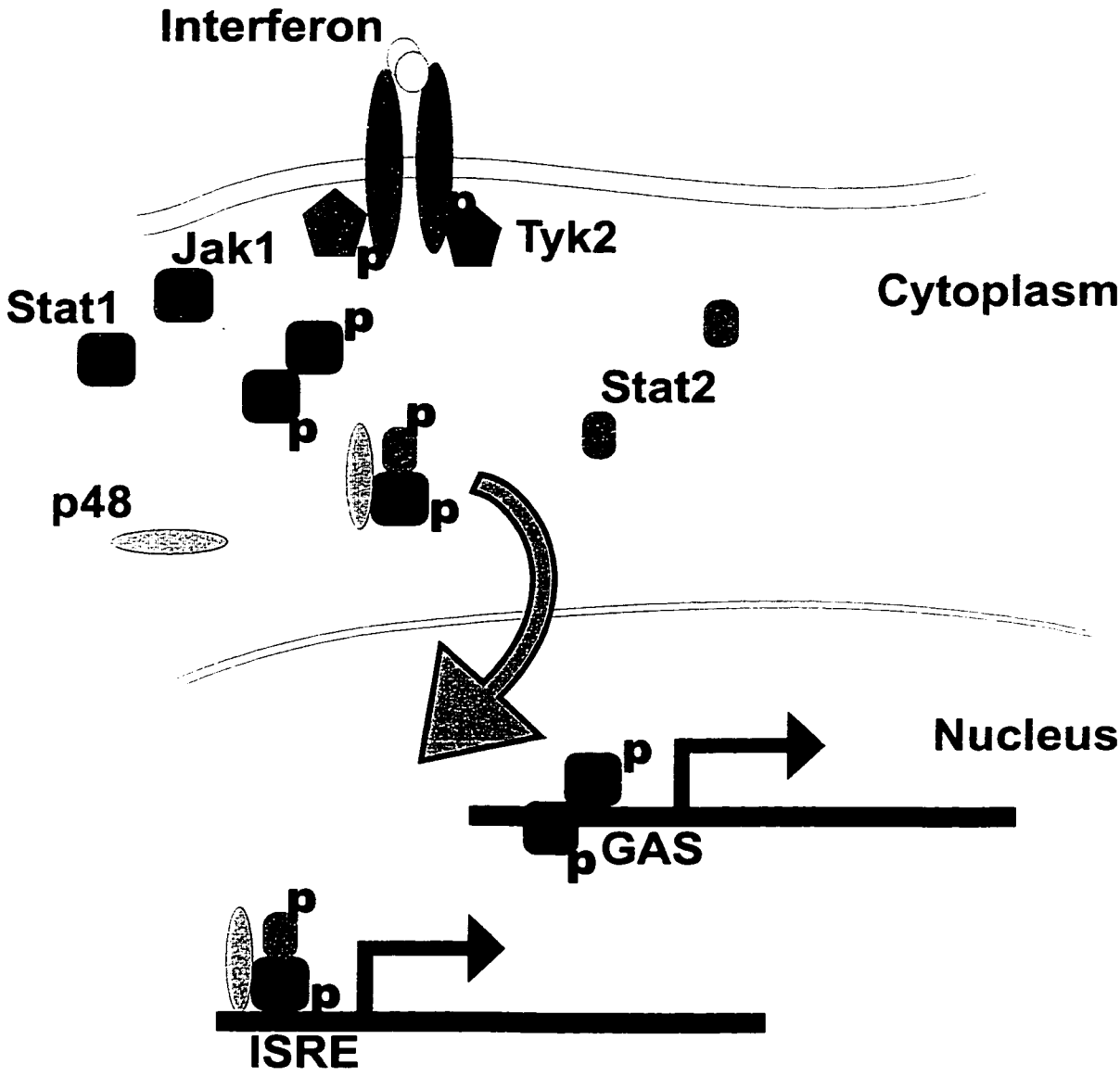
Type I IFNs are secreted by almost all cells in response to various biological stimuli such as viruses, bacteria, mycoplasma, as well as a host of cytokines and growth factors. IFNs α, β and ω bind to their common type I receptor, present on almost all cells, which initiates a signaling cascade responsible for the activation of a variety of IFN inducible genes. The type I receptor has two subunits, IFNAR1 and IFNAR2 which associate upon binding type I IFNs and activate the cytosolic tyrosine kinases: Jak1 and Tyk2 (Figure 6). Following activation, Jak1 and Tyk2 phosphorylate members of the STAT family of transcription factors which form hetero or homodimers and translocate to the nucleus. Once in the nucleus, STAT transcription complexes bind to sequence elements in the promoters of type I IFN induced genes and stimulate the transcription of these genes (185).

Another major signaling pathway activated by type I IFNs involves the insulin receptor substrate (IRS) system. IRS-1 and IRS-2 are rapidly phosphorylated on tyrosine in response to type I IFN stimulation, creating binding sites for the Src homology 2 (SH-2) domains of several signal transduction molecules such as PI-3' kinase and the Src kinase family members, Fyn and Lyn(199). Activation of PI-3' kinase is believed to be required for the proper activation of the STAT transcription complex and facilitates its complement of full

Figure 6. The IFN pathway

Diagram representing the interferon signaling pathway. Interferon (IFN) binds to its receptor and initiates a signaling cascade involving the JAK protein kinases and the Stat family of transcription factors. Following activation by phosphorylation, Stat oligomers translocate to the nucleus where they recognize and bind to GAS (interferon gamma activated sequence) or ISRE (interferon stimulated response element) DNA motifs to stimulate the transcription of IFN inducible genes.

Figure 6


Interferon
Jak1
Tyk2
Stat1
Cytoplasm
Stat2
p48
Nucleus
GAS
ISRE
p

functions(52). The biological effects of the activation of IRS proteins via type I IFNs is still unclear, although mice lacking both IRS-1 and IRS-2 are resistant to the anti-proliferative effects of type I IFNs (185).

Type I IFNs also signal through SH-2 and SH-3 domain containing adaptor proteins which link this signal cascade to other signaling pathways involving the G-protein Rap-1. In response to type I IFNs, Tyk-2 activates an adaptor protein CBL which binds and activates the Crk adaptor proteins CrkL and/or CrkII (169). These adaptor proteins interact with and stimulate the guanine nucleotide exchange C3G, which in turn activates the Rap-1 G-protein (51). This may explain why IFNs are growth inhibitory agents since Rap-1 in known to activate primarily growth inhibitory kinase cascades (93).

Type II Interferons

IFNγ is produced only by lymphocytes such as T cells and natural killer cells in response to IL-2 or antigen-mediated stimulation (174). Signalling through the type II IFN receptor proceeds very similarily to the type I signal cascade. Janus family tyrosine kinases Jak-1 and Jak-2, resident at the cytoplasmic portion of the type II receptor, are activated subsequent to the ligation of IFNγ with the two receptor subunits which make up the type II receptor; IFNGR1 and IFNGR2. These Jaks then phosphorylate STAT-1proteins which subsequently homodimerize and translocate to the nucleus where they transactivate the transcription of type II IFN induced genes.

IFNγ also signals through the CBL/Crk pathway similarily to type I IFNs(147), but do not activate the IRS signaling system(148).

Interferon induced antiviral pathways

Following the activation of the type I and type II IFN receptors, a cascade of signal transduction events leads from the cytoplasm into the nucleus which leads to the induction of a variety of genes required for antiviral defence. The four major IFN mediated antiviral systems which have been identified include: the Mx proteins, the 2'-5' oligoadenylate synthase pathway, nitric oxide synthase and the double-stranded RNA dependent protein kinase, PKR (Figure 7). Each of these pathways is stimulated to varying degrees by both type I and type II IFN, although most respond better to type I stimulation (185).

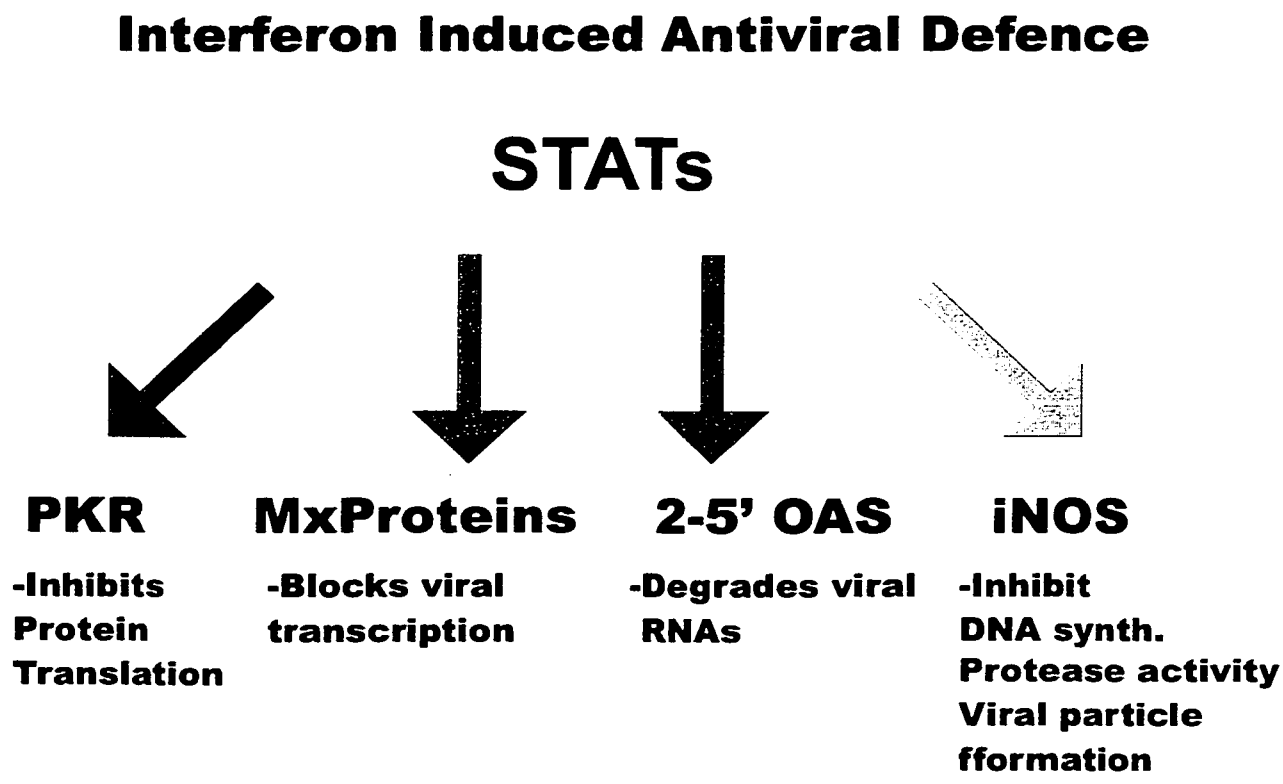
The Mx proteins members of the dynamin superfamily of large guanosine triphosphatases which are believed to inhibit viral replication by binding viral polymerase proteins and abolishing normal viral polymerase function(140). Interaction of Mx with viral targets is probably a GTP-dependent process as GTPase mutants are ineffective at blocking viral replication. Mx proteins have been demonstrated to confer resistance to influenza virus and vesicular stomatitis virus but not to other viruses (140).

Inducible nitric oxide synthase (iNOS) catalyses the production of nitric oxide from L-arginine. This chemical has pleiotropic effects in the host organism as a neurotransmitter, but has also been implicated in the defence against viral infection. It is believed to mediate its antiviral effects by inhibiting late stages of viral replication(159) including protein synthesis (72, 91)viral protease activity (170), viral DNA synthesis (72, 118)and viral particle

Figure 7. Interferon stimulated antiviral pathways

Interferon is responsible for the activation of a variety of antiviral mediators. The four best characterized are summarized in this figure together with their suspected modes of action. See text for details.

Figure 7


Interferon Induced Antiviral Defence
STATs
PKR
-Inhibits Protein Translation
MxProteins
-Blocks viral transcription
2-5' OAS
-Degrades viral RNAs
iNOS
-Inhibit DNA synth. Protease activity Viral particle fformation

formation (72).

2',5'-Oligoadenylate synthetase (2',5'-OAS) was discovered and characterized as an interferon (IFN)-induced enzyme that in the presence of double-stranded (ds) RNA converts ATP into 2',5'-linked oligomers. These short oligomers then activate RNAse L which degrades viral RNA and host rRNA (156). The 2',5'-OAS system is thought to act as an antiviral mechanism not only by directly degrading viral RNAs, but also by inducing apoptosis of infected host cells(24)

Chapter 2 In vivo regulation of SR proteins and alternative pre-mRNA splicing by the Clk family of protein kinases

Summary

Controlled expression of cellular and viral genes through alternative precursor messenger RNA (pre-mRNA) splicing requires serine/arginine-rich (SR) proteins. The Clk1 kinase, which phosphorylates SR proteins, is regulated through alternative splicing of the *Clk1* pre-mRNA, yielding mRNAs encoding catalytically active and truncated inactive polypeptides (Clk1 and $Clk1^T$, respectively). We present evidence that Clk1 and $Clk1^T$ proteins regulate the splicing of *Clk1 in vivo*. The peptide domain encoded by the alternatively spliced exon of *Clk1* is essential for the regulatory activity of the Clk1 kinase. We further show that Clk1 and other members of the Clk family of kinases (Clk2 and Clk3) regulate the alternative splicing of a viral pre-mRNA based reporter minigene, *in vivo*. This is the first direct demonstration of an *in vivo* link between alternative splicing and protein kinase activity.

Introduction

SR proteins are essential for both general splicing and the regulation of alternative splicing (21, 55, 116, 211). SR proteins accumulate within sub-nuclear domains called speckles where they are believed to be stored or pre-assembled into spliceosomes (56, 164, 182). The Clk kinases are able to phosphorylate SR proteins and cause their redistribution within the nucleus implicating these enzymes in the regulation of SR protein splicing activity (32, 47, 66, 132). The objective of the work presented in this section was to examine the relationship between the Clk family of kinases and the SR protein factors. We also wished to determine whether Clks play a role in alternative splice site selection. This required the

development of an assay to monitor the *in vivo* splicing of one or more reporter minigenes following the expression of Clk family kinases or mutants thereof. Our findings are consistent with a role for Clk kinases as an interface between signal transduction pathways and the splicing machinery.

Materials And Methods

Plasmid Construction

GFP-ASF was a gift from Dr. David Spector's lab(127). Pece-MClk1 and pece-MClk1T, peceMClk1$^{\Delta 130\text{-}158}$, CMV/CR-1 and CMV/CR-2, pece-MClk2, pece-MClk3 were all generated by Peter Duncan in our lab and are described elsewhere(46). GFP-Clk1 and GFP-Clk1^{T} were constructed by fusing the EcoR1 fragments from pece-Mclk1 or pece-Mclk1T, respectively(45), to a EcoR1 digested pEGFP-C1 vector (Clontech). The resulting construct creates a N-terminal fusion of GFP with Clk1 or Clk1^{T}. PCR was used to introduce a HindIII site in the 5' end and a AgeI site in the 3' end of Clk2, 3 and 4. The HindIII/AgeI fragments of Clk 2, 3 and 4 were subsequently cloned into either pEGFP-C1 or pEGFP-N1 vectors digested with HindIII/AgeI, to create N and C terminal fusion with GFP, respectively. BFP N and C terminal fusions of Clk1, 2, 3, 4 and Clk1T were constructed by swapping the GFP from the corresponding constructs described above with a PCR product encoding BFP.

Live subnuclear localization of GFP and BFP Clks

Fluorescently tagged Clk fusion constructs were transiently transfected into Cos-1 cells grown on gelatin-coated coverslips, using lipofectamine (as per manufacturer's

instructions; Gibco BRL). Coverslips were flipped onto a 30 µl drop of live mounting medium (phenol red-free DMEM/F12 HAM culture media supplemented with 5 % FBS) and sealed with molten 2% agarose. Slides were kept at 37 ^{0}C and visualized promptly using a Zeiss Axiophot epifluorescence microscope. Images were captured using a CCD camera. No further manipulation of the images was required.

FRET imaging

Cells were plated on gelatin-coated coverslips and transfected with various GFP or BFP fusion constructs using lipofectamine (as per manufacturer's instruction; Gibco BRL). After 16 hours, the coverslips were washed with PBS and fixed with either methanol or 4% paraformaldehyde. Fixed samples were mounted in 50% phenol red-free medium and glycerol mixed with antifade reagent (Prolong; Molecular probes). Images of the fluorescent cells were obtained using a Zeiss 410 laser scanning confocal microscope (LSCM). BFP fusion proteins were visualized by excitation at 354 nm and detection at 440 nm with a 360 nm dichroic filter. GFP fusions were observed by excitation at 488 nm and detection at 525 nm with a 488 nm dichroic filter. The FRET signal was measured by excitation at 354 nm and detection at 525 nm with a 488 nm dichroic. The resulting images were pseudo-coloured for convenience using Corel Photopaint software (Corel Corp.). Photo-bleaching was performed by scanning a selected region of interest using an un-attenuated laser for an extended period of time until no detectable fluorescence was apparent.

Transfections, protein and RNA isolations

COS-1 cells or 293T cells (44) (60mm dish) were transfected as indicated using Lipofectamine (Gibco BRL) as per the manufacturer's instructions. Filler plasmid DNA was

used where applicable to equalize total concentrations of DNA for each transfection. Cells were harvested in phosphate buffered saline (PBS) after 24 hours and treated as follows. Protein lysates were generated from one quarter of the cells by the addition of SDS sample buffer. Total RNA was extracted from the remaining three quarters using RNeasy purification (Qiagen).

Immunoblots

Immunoblots were probed with 9E10 anti-Myc (48) and E7 anti-β-tubulin (obtained from the ATCC) monoclonal antibodies and visualised with horseradish peroxidase-conjugated anti-mouse antibody and chemiluminescence. Alternatively, anti-HA 12CA5 monoclonal antibody (BABCO Inc.) was used to detect HA-Clkl polypeptides.

Immunohistochemistry, confocal microscopy

For immunofluoresence, COS-1 cells were transfected, fixed and stained as previously described (45). Monoclonal antibody 104 (164) was used to detect SR proteins, while 9E10 anti-Myc monoclonal antibodies detected full length and mutant M-Clk1 proteins following transfection. Cells were viewed by confocal microscopy using an upright Leica Confocal Laser Scanning Microscope equipped with a 55 mW krypton/argon air-cooled laser and a 63X Plan Apo oil immersion lens.

Reverse transcription-coupled PCR

For CMV/*Clk*1, CMV/*Clk*1^{K190R}, CMV/*CR*-1 and CMV/*CR*-2, 100 ng RNA was processed for RT-PCR by reverse transcription followed by amplification using the following primers: $^{5'}$tggtaggagtggaagaag$^{3'}$ (corresponding to positions 397-414 in the *Clkl* cDNA (78))

and $^{5'}$gatggctggcaactagaa$^{3'}$, directed against the Bovine Growth Hormone polyadenylation signal of the pcDNA3 vector. PCR conditions, including number of cycles and template concentrations, were optimized to maintain linearity during amplification. Linearity was monitored by serial dilution of the template cDNAs, demonstrating a corresponding decrease in signal, as well as consistent ratios between bands of interest (EB^+/EB^-). PCR products were separated on 1.5% agarose gels, stained using SYBR Green I (Molecular Probes), visualised and quantitated using the STORM fluorescence imager (Molecular Dynamics). The identity of the spliced CR-1 mRNAs was confirmed by direct sequencing of the PCR products in Figure 2, whereby the larger PCR product is derived from spliced RNA containing exon EB and the smaller product corresponds to spliced RNA in which exon EB has been skipped. RT-PCR analysis to detect EIA specific mRNAs was as described above, using EIA specific primers (205).

Results

Intracellular localization of GFP and BFP fusion proteins of Clks 1-4 and Clk1^T

The primary sequences of each of the Clk family members have been shown to contain SV40-like nuclear localization sequences in the N-terminal domain, predicting nuclear expression of these proteins. To observe the localization of these kinases, N-terminal and C-terminal fusions of each kinase with green (GFP) or blue (BFP) variants of green fluorescent protein were constructed. These fusion protein constructs were transiently transfected into a variety of cell lines to confirm earlier results obtained by indirect immunofluorescence for the localization of Clk1 and Clk1^T (32), and to extend these studies to Clk2, Clk3 and Clk4. As in these earlier studies, all 4 Clk kinases were found in the

nucleus of transiently transfected live Cos-1 cells (Figure 1, panels A-D). Furthermore, Clks 1, 2 and 3 all appeared to have similar sub-nuclear localization patterns. All three kinases appeared nucleoplasmic with a few punctate regions of expression scattered throughout the nucleus. Clk4 however, appeared to have no nucleoplasmic staining, with very many small symmetrical punctate regions of expression (Figure 1, panel D). The truncated isoform of Clk1 termed Clk1^{T} also differed in its sub-nuclear localization from that observed for the full length Clk1 kinase. Clk1^{T} appeared to localize to several large punctate regions within the nucleus with little or no nucleoplasmic staining (Figure 1, panel E). These experiments were all carried out in NIH 3T3, Hela, and 293T cell lines, with similar results (data not shown).

Truncated Clks Co-localize with SR proteins in the nucleus

The staining pattern of GFP-Clk1^{T} expressed in Cos-1 cells described above was reminiscent of the sub-nuclear localization described for SR proteins(163-165). Using a monoclonal antibody recognizing a phosphoepitope common to all SR proteins (mab-104), we examined the localization of SR proteins in cells transiently transfected with Myc epitope tagged Clk1^{T}, Clk2^{T} or Clk3^{T}. SR proteins are known to localize to discrete punctate regions known as nuclear "speckles" (163-165). In all cases, the truncated Clks appeared to co-

Figure 1. Subnuclear localization of the Clk kinases.

GFP and BFP N-terminally tagged Clks were transiently expressed in Cos-1 cells and observed live. GFP tagged Clk1 kinase (A), GFP -Clk2 (B) and BFP-Clk3 (C) appear predominantly nucleoplasmic, often with several punctate regions of fluorescence. (D) BFP-Clk4 and GFP-Clk4 (inset) show a different pattern of subnuclear localization with no nucleoplasmic fluorescence and 30-50 very small, punctate regions of fluorescence designated here as "microspeckles". (E) GFP-Clk1^{T} appears confined to several large punctate regions within the nucleus with no nucleoplasmic staining.

Figure 1

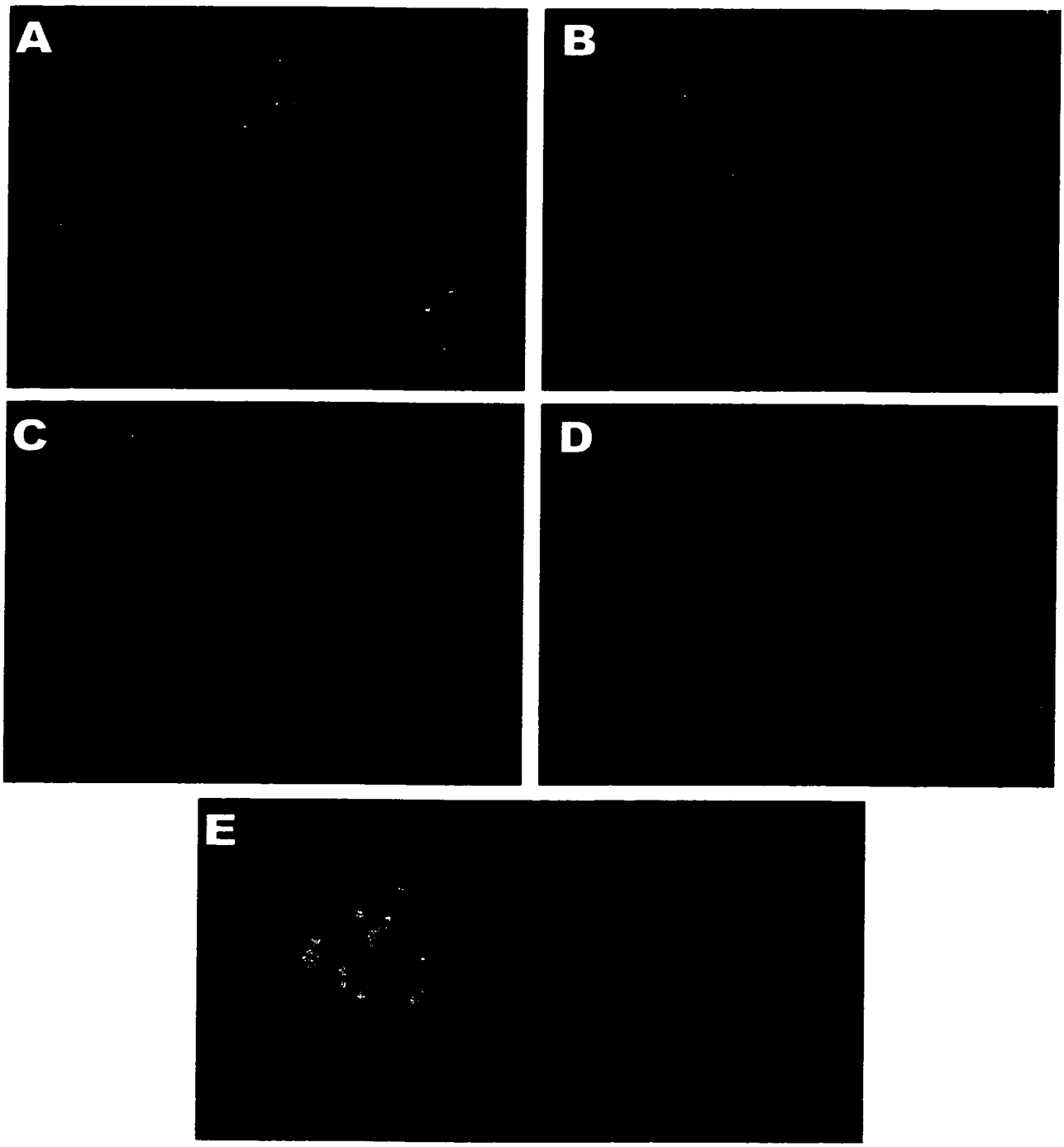
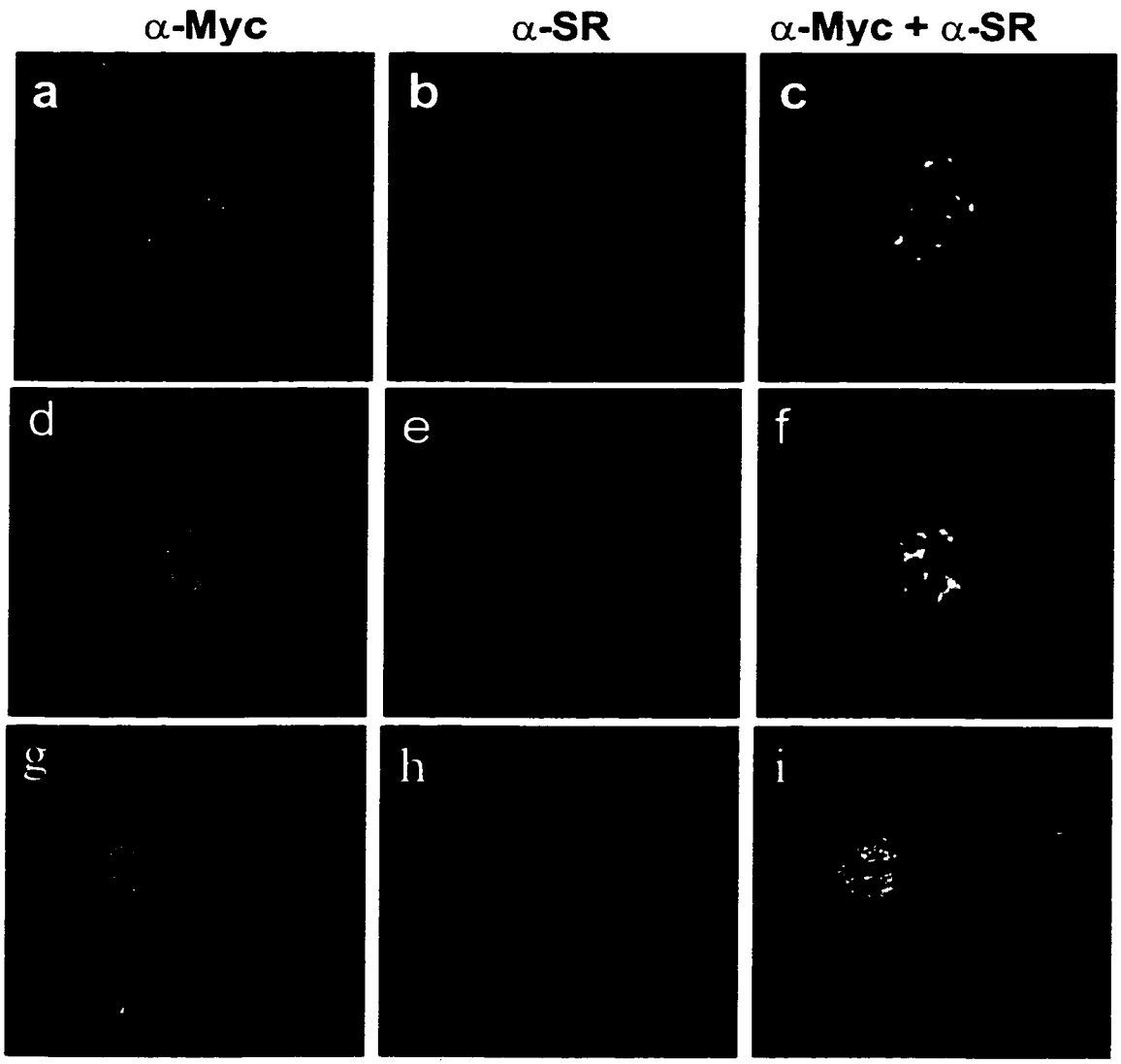


Figure 2. Truncated Clks co-localize with SR proteins

Indirect immunofluorescent staining and confocal microscopy of transfected COS-1 cells using the anti-Myc 9E10 monoclonal antibody (mAb) (α-Myc, panels a,d,g), anti-SR 104 mAb (α-SR, panels b,e,h) or an overlay of the two signals (α-Myc+α-SR, panels c,f,i). Alignment of green and red signals appears yellow. Cells were transfected with pECE expression vector encoding $Clk1^T$ (panels a-c), $Clk2^T$ (panels d-f) or $Clk3^T$ (panels g-i)

Figure 2



localize with SR proteins in these "speckles" (Figure 2, panels a-g). $Clk3^T$ however, appeared much more nucleoplasmic in staining with only a few "speckles", but each of these appeared to co-localize with a corresponding SR protein containing speckle (Figure 2, panels g-i).

Full Length Clks can release SR proteins from nuclear speckles

Full length, catalytically active Clks 1, 2 or 3 were transiently transfected into Cos-1 cells to determine their effects on the sub-nuclear localization of SR proteins. Using the anti-SR monoclonal antibody mab-104, we observed that in cells expressing any of the three Clks kinases, SR proteins were no longer confined to discrete nuclear speckles, but appeared nucleoplasmic in their staining pattern (Figure 3, *panels a-i*). In these same cells, the Clk kinases were also nucleoplasmic as observed previously. Clk3 demonstrated a lesser ability to modify SR protein localization than did Clks 1 and 2. This is apparent in figure 3, panel g where a cell expressing less Clk3 (less intense green fluorescence) was not able to re-localize SR proteins (Figure 3, panel h), as is the case for higher expressors (faint α-Myc staining; Figure 3, *panels a & d*) fail to "de-speckle" SR proteins (Figure 3, *panels a-f*). This phenomenon was not observed with lower expressors of Clks 1 and 2 (data not shown).

Clks co-localize in the nucleus

The sub-nuclear localization of Clks1 2 and 3 was demonstrated to be similar and therefore we predicted that they would perhaps co-localize in the nucleus. To examine this we transiently expressed pairs of GFP and BFP tagged Clks corresponding to each possible combination (ie GFP-Clk1 with BFP-Clk2, GFP-Clk3 with BFP-Clk2 etc...) and observed

Figure 3. Clk kinases relocalize SR proteins to the nucleoplasm

Indirect immunofluorescent staining and confocal microscopy of transfected COS-1 cells using the anti-Myc 9E10 monoclonal antibody (mAb) (α-Myc, panels a,d,g), anti-SR 104 mAb (α-SR, panels b,e,h) or an overlay of the two signals (α-Myc+α-SR, panels c,f,i). Alignment of green and red signals appears yellow. Cells were transfected with pECE expression vector encoding Clk1 (panels a-c), Clk2 (panels d-f) or Clk3 (panels g-i).

Figure 3

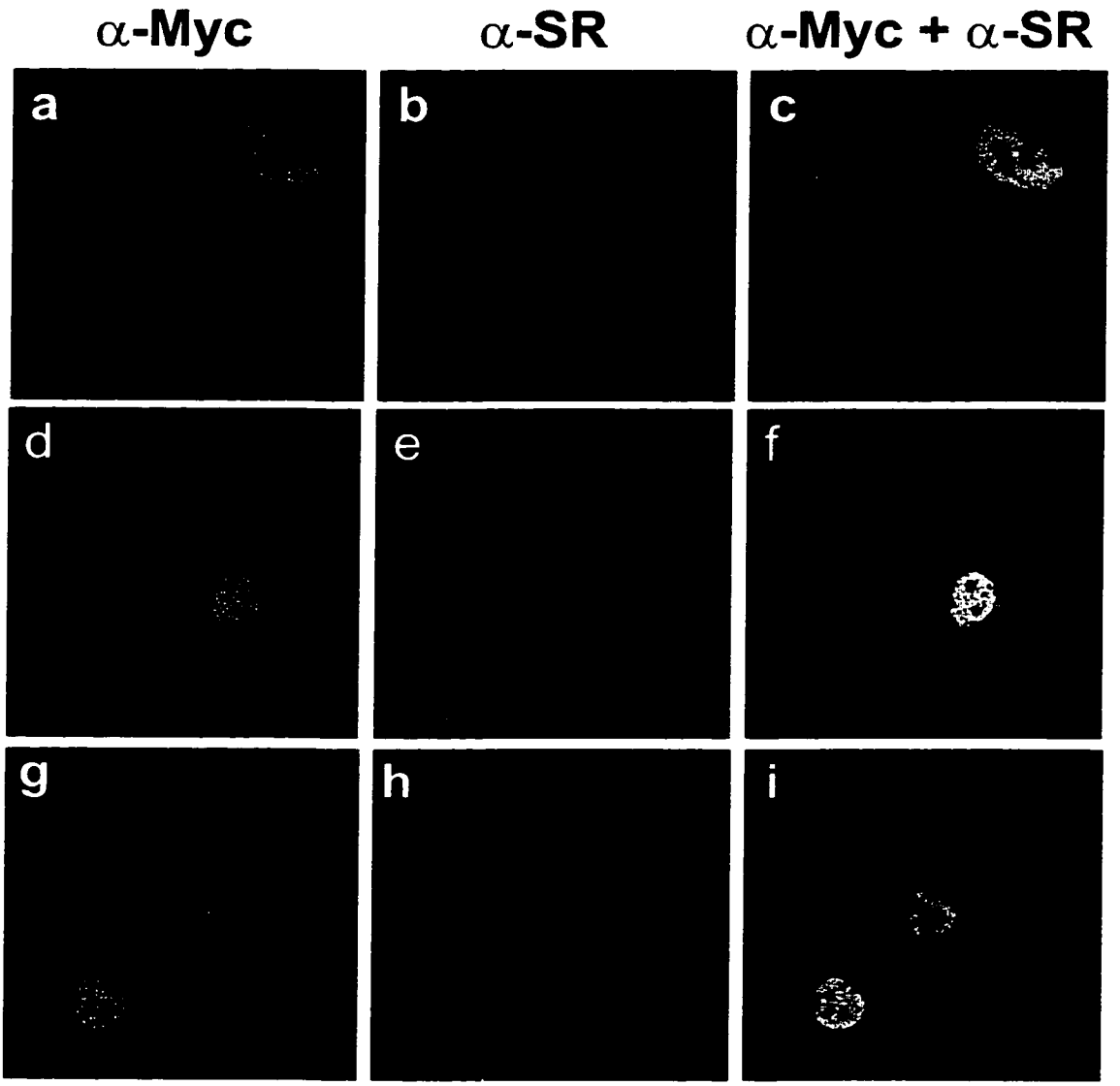
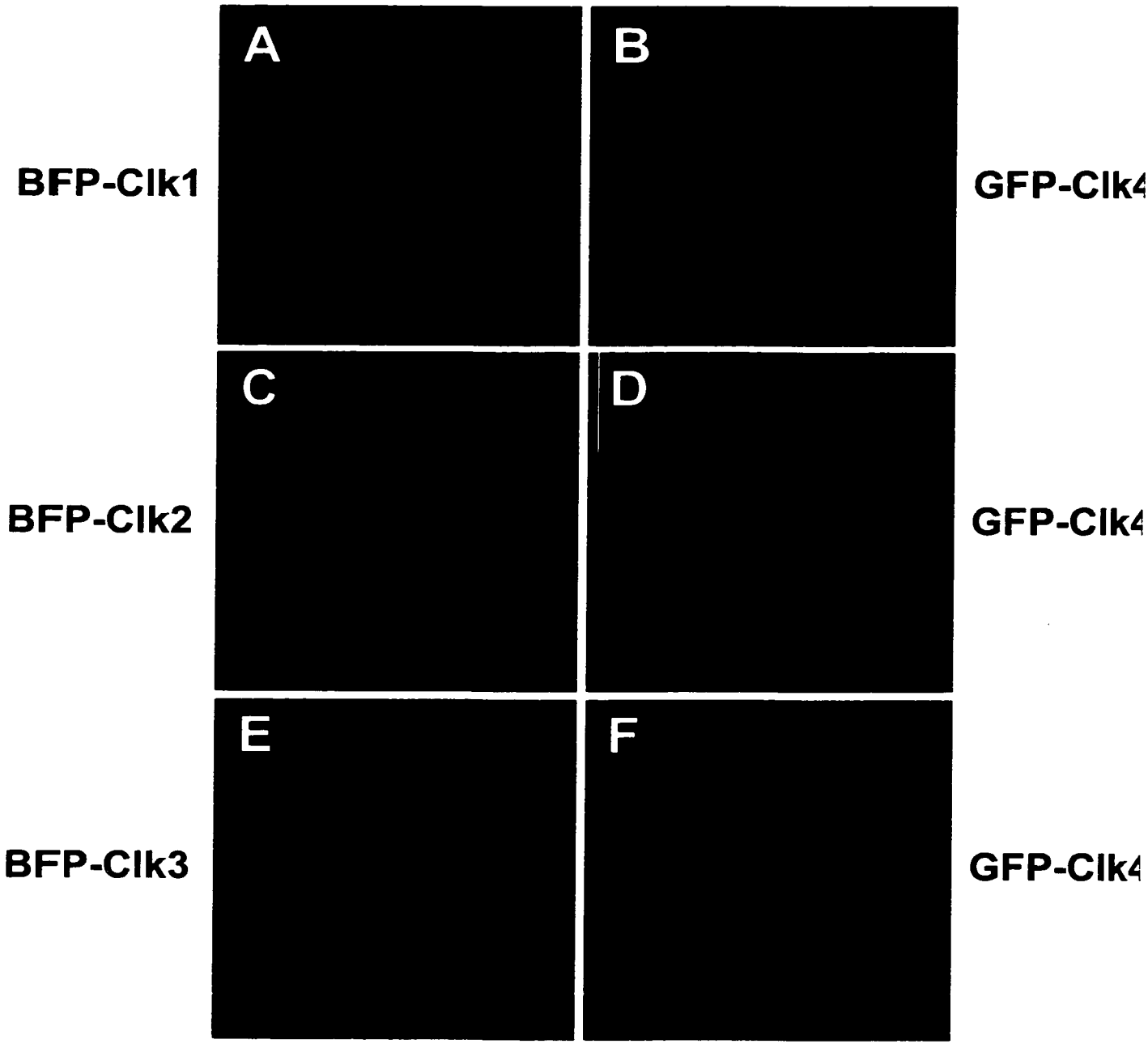


Figure 4. Clk kinases co-localize in the nucleus

Pairs of BFP tagged and GFP tagged Clks were transiently co-expressed in Cos-1 cells and visualized live using epifluorescence microscopy. BFP-Clk1 (A) and GFP-Clk4 (B) were coexpressed. BFP-Clk2 (C) and GFP-Clk4 (D) were coexpressed. BFP-Clk3 (E) and GFP-Clk4 (F) were co-expressed. In each case BFP tagged Clk1, 2 or 3 colocalized with GFP–Clk4 when expressed in the same cell.

Figure 4



their sub-nuclear fluorescence. In all cases, GFP and BFP Clks expressed in the same cell, demonstrated identical fluorescence patterns. Figure 4, panels a-f, is a representation of the data showing BFP-Clk1, 2 or 3 co-expressed in Cos-1 cells with GFP-Clk4. It is apparent from these images that no matter which kinases are expressed, they co-localize in discrete "speckle-like" regions or in a more nucleoplasmic pattern, or a combination of both. The images shown here are merely representative of common patterns seen after examining several hundred samples and are not meant to specify the only fluorescence pattern seen with these specific kinase pairs. For example, BFP-Clk3 and GFP-Clk4 appear completely nucleoplasmic in figure 4, panels e & f; but in other samples they have had the appearance of "Clk4-like microspeckles", or a combination or both. What is true of every sample studied to date is that both GFP and BFP fluorescence patterns are identical in any given cell expressing any given pair of kinases demonstrating co-localization of these kinases *in vivo*.

A corollary of these observations is that Clks appear to be able to alter each other's sub-nuclear localization. For example, Clk4 normally appears as "microspeckles", while Clk3 appears nucleoplasmic with a few punctate speckles (Figure 4 panels D & C, respectively). However, when they are co-expressed, Clk 4 is nucleoplasmic with no apparent speckling pattern (Figure 4, panel f). This was observed in the other direction as well, for example, Clk 1, 2 or 3 when coexpressed with Clk4 appeared to localized to "Clk4-like microspeckles" with little or no nucleoplasmic staining (data not shown).

Truncated Clk1 can localize Clks to nuclear speckles

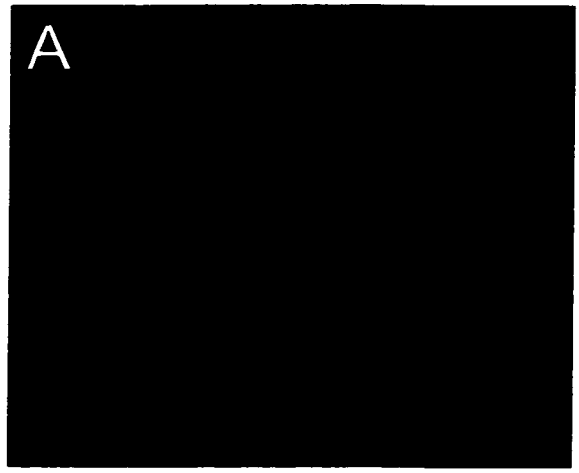
Expression of the truncated Clk1 GFP fusion protein in combination with any

Figure 5. Clk1^{T} can localize Clk4 to nuclear speckles

Pairs of BFP tagged and GFP tagged Clks were transiently co-expressed in Cos-1 cells and visualized live using epifluorescence microscopy. (A) BFP-Clk4 expressed alone demonstrates characteristic "microspeckle"pattern. (B) GFP-Clk1^{T} expresses alone shows nuclear speckle pattern of localization. (C) When co-expressed BFP-Clk4 appears to have re-localized to "Clk1^{T}-like" nuclear speckles.

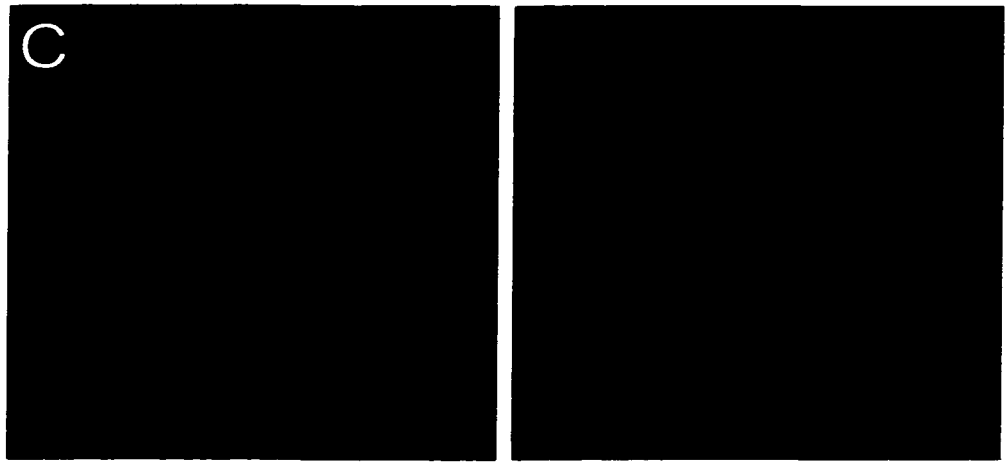
Figure 5

BFP-Clk4 alone



GFP-Clk1^{T}alone

Co-expressed



of the full length Clk kinases (BFP-Clks1-4) demonstrates the ability of Clk1T to localize Clks to nuclear speckles. An example of this is shown in figure 5 where expression of BFP-Clk4 alone in Cos cells results in the predicted "microspeckle" pattern observed with this kinase (Figure 5, panel a). However, when co-expressed with GFP-Clk1T, Clk4 localizes to fewer, larger punctate regions reminiscent of classical nuclear speckles (Figure 5, panel c). Clk1T in these same cells has an identical staining pattern, indicative of their co-localization (Figure 5, panel d). The same observations were made when Clk1T was co-expressed with any other full length Clk kinase (data not shown).

SR proteins interact in vivo

Endogenous SR proteins have been shown to co-localize in the nucleus in previous reports using a variety of antibodies directed at single SR proteins or the entire family (164, 165, 182). We wanted to determine if SR proteins interacted directly *in vivo*. To do this we employed fluorescence resonance energy transfer (FRET) to detect the close association of GFP and BFP tagged SR proteins co-expressed transiently in Cos-1 cells (Figure 6). The principle of FRET states that when two fluorescent molecules, a donor and an acceptor, are brought into close proximity, there is the potential for non-radioactive transfer of energy between them. The distance between these two fluors must be less than 100 Å. When the donor is activated at its excitation frequency, the acceptor fluor, if it is close enough, will become excited by the transfer of energy from the donor and emit a photon at its emission frequency. By measuring the number of photons emitted by the acceptor fluor following excitation at the donor fluor, one can determine the distance between these molecules. Another principle of FRET takes advantage of the

Figure 6. Fluorescence energy resonance transfer

Schematic representation of the two principles of FRET used to Fluorescent Resonance Energy Transfer (FRET) detect the interaction of two molecules which are <100Å apart. (A) FRET is the non-radioactive transfer of energy from one fluorescent molecule to another. FRET can occur only if these fluors are in very close proximity (<100Å). To determine if two molecules are sufficiently close, one can excite one of the fluors at its specific excitation wavelength (Blue 365 nm) and measure the photons emitted at the emission wavelength of the other fluor (Green 525 nm). If photons are detected, FRET is occurring and the molecules are said to be interacting. (B) Another property of FRET states that two proximal fluors (<100Å apart) will quench each other's fluorescence. We can determine if two molecules are interacting by measuring the fluorescence of one fluor at its excitation and emission wavelengths (Green: ex.488 nm; em. 525 nm) before and after photo-destruction of the other fluor (Blue). If there is an increase in fluorescence after photo-bleaching then FRET must be occurring and the two molecules are said to be interacting (<100Å apart). An "X" in the red "interaction domain" of the GFP fusion protein is meant to denote a mutation resulting in the loss of this putative interaction.

Figure 6

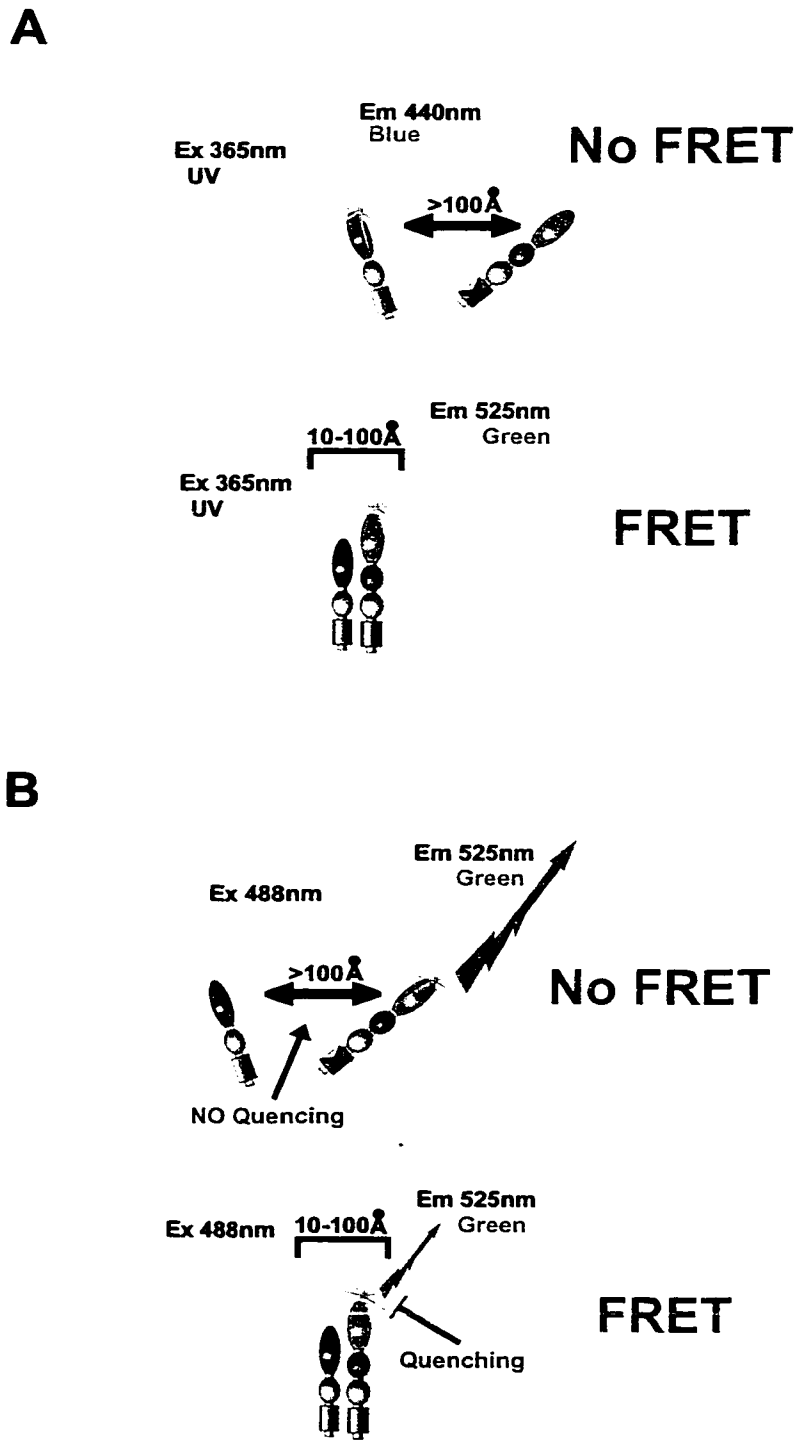

A
Em 440nm
Blue
Ex 365nm
UV
No FRET
>100Å
Em 525nm
Green
10-100Å
Ex 365nm
UV
FRET
B
Em 525nm
Green
Ex 488nm
>100Å
No FRET
NO Quencing
Em 525nm
Green
Ex 488nm
10-100Å
FRET
Quenching

Figure 7. Detection of SR proteins interacting *in vivo* by FRET

BFP-SRp20 was transiently co-expressed with GFP-ASF in Cos-1 cells and assayed for FRET. (A) Fluorescence of BFP-SRp20 as measured by BFP filter set, (B) GFP-ASF as visualized using GFP filter set, (C) FRET signal as measured using the chimeric FRET filter set. An abundant FRET signal was observed indicating the two molecules to be less than 100Å apart. The measurements were taken again following the photo-destruction of BFP fluorescence in the lower half of the cell (D, E, and F, respectively). The increase in GFP fluorescence in the lower portion of the cell in panel (E) is indicative of FRET mediated quenching between the two molecules, again indicating an *in vivo* interaction between these fluorescently tagged SR proteins.

Figure 7

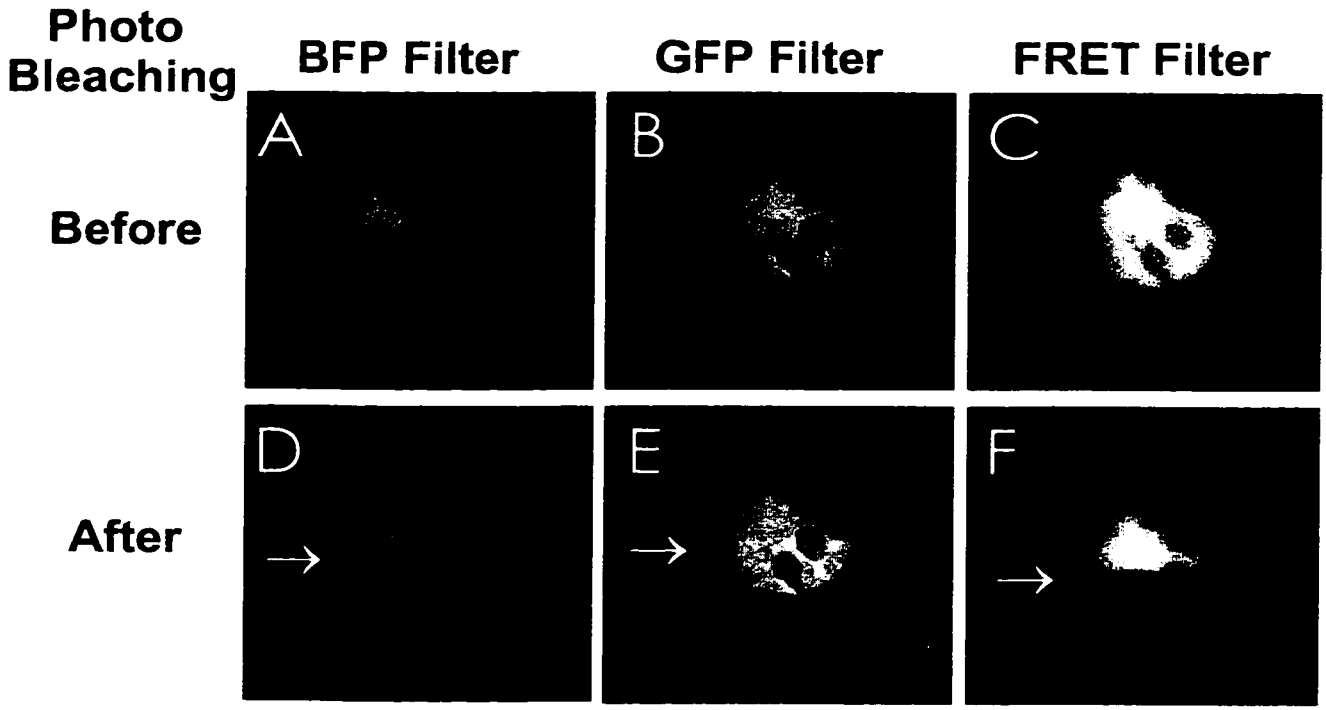


Photo Bleaching
BFP Filter
GFP Filter
FRET Filter
A
B
C
Before
D
E
F
After

Figure 8. Summary of FRET experiments

Pairs of BFP and GFP tagged Clks and their substrates were expressed in various cell lines and assayed for FRET. A matrix is represented here to summarize the combinations of fluorescently tagged proteins assayed for interactions using our FRET assay. Within each square is listed the cell line which was used for each particular experiment. Cos is Cos-1; 3T3 is NIH 3T3 murine fibroblasts; 293 is 293T cells. B20 is BFP-SRp20; B75 is BFP-SRp75; Bmusculin is BFP-musculin; BClks are BFP-Clks; ClksB are Clks-BFP and similarily GClks are GFP-Clks and ClksG are Clks-GFP. Dark shaded boxes represent positive FRET interactions; all others are FRET negative.

Figure 8

	B20	B75	Bmusculin	BClk1	BClk1T	Clk2B	Clk3B	Clk4B
GClk1	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
Gclk1T	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
GClk2	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
GClk3	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
GClk4	COS, 3T3	COS, 3T3	COS, 3T3	COS, 3T3	COS, 3T3	COS, 3T3	COS, 3T3	COS, 3T3
Clk2G	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
Clk3G	COS 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
Clk4G	COS, 3T3	COS, 3T3	COS, 3T3	COS	COS	COS	COS	COS
Gasf	COS, 3T3	COS, 3T3	COS 3T3	COS, 293	COS, 293	COS, 293	COS, 293	COS, 293

Legend FRET | No FRET

quenching properties of this physical interaction. When two fluorescent molecules, a donor and an acceptor, are brought into close proximity, each will quench the other's emissions. Therefore, one can determine the distance between these molecules by measuring the increase in fluorescence following the photo-destruction of the quenching fluor. We have used both of these principles to determine if FRET is taking place between GFP-ASF and BFP-SRp20 co-expressed in Cos-1 cells. In this series of images, we see GFP-ASF (Green; acceptor) and BFP-SRp20 (Blue; donor) expressed in the same COS-1 cell (Figure 7, panels A&B). The fluorescence of BFP-SRp20 and GFP-ASF was measured individually followed by the fluorescence using a FRET detecting filter set (Figure 7, panels a-c). A strong FRET signal was observed demonstrating that these two proteins must be less than 100 Å apart. Following the laser ablation BFP-SRp20 fluorescence in the lower half of the cell (Figure 7, panel c), an increase in green fluorescence was observed in this lower section (Figure 7, panel d) indicating the FRET mediated quenching of GFP by BFP. This would again indicate that these two molecules are less than 100 Å apart, and since 100 Å is about the diameter of the average 50 kDa globular protein, we conclude that these proteins are interacting directly in these cells.

We extended these studies to monitor the interactions of Clk kinases with themselves and their suspected substrates in both fixed and live cells. We have created chimeric proteins of autofluorescent GFP and BFP proteins fused to Clk family members and their suspected targets in an effort to detect their direct interactions *in vivo*. Figure 8 shows the combinations of fluorescently tagged proteins assayed in this way, along with the cell type(s) in which thay were assayed. No detectable interactions were observed using FRET other than the

aforementioned SR protein interactions.

The Clk1 kinase regulates pre-mRNA splicing.

Our lab has demonstrated previously that the *Clk1* pre-mRNA is alternatively spliced (45) and its protein product the Clk1 kinase, interacts with SR proteins *in vitro*(32). We set out to determine if the Clk1 kinase (78) can elicit changes in splicing of its own pre-mRNA. To this end we constructed a *Clk1* mini-gene which contains two introns flanking the alternatively spliced exon, referred to in the following as EB (Figure 9, panel a). Splicing of the mini-gene to retain exon EB (exon inclusion) generates an mRNA encoding full-length catalytically active Clk1 whereas splicing to exclude exon EB (exon skipping) generates an mRNA encoding $Clk1^{T(45)}$. When expressed in COS-1 cells the Clk1 mini-gene leads to the production of predominately $Clk1^{T}$ protein as determined by immunoblot analysis (Figure 9, panel b, lane 2). In contrast, when a point mutation is introduced within the ATP binding fold of the catalytic domain of the Clk1 mini-gene, rendering the full-length Clk1 protein catalytically inactive ($Clk1^{K190R}$), significantly more full-length protein is produced (Figure 1B, lane 3). Analysis of the mRNA produced from the two mini-genes, by reverse transcription and quantitative PCR amplification (RT-PCR), supported the immunoblot results. As can be seen in figure 9 (panels c and d, lane 2), cells transfected with the wild type *Clk1* mini-gene predominantly express mRNA encoding $Clk1^{T}$ protein. The catalytically inactive $Clk1^{K190R}$ mini-gene construct produced significantly more mRNA encoding full-length $Clk1^{K190R}$ protein (Figure 9, panels c and d, lane 3). These results are consistent with the idea that the catalytic activity of the Clk1 kinase regulates the splicing of the *Clk1* pre-mRNA.

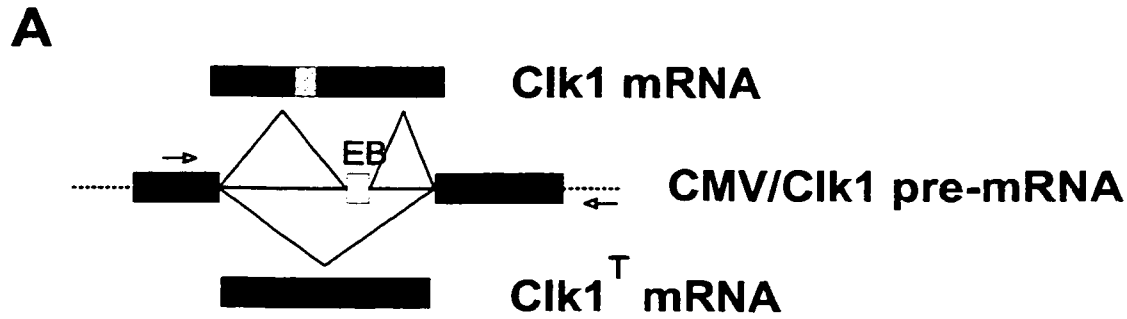
To determine the relative contributions of the Clk1 and Clk1^{T} proteins to the alternative splicing of *Clk1* pre-mRNA, two smaller mini-gene constructs were created (CMV/*CR-1* and CMV/*CR-2*, Figure 10, panel a). The *CR-1* mini-gene contains a large deletion within the catalytic domain and has the capacity to produce only truncated Clk1 proteins and no full length kinase while the *CR-2* mini-gene contains a translational stop codon following the Myc epitope tag which prevents the production of any Clk1 related proteins. The *CR-1* mini-gene was co-transfected into the human cell line 293T along with increasing amounts of an expression vector encoding a haemaglutinin epitope tagged version of Clk1 (HA-Clk1(22)). The RNA products of the *CR-1* reporter were analysed by quantitative RT-PCR amplification (Figure 10). As can be seen in figure 10, panel b, lane 2, transfection of the *CR-1* reporter on its own, gave rise to mRNA products in a ratio similar to that seen with *Clk*^{K190R} mini-gene (compare Figure 10, panel b, lane 2 and Figure 10, panel c, lane 3). Co-transfection of the HA-Clk1 expression vector promoted exon EB skipping. The ratio of exon EB inclusion to exon EB skipping decreased as the HA-Clk1 protein level increased (Figure 10, panel b, lanes 2-5) This experiment is representative of five separate transfections which gave 6-10 fold decreases in the ratio of exon EB inclusion:exon EB skipping,(EB^{+}/EB^{-}). Thus, overexpression of Clk1 kinase *in vivo* can promote exon EB skipping in COS-1 and 293T cells.

Co-transfection of *CR-1* with HA-Clk1^{K190R} resulted in a modest (2-3 fold) but reproducible increase in the ratio of EB^{+}/EB^{-} (Figure 10, panel b, lanes 6-9). One interpretation of this result is that the catalytically inactive Clk1^{K190R} protein can act as a dominant negative inhibitor of the endogenous 293T human Clk1 protein kinase and thus

Figure 9. Catalytic activity of Clk1 kinase regulates splicing of *Clk1* pre-mRNA *in vivo*.

(A) Schematic representation of a portion of the *Clk1* pre-mRNA mini-gene and the spliced mRNA products. The alternatively spliced exon (EB) and primers used for amplification by RT-PCR are shown. CMV, cytomegalovirus promoter. (B) Clk1 and $Clk1^{T}$ proteins expressed in COS-1 cells following transfection of CMV vector (lane 1), CMV/*Clk1* pre-mRNA (lane 2) and CMV/$Clk1^{K190R}$ pre-mRNA (lane 3). Clk1 proteins were detected by anti-Myc mAb immunoblot. Anti-β-tubulin mAb was used to control for equal loading of proteins. Positions of molecular mass markers (kDa) are indicated to the left of the panel. WT, wild-type kinase. (C) Detection of *Clk1* mRNAs expressed following RT-PCR amplification; lanes are as in (B). Positions of molecular size standards (kb) are indicated to the left of the panel. (D) Ratio of *Clk1* mRNAs shown in (C); lanes are as in (B).

Figure 9


A
Clk1 mRNA
EB
CMV/Clk1 pre-mRNA
Clk1T mRNA

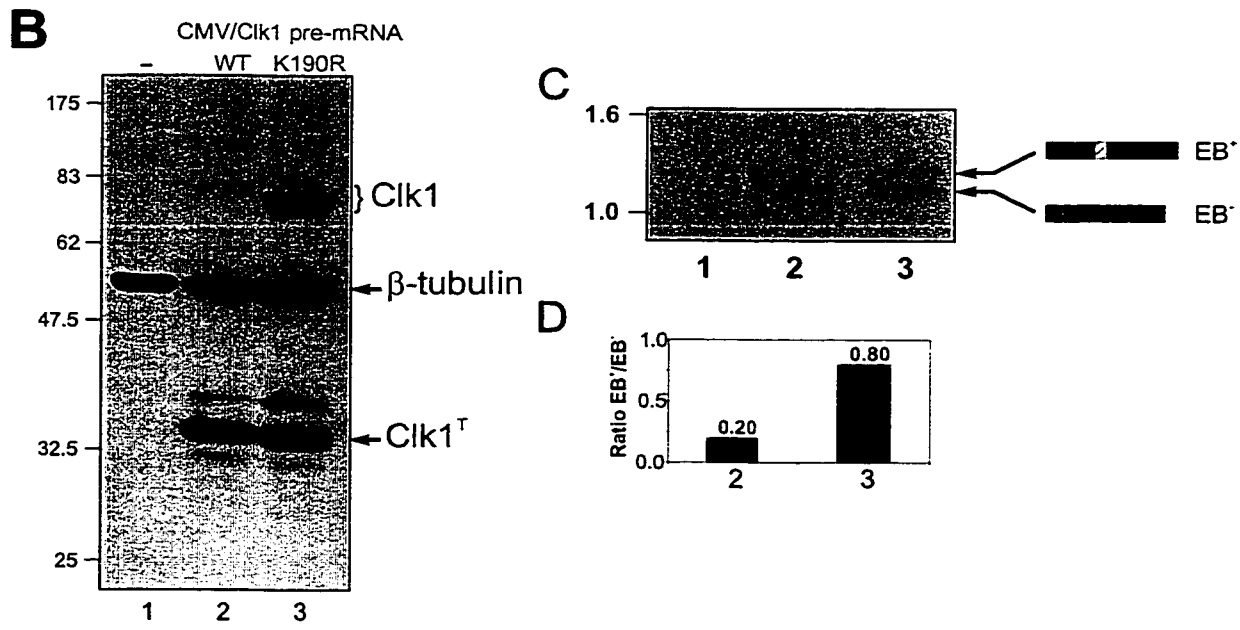
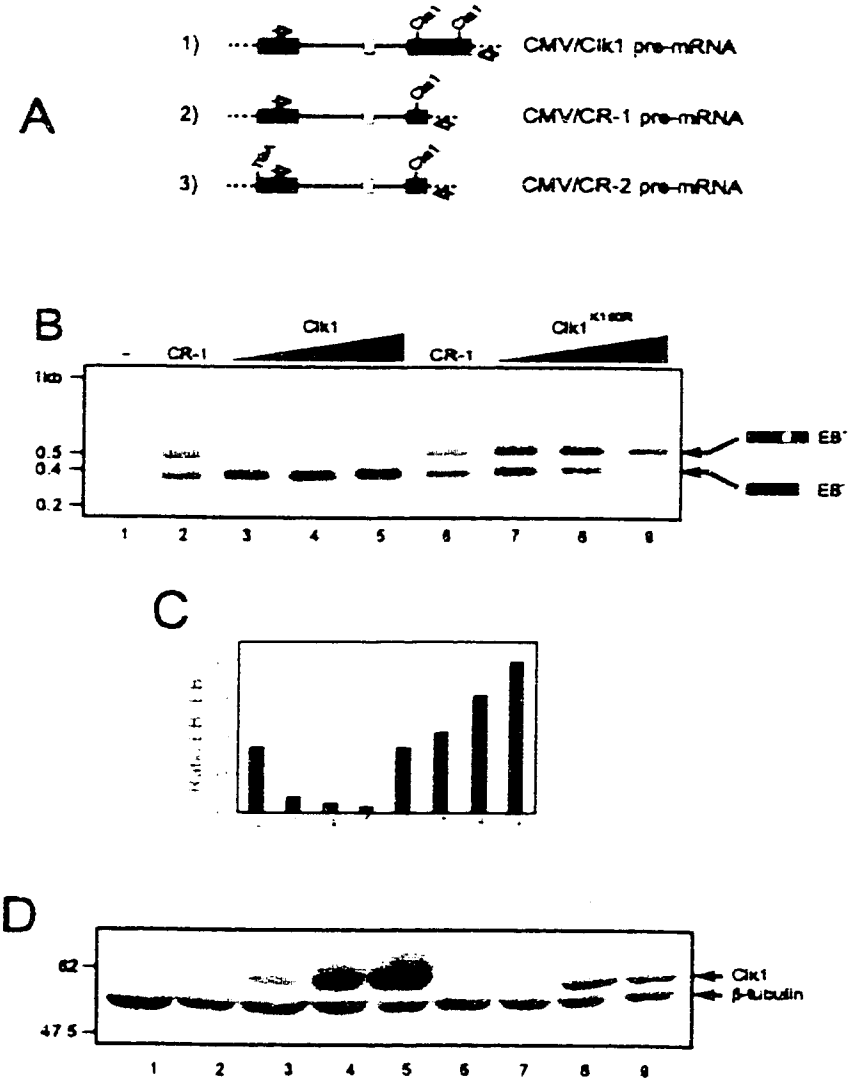

B
CMV/Clk1 pre-mRNA
-
WT
K190R
175
83
62
47.5
32.5
25
Clk1
β-tubulin
Clk1T
1
2
3
C
1.6
1.0
EB+
EB-
1
2
3
D
Ratio EB+/EB-
1.0
0.5
0.0
0.20
0.80
2
3

Figure 10. Catalytically active Clk1 promotes exon skipping of *Clk1* pre-mRNA *in vivo*.

(A) Schematic representation of *Clk1* mini-genes. The primers used for amplification by RT-PCR are indicated. (B) Pattern of *Clk1* alternative splicing upon cotransfection of *Clk1* and *Clk1*^{K190R} expression vectors in 293T cells. Lane 1, CMV vector, lanes 2-9 2.5μg CMV/*CR-1* + 0.5, 2.5, 5μg CMV/*HA-Clk* (lanes 3-5) or CMV/*HA-Clk*^{K190R} (lanes 7-9), respectively. Positions of molecular size standards (kb) are indicated to the left of the panel. (C) Ratio of *Clk1* mRNAs shown in (B); lanes are as in (B). (D) Detection of Clk1 proteins expressed by anti-HA mAb immunoblot; lanes are as in (B). Positions of molecular mass markers (kDa) are indicated to the left of the panel.

Figure 10


A
1)
CMV/Clk1 pre-mRNA
2)
CMV/CR-1 pre-mRNA
3)
CMV/CR-2 pre-mRNA
B
-
CR-1
Clk1
CR-1
Clk1 K190R
1kb
0.5
0.4
0.2
EB+
EB-
1 2 3 4 5 6 7 8 9
C
D
62
47.5
Clk1
β-tubulin
1 2 3 4 5 6 7 8 9

favours exon EB inclusion.

The Clk1^{T} protein affects splicing

We have previously suggested that Clk1^{T} may be a natural antagonist to Clk1 activity(45). Since the *CR-1* reporter construct retains the coding capacity to produce Clk1^{T}, we compared the spliced products generated from *CR-1* and *CR-2*, which produces no Clk1 related proteins. In figure 11, panel a, it is evident that increasing amounts of *CR-1* transfected per cell favours exon EB inclusion. We believe that this is a result of an increase in Clk1^{T} protein production per transfected cell. To test this idea a fixed amount of *CR-1* was transfected along with increasing amounts of a Clk1^{T} expression vector and again we observed an increase in exon EB inclusion (Figure 11, panels c and d). In contrast, increasing amounts of the *CR-2* reporter, which cannot produce Clk protein, does not alter the EB^{+}/EB^{-} ratio (Figure 11, panels a and b). Thus, overexpression of catalytically active Clk1 favours skipping of exon EB, while conversely, Clk1^{T} promotes exon EB inclusion.

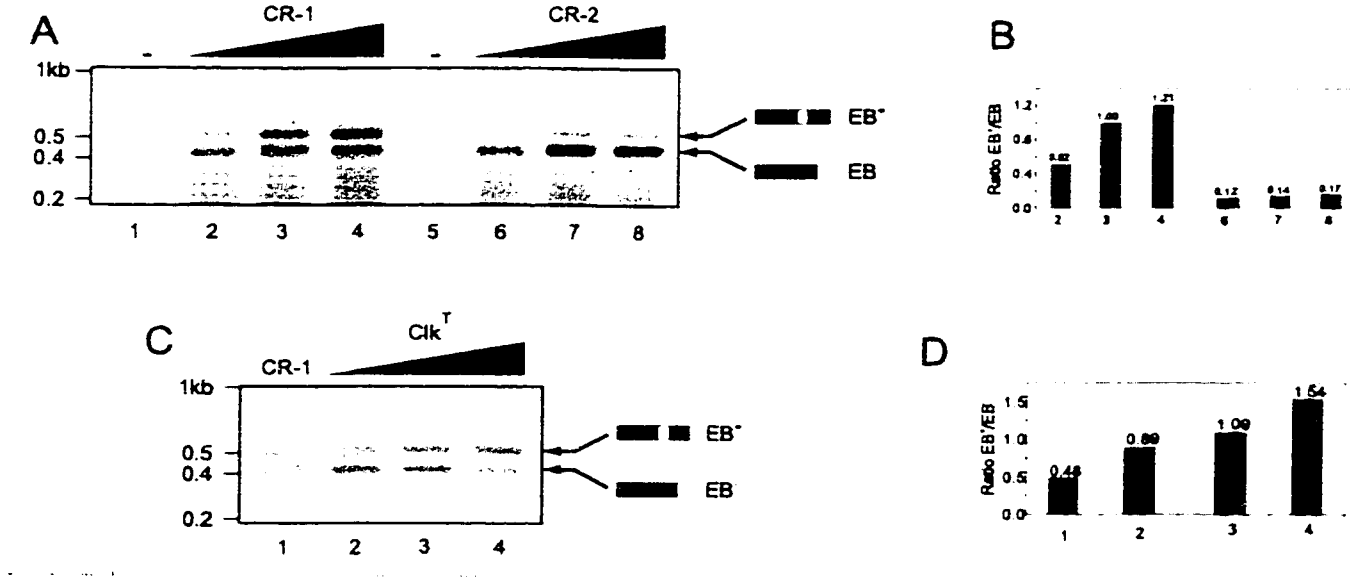
The exon EB domain affects Clk pre-mRNA splicing and SR protein re-distribution

We examined whether regions other than the catalytic domain of Clk1 are involved in the promotion of exon EB skipping. In particular, since portions of exon EB are conserved between the three known Clk family members(45, 70) we created a deletion mutant of Clk1 lacking exon EB (herein referred to as Clk1$^{\Delta 130\text{-}158}$). The Clk1$^{\Delta 130\text{-}158}$ protein expressed in bacteria displayed considerable catalytic activity indicating that it retains a functional kinase domain (P.I. Duncan, D.F. Stojdl, R.M. Marius, J.C. Bell, data not shown). Co-transfection of *CR-1* with Clk1$^{\Delta 130\text{-}158}$ in 293T cells resulted in a splicing pattern similar to that seen with Clk1^{K190R} and Clk1^{T} (Figure 12, panels a and b).

Figure 11. Clk1^{T} promotes exon inclusion of *Clk1* pre-mRNA *in vivo*.

(A) Increasing expression of *CR-1* but not *CR-2 Clk1* pre-mRNA promotes exon inclusion. 293T cells were transfected with 0 (lanes 1,5), 0.5 (lanes 2,6), 2.5 (lanes 3,7) or 5μg (lanes 4,8) CMV/*CR-1* (lanes 1-4) or CMV/*CR-2* (lanes 5-8). Positions of molecular size standards (kb) are indicated to the left of the panel. (B) Ratio of *Clk1* mRNAs shown in (A); lanes are as in (A). (C) Pattern of *Clk1* alternative splicing upon cotransfection of Clk1^{T} expression vector. Lanes 1-4, 2.5μg CMV/*CR-1* + 0, 0.5, 2.5, 5μg pECE/*M-Clk1*^{T} (4), respectively. Positions of molecular size standards (kb) are indicated to the left of the panel. (D) Ratio of *Clk1* mRNAs shown in (C); lanes are as in (C).

Figure 11


A
CR-1
CR-2
1kb
0.5
0.4
0.2
1
2
3
4
5
6
7
8
EB*
EB
B
Ratio EB*/EB
C
CR-1
Clk T
1kb
0.5
0.4
0.2
1
2
3
4
EB*
EB
D
Ratio EB*/EB
0.48
0.89
1.09
1.54
1
2
3
4

We have shown here (Figure 12, panel D, a-c) and previously (32) that Clk1 kinase can alter the subnuclear distribution of SR proteins. $Clk1^{T}$ co-localizes with SR proteins in nuclear speckles but does not affect their distribution (Figure 12, panel D, d-f). The $Clk1^{\Delta 130-158}$ protein, which was predominantly nucleoplasmic, like Clk1, but was also found at a low but detectable level in speckles, did not significantly affect SR protein speckles (Figure 12, panel D, g,h,i). Thus we conclude that the catalytic activity of Clk1 is necessary, however not sufficient for influencing splicing and SR protein distribution. Furthermore the peptide motif encoded by exon EB is essential for both SR protein re-distribution and exon EB skipping.

Regulation of E1A pre-mRNA splicing by Clk1

To determine whether Clk1 can regulate alternative splicing of other primary transcripts, we cotransfected COS-1 cells with Clk1 and an adenovirus E1A reporter gene which is capable of producing multiple mRNAs (9S, 12S and 13S) through the use of alternative 5' splice sites (21, 205). The adenoviral E1A protein is expressed as a variety of isoforms during the virus' life cycle. For the purposes of this study, we have concerned ourselves with the diversity generated through the competition of alternative 5' splice sites for a unique 3' splice site. The E1A mini-gene used in this study produces primarily mRNAs corresponding to the adenoviral E1A protein isoforms: 9S, 12S and 13S. The 5' splice donor which gives rise to the 9S isoform is considered to be more distal as compared to 12S and 13S splice donors sites, which are more proximal to the 3' splice acceptor site. Transfection of the E1A mini-gene alone generated multiple RNA species characteristic of the utilisation of alternative 5' splice sites as has been shown previously (Figure 13, panel A, lane 2). The

presence of cotransfected, catalytically active Clk1 resulted in a shift in splicing favouring use of the most distal 5' splice site (9S RNA isoform) (Figure 13, panel A, lane 3), characteristic of the late phase of adenovirus infection (21, 205).

Figure 12. The peptide motif encoded by the *Clk1* alternatively spliced exon is necessary for exon skipping activity.

(A) Pattern of *Clk1* alternative splicing upon cotransfection of $Clk1^{\Delta 130\text{-}158}$ expression vector. Lane 1, CMV vector, lanes 2-5 0.1μg CMV/*CR-1* + 0, 2.5 and 5μg pECE/*M-*$Clk1^{\Delta 130\text{-}158}$, respectively. Positions of molecular size standards (kb) are indicated to the left of the panel. (B) Ratio of *Clk1* mRNAs shown in (A); lanes are as in (A). (C) Detection of $Clk1^{\Delta 130\text{-}158}$ protein expression by anti-Myc mAb immunoblot; lanes are as in (A). Positions of molecular mass markers (kDa) are indicated to the left of the panel. (D) Nuclear localisation of Clk1 isoforms and their effects on the distribution of SR proteins. Indirect immunofluorescent staining of transfected cells using the anti-Myc mAb (α-Myc, panels a,d,g) and anti-SR mAb (α-SR, panels b,e,h) or an overlay of the two signals (α-Myc + α-SR, panels c,f,i). Cells were transfected with pECE/*M-Clk1* (panels a-c), pECE/*M-*$Clk1^{T}$ (panels d-f) or pECE/*M-*$Clk1^{\Delta 130\text{-}158}$. Magnification 63X.

Figure 12

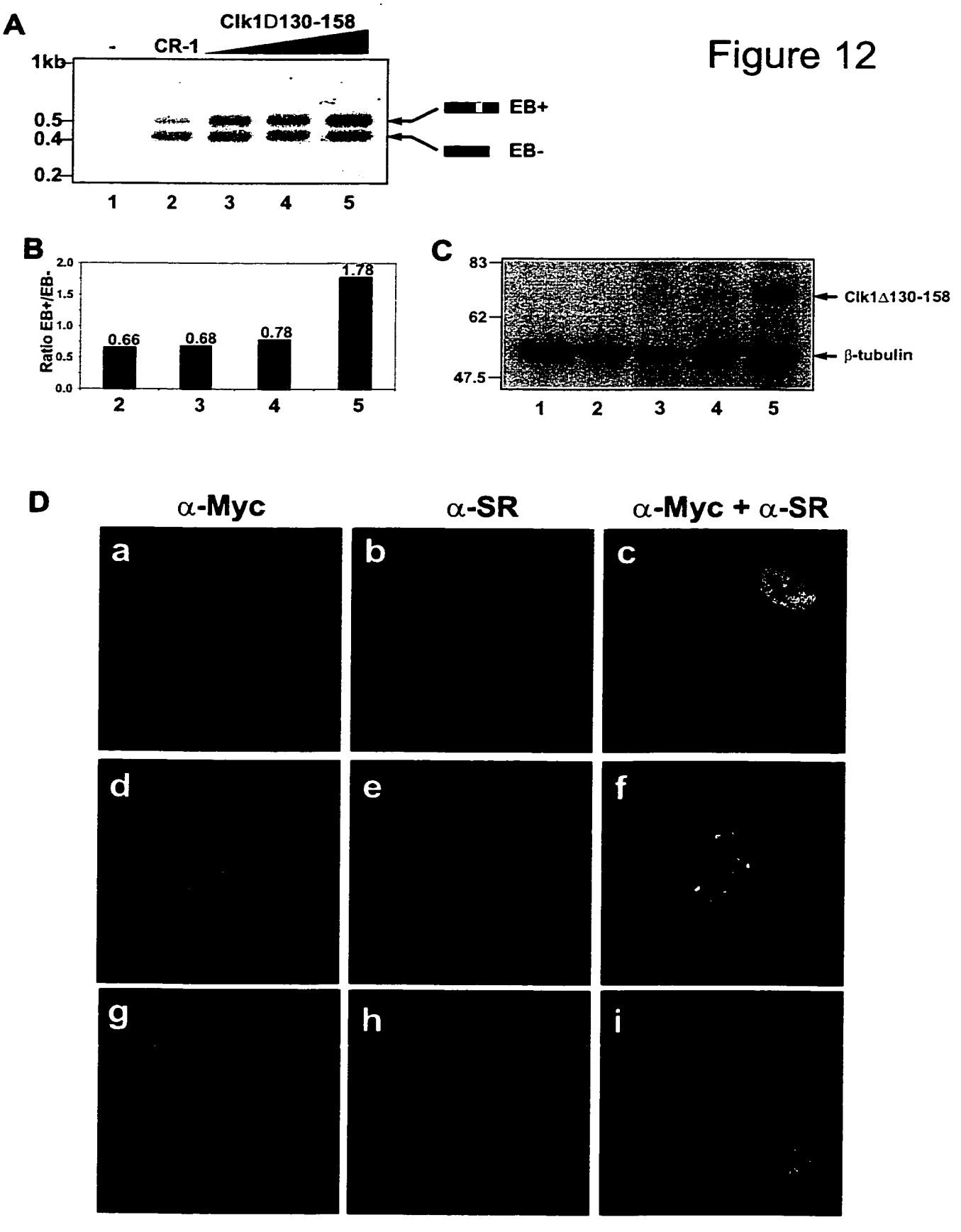
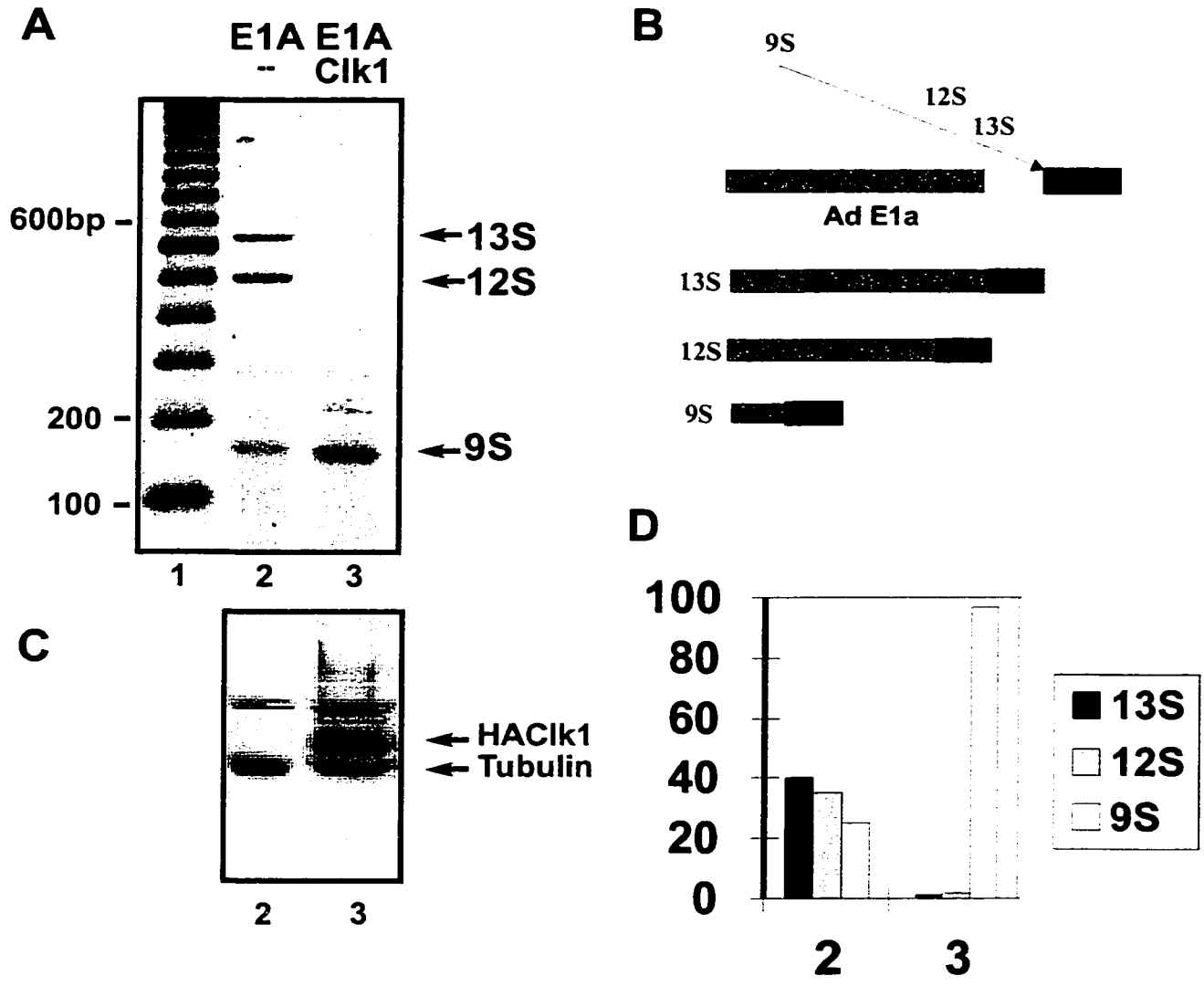

A
Clk1D130-158
-
CR-1
1kb
0.5
0.4
0.2
EB+
EB-
1
2
3
4
5
B
Ratio EB+/EB-
2.0
1.5
1.0
0.5
0.0
0.66
0.68
0.78
1.78
2
3
4
5
C
83
62
47.5
Clk1Δ130-158
β-tubulin
1
2
3
4
5
D
α-Myc
α-SR
α-Myc + α-SR
a
b
c
d
e
f
g
h
i

Figure 13. Effect of Clk1 transient overexpression on alternative splicing of adenovirus E1A pre-mRNA.

(B) Shows a schematic representation of the three possible splicing products. (A) Pattern of E1A alternative splicing in COS-1 cells upon transfection of E1A mini-gene (lane 2) or co-transfection with pECE/HA-Clk1 expression vector (lane 3) as detected by RT-PCR. Positions of molecular size standards (kb) are indicated to the left of the panel, corresponding to the markers in lane 1. (C) Analysis of protein lysates showed expression of Clk1 in pECE/M-Clk1 transfected cells (lanes are as in panel A). (D) The 13S, 12S and 9S mRNA isoforms were quantified and the percentage of each isoform is shown (lanes are as in panel A).

Figure 13


A
E1A -
E1A Clk1
600bp
200
100
13S
12S
9S
1
2
3
B
9S
12S
13S
Ad E1a
13S
12S
9S
C
HAClk1
Tubulin
2
3
D
100
80
60
40
20
0
13S
12S
9S
2
3

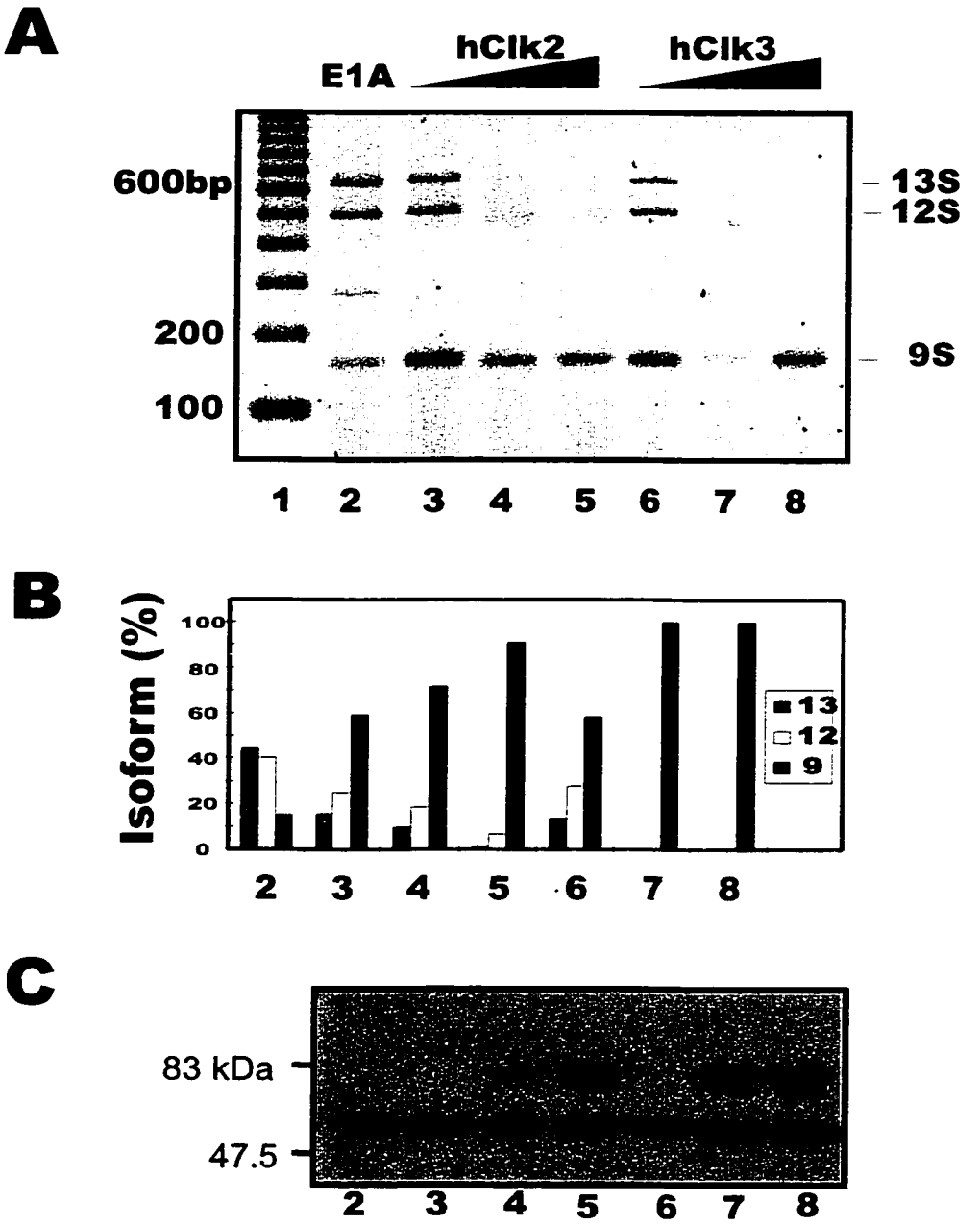
Clk2 and Clk3 regulate splicing in vivo

To determine the *in vivo* effects of Clk2 and Clk3 we transiently transfected increasing concentrations of Clk2 or Clk3 expression vectors along with an adenovirus E1A splicing reporter construct. E1A pre-mRNA spliced products were assessed by RT-PCR analysis as described in Materials and Methods. COS-1 cells expressing only the E1A reporter produced predominantly 12S and 13S RNAs (Figure 14, panels A & B, lane2), representing proximal splice site selection. As was the case for Clk1, expression of Clk2 or Clk3 resulted in a shift from the proximal 12S and 13S 5'splice donors, to the more distal 5' splice site, thereby producing considerably more 9S RNAs than in cells transfected with the reporter alone (Figure 14, panel A & B, lanes 3-8). An immunoblot of corresponding samples, probed with anti-Myc antibody, confirmed the expression of Clk proteins in cells transfected with Clk expression constructs (Figure 14, panel C).

Figure 14. Clk2 and Clk3 also modulate E1A alternative splicing *in vivo.*

Cos-1 cells were transfected with CMV/*E1A* mini-gene reporter alone (*lane 1*), or cotransfected increasing amounts of plasmid (0.1, 1.0 and 3.0μg DNA) encoding Clk2 (*lanes 3,4,5*) or Clk3 (*lanes 6,7,8*), respectively. Cells were harvested in PBS (see Materials and Methods) and total RNA and proteins isolated. *E1A*-specific RNAs were amplified by RT-PCR, resolved by gel electrophoresis, stained with SYBR Green I and visualised by fluorimagery (A). Positions of molecular size standards (kb) are indicated to the left of the panel. Ratio of *E1A* mRNAs shown in A (B). Clk expression levels were determined from aliquots of cell lysates (C). Proteins were resolved by SDS-PAGE and transfered to nitrocellulose. Proteins were visualized by immunoblot analysis and enhanced chemiluminescence with the anti-Myc and anti-β-tubulin antibodies. Positions of molecular mass markers (kDa) are indicated to the left of the panel.

Figure 14


A
E1A
hClk2
hClk3
600bp
200
100
13S
12S
9S
1 2 3 4 5 6 7 8
B
Isoform (%)
100
80
60
40
20
0
13
12
9
2 3 4 5 6 7 8
C
83 kDa
47.5
2 3 4 5 6 7 8

Discussion

Alterations in SR protein concentration are thought to be critical to the control of alternative splicing (21, 55, 114, 116, 197, 211). Indeed, exon skipping and inclusion can be modulated by both the concentration of SR proteins and the placement of the splicing enhancers to which they bind within the pre-mRNA (86, 99). We suggest as one possibility that the Clk kinases phosphorylate a SR protein(s) facilitating its release from storage sites and thus increasing its effective nucleoplasmic concentration and availability to participate in the splicing reaction. This would be consistent with our observations that the subnuclear distribution of SR proteins can be regulated *in vivo* by Clk kinase activity.

An increasing volume of information on subnuclear structures has evolved in the past several years (reviewed in163, and182). SR proteins and indeed many splicing factors have been found to concentrate in nuclear structures called speckles, or interchromatin granule clusters (IGCs). However, these same factors are required at the sites of active transcription, in order to perform their role(s) in pre-mRNA processing. Several reports have now established that most transcription appears not to occur in these IGCs (127, 133, 213, 215). Therefore, factors required for splicing must be recruited from these IGCs, and it appears that phosphorylation is required for this process. For SR proteins with 1 RRM and 1 RS domain (SRp20 and SC35) the RS domain is sufficient to target the protein to the IGCs. For larger SR proteins which have 2 RRMs and a RS domain, it appears that the RS domain is not necessary or sufficient for IGC localization (20). As long as 2 of these 3 domains are present (ie. deletion of only one of these domains at a time), the protein can still localize to IGCs,

interestingly however, the mutant SR protein is not recruited to active transcription sites (126). Moreover, release from speckles seems to be dependent on the presence of a RS domain and more specifically, one which can be phosphorylated. Mutants of SF2/ASF which have serine to glycine substitutions in their RS domain, rendering these proteins refractory to phosphorylation, are found to localize to speckles, but are not released from speckles upon induction of transcription (126). We demonstrate here the Clk family of kinases (Clk 1, 2, 3) are able to release SR proteins from IGCs. Furthermore, we show catalytically inactive mutants of Clks (Clk^Ts), when overexpressed in cultured cells, localize SR proteins and Clks into enlarged speckles. It has been shown that S/T phosphatase PP1activity is required for the localization of SR proteins to enlarged speckles following transcriptional inhibition; possibly representing the shuttling of SR proteins back to the speckles (127). These observations again demonstrate that for SF2/ASF at least, the RS domain is not required for localization to IGCs, but that recruitment from nuclear speckles to the sites of active transcription is dependent on phosphorylation of serines in the RS domain. We believe the Clk kinases, and perhaps other SR protein kinases, are responsible for the release of SR proteins following some sort of recruitment signal.

We and others (20, 126) have speculated that recruitment of SR proteins to and from speckles requires two separable events. Phosphorylation of the RS domain facilitates the release from speckles, however, migration to the sites where these factors are required, is likely through an interaction with another party. Perhaps a specialized transporter of some kind, or a constituent of the transcriptional machinery fulfills this function. This interaction may be mediated by the RRM(s) of the SR protein, through either a protein-RNA interaction

or protein-protein interaction. In this respect it is interesting to note that proteins without an RS domain can still localize to IGCs by "piggybacking" on other proteins which do. Hedley *et al.*(74) demonstrated this using a Tra1 mutant with no RS domain. This mutant was still found to migrate to speckles but only if this mutant was able to bind a full-length Tra2 protein.

The phosphorylation status of SR proteins also seems to be important for their ability to function as splicing enhancers. Xiao and Manley (203) showed that phosphorylated ASF/SF2, but not mock-phosphorylated ASF/SF2, activates the splicing of HIV tat pre-mRNA *in vitro*. Phosphorylated ASF/SF2 was able to efficiently complement an SR protein-deficient HeLa S100 extract by promoting the splicing of an adenovirus-2-derived pre-messenger RNA, whereas unphosphorylated ASF/ SF2 could not (22). Furthermore, dephosphorylation of SF2/ASF by the phosphatase PP2 blocks the ability of this SR protein to function as a silencer of splicing on an Ad virus pre-mRNA (86). In a more recent report by the same group, an Ad virus protein E4 orf4 was found to trigger this dephosphorylation event, leading to the hypothesis that the virus can usurp the splicing machinery to regulate its gene expression (87). Phosphorylation could modulate the RNA binding properties of SR proteins, potentially modifying their target specificity. Indeed, one group has reported that phosphorylation of SR proteins improves the specificity of RNA binding by diminishing the non-specific RNA interactions of the unphosphorylated RS domain with RNA targets. This group has also reported an RNA sequence identified by *in vitro* selection, to which SRp40 could bind, but only after the protein was phosphorylated by exposure to nuclear extracts or purified Clk1 kinase (203). Another possibility is that protein-protein interactions between

SR proteins and other consituents of the spliceosome may be affected by RS domain phosphorylation. Xiao et al. (203) have demonstrated that phoshorylated ASF/SF2 bound more efficiently to U1 70K than did unphosphorylated ASF/SF2. A more recent report from the same authors demonstrates that phosphorylation differentially affects the ability of ASF/SF2 to bind a variety of putative partners. Phosphorylation was shown to again have a positive affect on the interaction of ASF/SF2 with U1 70K, while not affecting its interaction with U2AF35. Interestingly, phosphorylated ASF/SF2 was shown to bind itself, SRp40, and hTra2 poorly compared to mock-phosphorylated ASF/SF2. This brings up the intriguing possibility that phosphorylation may play a role as a molecular switch, whereby phosphorylation modifies a cohort of interactions available to ASF/SF2 depending upon the stage of splicing and the requirements of the splicing machinery at that time (203). This agrees well with the afore mentioned role of SR proteins in the maturation of the spliceosome and the requirement of regulated phosphorylation/dephosphorylation cycles during this process.

Thus the Clk kinases could regulate both the effective concentration of SR proteins and their activity in the splicing reaction. In support of this idea are our results with the E1A reporter constructs. Previously it has been demonstrated that increases in SR protein concentration causes changes in splicing and, in particular, overexpression of ASF appears to favour use of the proximal 5' splice site in the E1A gene (21, 115). Since overexpression of Clk family members favours the distal 5' splice site, we conclude that while Clks can cause re-distribution of SR proteins they may also modulate the activity of certain SR proteins in the splicing reaction. We suggest that the in vivo effects of Clks on SR proteins are via direct

phosphorylation however we cannot exclude the possibility of indirect effects.

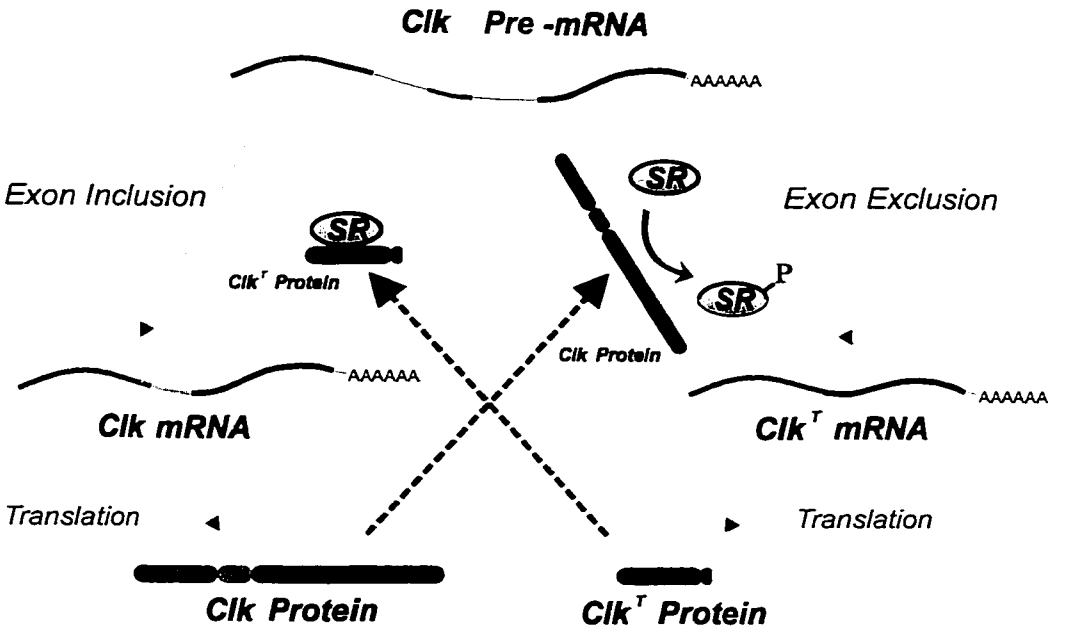
The alternatively spliced exon EB encodes a protein motif which is essential for regulation of alternative splicing by the Clk1 kinase. Thus while a catalytically active Clk1 kinase domain is necessary for this enzyme to modulate splicing, other Clk domains are also required. It may be that exon EB is essential for substrate recognition by the Clk1 kinase. Indeed portions of the EB motif are conserved between all four Clk kinase family members (45, 70). We have found that a synthetic peptide corresponding to the domain encoded by exon EB is an excellent *in vitro* substrate of the Clk1 kinase and that some of the Clk1 autophosphorylation sites are found within this exon (Duncan and Bell, unpublished). Perhaps, like the interaction of ASF with certain SR proteins, Clk interactions are regulated by phosphorylation within this region. Indeed, a recent report has identified a conserved serine residue within this EB exon that is critical for the activity of these kinases(131).

Finally, the results presented here suggest a putative autoregulatory mechanism whereby Clk controls its activity through an alternative splicing event. As shown in the model presented in figure 15, increased expression of Clk1 protein influences splicing to generate transcripts encoding $Clk1^{T}$ protein. Conversely, increased $Clk1^{T}$ protein favours splicing to generate transcripts encoding catalytically active Clk1 kinase. Our lab has shown that $Clk1^{T}$ and Clk1 can form heterodimers *in vitro* and suggest that such complexes have either decreased kinase activity or altered substrate recognition properties (Duncan and Bell, unpublished). Thus far we have been unable to demonstrate an *in vivo* interaction between Clk1 and Clk1T using FRET, but due to technical limitations of the assay, this does not preclude

Figure 15. The Clk kinase regulates splicing of its own pre-mRNA.

Splicing of the Clk1 pre-mRNA results in variants either coding for catalytically active (Clk1 kinase) or catalytically inactive ($Clk1^T$) isoforms. SR proteins (SR) are known to be involved in alternate splice site selection. The Clk1 kinase phosphorylates a SR protein(s) which then favours exon exclusion, leading to a splice variant coding for the truncated $Clk1^T$ protein. $Clk1^T$ can associate, but is not able to phosphorylate SR proteins, and therefore, exon inclusion is favoured. The resulting splice variant codes for the full-length catalytically active Clk1 kinase. As this form accumulates, the SR protein(s) is phosphorylated, leading to the eventual decrease in Clk1 in favour of $Clk1^T$.

Figure 15


Clk Pre -mRNA
AAAAAA
Exon Inclusion
Exon Exclusion
SR
SR
P
SR
Clk^T Protein
Clk Protein
AAAAAA
AAAAAA
Clk mRNA
Clk^T mRNA
Translation
Translation
Clk Protein
Clk^T Protein

this putative interaction from occurring *in vivo*. It is known that at least at the level of mRNA expression, Clk1 and Clk1^T isoforms are co-ordinately expressed and their relative abundances varies between cell and tissue types (Stojdl and Bell, unpublished). While the physiological role of Clk1:Clk1^T heterodimers remains to be established, the results presented here are consistent with a mechanism of pre-mRNA alternative splicing regulation dependent upon Clk1 kinase activity (Figure 15). This key regulatory role for the Clk1 kinase suggests that this enzyme could function as an interface between signal transduction pathways and the mRNA splicing machinery. We speculate the catalytic activity of Clks could be modulated by other phosphatases or kinases which are themselves components of signal transduction pathways, representing a cascade of modifiers responsible for the regulation of splicing. This perhaps demonstrates an example of phosphorylation as a modulator of gene expression by regulation of alternative splice site selection. Interestingly, the remaining members of the Clk family of kinases all undergo the same alternative splicing event as the prototypical Clk1 kinase, and each has demonstrated the ability to modulate splice site selection of an exogenously expressed viral transcript. An intriguing question remains: do Clk1, Clk 2, Clk3 and Clk4 each modulate only their own splicing or can they regulate the splicing of all Clk family members, thereby forming a network of regulation?

Chapter 3 The murine double-stranded RNA dependent protein kinase, PKR is required for resistance to VSV.

Summary

IFN induced anti viral responses are mediated through a variety of proteins, including the double-stranded RNA dependent protein kinase, PKR. Here we show that fibroblasts derived from $PKR^{-/-}$ mice are more permissive to VSV infection than wild type fibroblasts, and demonstrate a deficiency in IFN-α/β mediated protection. We further show that mice lacking PKR are extremely susceptible to intranasal VSV infection, succumbing within days after instillation with as few as 50 infectious viral particles. Again type I IFNs were unable to rescue $PKR^{-/-}$ from VSV infection. Surprisingly, intranasally infected $PKR^{-/-}$ mice did not die from pathology of the central nervous system, but rather from acute infection of the respiratory tract; demonstrating high viral titres in the lungs when compared to similarly infected wild type animals. These results confirm the role of PKR as the major component of IFN mediated resistance to VSV infection. Since previous reports have shown PKR to be unessential for survival in animals challenged with encephalomyocarditis virus, influenza and vaccinia virus (3, 206), our findings serve to highlight the premise that host dependence on the various mediators of IFN induced anti viral defences is pathogen specific.

Introduction

Interferons (IFN) have for some time now been recognized as the cytokines responsible for conferring an antiviral state to cells of the host. These effects are mediated by the gene products, activated or induced following the interaction of IFNs with their cognate receptor(s). Several pathways have been identified, the most actively studied of which include the Mx proteins, 2-5' adenylate synthase/RNaseL system, inducible nitric oxide

synthetase, and double-stranded RNA activated protein kinase PKR. Each of these systems affects the viral life cycle in different fashions. The Mx proteins for example, members of the dynamin superfamily, are thought among other things to inhibit the transport of virus nucleocapsids into the nucleus, thereby preventing viral transcription(95). Influenza, vesicular stomatitis virus (VSV), and measles are just some of the viruses demonstrating Mx protein dependent growth suppression (68). Interestingly, most laboratory strains of mice possess naturally occurring mutations in the two murine Mx genes cloned to date, and have shown an increased susceptibility to influenza and VSV infections as compared with wild mice (83, 84). The 2'-5' adenylate synthetase/RNase L pathway represents a multienzyme system which is activated upon the binding of double stranded RNA (dsRNA) to 2'-5' adenylate synthetase, which in turn produces 2'-5'-oligoadenylates (2'-5' A) that stimulate RNase L to degrade viral RNAs (89). RNase L has been shown to be protective against encephalomyocarditis virus (EMCV) (216) and vaccinia virus (41). Cytokine induced nitric oxide synthase (iNOS) has been shown to be protective against several viruses including: vaccinia (88), herpes simplex-1 (112), VSV (14), and reovirus (141). iNOS is thought to inhibit late stages of viral replication including protein synthesis (72, 91), viral protease activity (170), viral DNA synthesis (72, 118) and viral particle formation (72). PKR is an IFN inducible serine/threonine protein kinase activated subsequent to the binding of dsRNA. Once activated, PKR inhibits protein translation by phosphorylating eukaryotic initiation factor-2α (eIF2-α). PKR has also demonstrated a role in both virally and stress induced apoptosis (40, 90, 190, 208) and may thereby represent a multifunctional antiviral protein.

Two distinct homozygous disruptions for the PKR gene have been described

previously from independent groups (3, 206). Yang *et al.* (206) challenged their $PKR^{-/-}$ mice with EMCV but found no difference in survival as compared with wild type animals. This group did however demonstrate a reduction in protective effect from pretreatment with either IFN-γ or the dsRNA analogue poly inosine-poly cytosine (p(I).p(C)). These $PKR^{-/-}$ mice demonstrated normal type I IFN responses, however, embryo fibroblasts (MEFs) from these animals showed impaired induction of type I IFN and reduced activation of NF-κB following p(I).p(C) treatment. Abraham et al. (3) found no deficiency in resistance to either influenza or vaccinia virus *in vivo*, while type I IFN signaling, haematopoiesis, apoptosis and eIF2-α phosphorylation were found to be indistinguishable in MEFs derived from $PKR^{-/-}$ or wild type mice.

The present study was undertaken to examine the role of PKR in the IFN mediated resistance to vesicular stomatitis virus (VSV) infection. $PKR^{-/-}$ mice and MEFs derived thereof were tested, with or without IFN pretreatment, for their ability to resist VSV infection. We show here that PKR does indeed play a crucial role in antiviral defense and may represent the dominant IFN mediated response to VSV in vivo, particularly in the respiratory system.

Materials and Methods

Virus

The Indiana serotype of VSV was used throughout this study and was propagated in L929 cells.

Assaying viral production in primary mouse embryo fibroblasts

Primary MEF cultures were established from PKR$^{-/-}$ (3) and Balb-C E13.5 embryos as described previously (106). For analysis of MEF susceptibility to VSV infection, MEFs were seeded to 80% confluence in 35 mm dishes containing 2 ml of α-MEM (Gibco/BRL; Canada) supplemented with 10% FBS and either not treated, or supplemented with various doses of IFN; then incubated for 16 hours. Virus diluted in medium to an appropriate multiplicity of infection (MOI) was added to the dishes and allowed to adsorb for 30 min. at 37° C. The dishes were subsequently rinsed 3 times with PBS, overlaid with 2 ml of medium and incubated for 8 hours. Virus laden media from these dishes were then titred as described previously (26) with minor modifications. Briefly, virus containing media were serially diluted in α-MEM medium supplemented with 10% FBS. Each virus inoculum was allowed to adsorb to a monolayer of L cells for 30 min. at 37° C and then overlaid with 0.5% agarose in medium. Following an overnight incubation at 37° C the agar was removed, the monolayers were fixed in 4% paraformaldehyde and stained with 0.5% methylene blue. Plaques were counted visually.

Analysis of viral protein production by ^{35}S in vivo labelling

MEFs were infected with VSV at an MOI of 3 pfu/cell as described above. At 2, 4, 6, and 8 hours post infection, the dishes were rinsed with PBS and the cells harvested and lysed directly into SDS sample buffer (186). One hour prior to each time point (ie at 1, 3, 5, and 7 hours pi), the cells were pulsed with 500 μCi of ^{35}S labelled methionine/cysteine (1000 Ci/mmol; ICN; CA, USA) in Met/Cys deficient media (Gibco/BRL; ON, Canada) supplemented with 1% FBS. The lysates were boiled and the labelled proteins were

separated by SDS PAGE and visualized using a phosphoimager (Molecular Dynamics; CA, USA).

Experimental infection of mice

8-10 week old female mice were used throughout. Anaesthetised mice were infected intranasally with VSV diluted in 50 µl of phosphate buffered saline (PBS) into the nares of each animal. Subcutaneous (SC) infection proceeded without prior anaesthetic, with a 50 µl dose of virus diluted in PBS. For intraveneous (IV) infection, mice were catheterized into the tail vein, injected with VSV diluted in 100 µl of PBS, then the catheter was subsequently flushed with another 100 µl of PBS to ensure a consistent dose. In all cases mice were monitored for up to 14 days after infection for weight loss, piloerection, group huddling, dehydration, respiratory distress and hind limb paralysis. Animals displaying hind limb paralysis or severe morbidity were scored as such and euthanized in the interests of animal welfare.

Interferon treatments of animals was by intraperitoneal injection of either murine IFN-α/β (gift from Joan Durbin, New York University School of Medicine, NY, USA) as indicated, 24 hours prior to viral infection.

Determination of tissue viral titres

Mice were sacrificed and organs were aseptically removed and snap frozen on dry ice. Specimens were homogenized in 2 ml of PBS on ice and the virus was titrated on L cell monolayers as described above.

Results

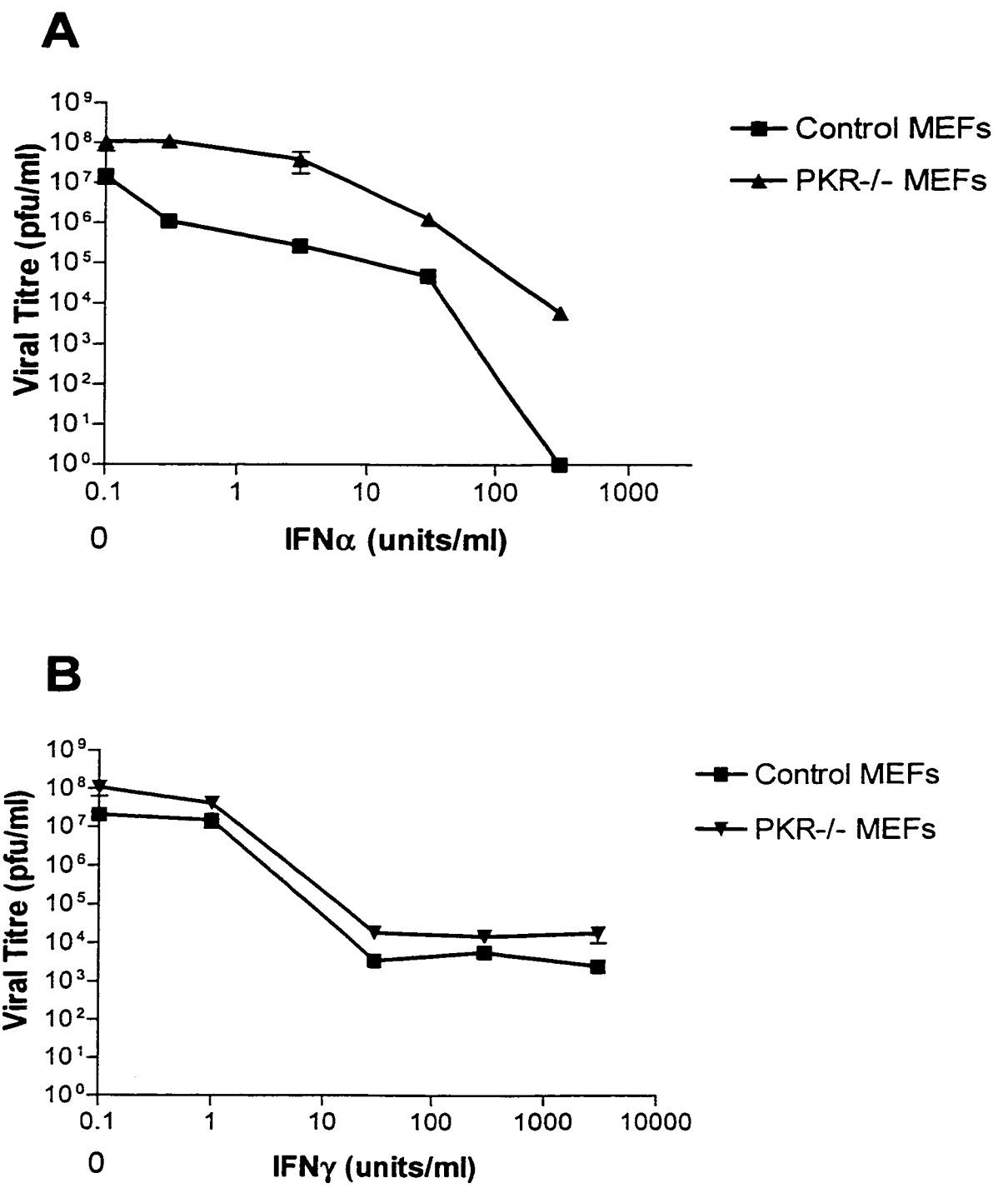
$PKR^{-/-}$ MEFs are more permissive to VSV infection when compared to control MEFs

Primary mouse embryo fibroblasts (MEFs) derived from $PKR^{-/-}$ mice and control mice were assayed for their permissiveness to VSV infection (Figure 1). IFN treated or untreated MEFs were infected with VSV at an MOI of 0.1 plaque forming units per cell (pfu/cell), and allowed to produce virus for 24 hours. The media from these dishes was then titrated to determine the number of infectious viral particles (pfu) produced from these cells, with or without pre-treatment with INF-α/β or IFN-γ. We observed a slight increase in the amount of virus produced from $PKR^{-/-}$ MEFs as compared with control MEFs (Figure 1, panels A & B), indicating that $PKR^{-/-}$ fibroblasts are moderately more permissive to VSV infection. Increasing doses of IFN-γ protected $PKR^{-/-}$ and control MEFs to a similar degree at each dose given, indicating no deficiency in the type II IFN pathway (Figure 1, panel B). We did, however, see a consistently greater viral titre produced from infected PKR-/- MEFs than from control MEFs, regardless of IFN-γ dose. Type I IFN mediated responses showed a marked deficiency in $PKR^{-/-}$ MEFs, as doses of IFN-α/β which completely protected control MEFs (7 log reduction in titre) were only able to reduce the titre in $PKR^{-/-}$ MEFs by 4 logs to 1×10^4 pfu/ml (Figure 1, panel A).

Figure 1. MEFs from $PKR^{-/-}$ animals show increase in VSV production and a type I IFN deficiency compared to control MEFs.

Primary embryo fibroblasts from $PKR^{-/-}$ and Balb-C mice (control) were either untreated or treated with various doses of IFNs 18 hours prior to their infection with VSV at an MOI of 0.1 pfu/cell. The infectious viral particles produced from these infections were titrated as described in the Material and Methods and are represented as pfu/ml. Untreated $PKR^{-/-}$ MEFs were slightly more productive for VSV infection (5-10x) when compared to untreated control MEFs (panels A & B). (A) Control MEFs showed dose dependent reductions in titre when pretreated with IFN-α/β, with 300 IU/ml providing complete protection. $PKR^{-/-}$ MEFs, however, responded less well producing between 1 to 4 logs more virus than control MEFs, depending on the dose of IFN given. (B) IFN-γ pretreatment protected both $PKR^{-/-}$ and control MEFs to a similar degree, however, $PKR^{-/-}$ MEFs consistently produced 2 to 5 fold more virus than controls regardless of IFN dose. Data represent means +/- SEM of triplicate experiments.

Figure 1


A
Viral Titre (pfu/ml)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0.1
1
10
100
1000
0
IFNα (units/ml)
Control MEFs
PKR-/- MEFs
B
Viral Titre (pfu/ml)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0.1
1
10
100
1000
10000
0
IFNγ (units/ml)
Control MEFs
PKR-/- MEFs

We also monitored the kinetics of infection in MEFs using ^{35}S methionine *in vivo* labelling following infection of MEFs at a dose of 3 or 10 MOI pfu/cell. For $PKR^{-/-}$ MEFs, (Figure 2) we detected the appearance of viral proteins at about 4 hour pi, with bands representing VSV G, N and M proteins increasing in intensity relative to host proteins as time progressed. Control MEFs also expressed viral proteins at 4 hours pi, but the intensity of these bands did not appear to increase appreciably during the remaining course of infection as it had for the $PKR^{-/-}$ MEFs. It appears from these experiments that although the onset of infection is similar between wild type and $PKR^{-/-}$ MEFs, control fibroblasts are capable of limiting the infection while in $PKR^{-/-}$ cells, the kinetics of VSV replication are unabated.

$PKR^{-/-}$ mice demonstrate an acute susceptibility to intranasal VSV infection

To further investigate the role of PKR in the defence against VSV, we infected $PKR^{-/-}$ mice intranasally (IN) at varying doses and monitored their survival alongside $PKR^{+/+}$ animals. Table 1 reveals the extreme sensitivity of $PKR^{-/-}$ mice to intranasal VSV infection, even down to the minimum dose of 50 pfu. None of the mice lacking PKR could survive beyond 5 days post infection (pi), while all wild type animals, treated at the highest dose tested, lived past this point. While the normal pathology of VSV infections in mice is manifested in the CNS, most notably as hind limb paralysis, curiously, all $PKR^{-/-}$ animals infected with VSV appeared to die of respiratory failure.

Interferon cannot rescue $PKR^{-/-}$ animals from intranasal VSV infection

Animals nullizygous for PKR and infected with VSV by the intranasal route,

demonstrate a significantly decreased survival rate compared to control mice (Figure 3; panel

Figure 2. SDS PAGE separation of ^{35}S labelled proteins synthesised in VSV infected MEFs from $PKR^{-/-}$ and control animals.

MEFs were either not infected (-), or infected with VSV at an MOI of 3 for 2, 4, 6, or 8 hours as described in the Materials and Methods. The infected cells at each time point were pulsed with ^{35}S methionine/cysteine for 1 hour prior to being harvested and lysed. VSV proteins (G, N and M) begin to appear in $PKR^{-/-}$ and control cells at 4 hours pi. While this pattern of labelled proteins remains constant in control cell up to 8 hours pi, viral proteins become the predominant translated proteins in the $PKR^{-/-}$ samples by 8 hours pi. Molecular masses are indicated in kDa.

Figure 2

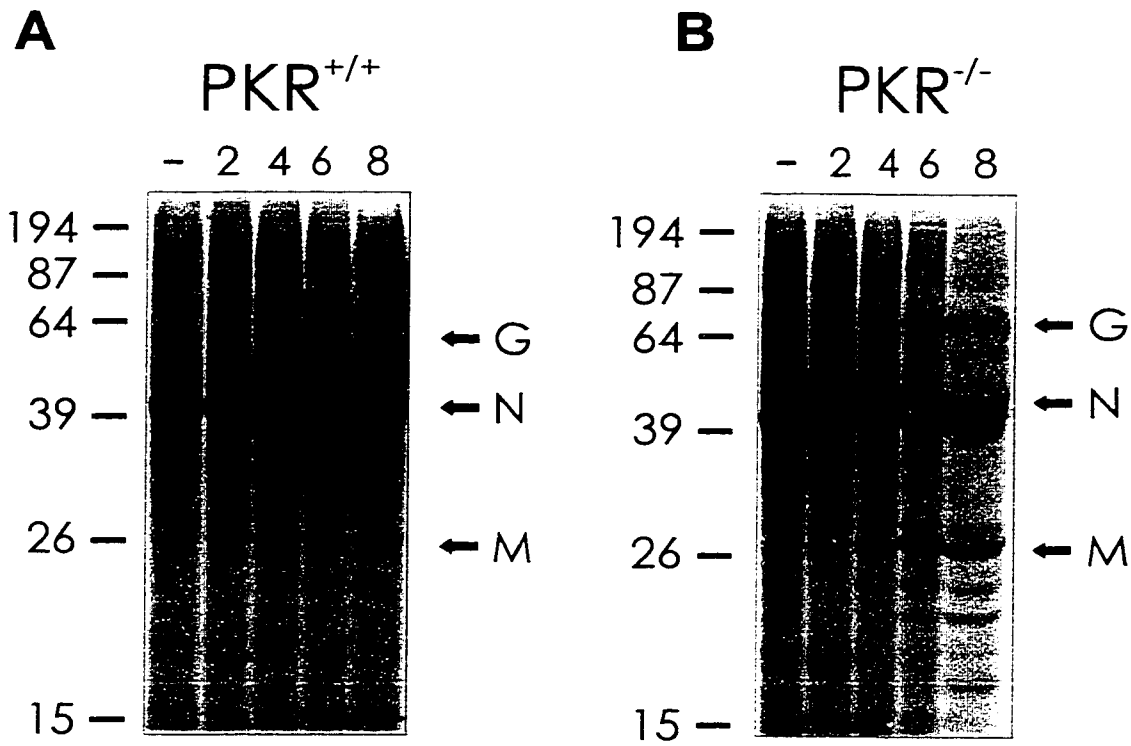


Table 1. $PKR^{-/-}$ or $PKR^{+/+}$ mice infected intranasally with varying doses of VSV.

$PKR^{-/-}$ and control mice ($Sty^{-/-}$; irrelevant knockout mice generated from the same strain as $PKR^{-/-}$ mice, used as a $PKR^{+/+}$ strain with very similar genetic background to $PKR^{-/-}$ animals) were infected intranasally at various doses and their survival monitored over time. All $PKR^{-/-}$ mice succumbed to the infection between days 2 and 5 depending on the dose, while control mice remained alive beyond this point.

Table I

Innoculum (pfu)	Survival at day 5
$PKR^{+/+}$ $5x10^4$	5/5
$PKR^{-/-}$ $5x10^4$	0/5
$PKR^{-/-}$ $5x10^3$	0/4
$PKR^{-/-}$ $5x10^2$	0/3
$PKR^{-/-}$ $5x10^1$	0/3

A, B). At this dose ($5x10^4$ pfu IN) all $PKR^{-/-}$ died fairly synchronously by day 4. All these animals exhibited piloerection and rapid breathing as early as 16 hours after infection, weight loss (3-5% total weight per day), and squinting of the eyes (by day 2). By day 4 post infection, most animals were in respiratory distress, many with severe distress as denoted by "crackles and pops" heard while breathing. If allowed to continue, the animal died within the hour. This is in stark contrast with wild type animals which all survived past day 14 and never exhibited the respiratory distress manifested by the knock out animals. Wild type mice sometimes showed mild piloerection, eye squinting, and weight loss, but appeared to recover from these symptoms by day 6 pi. To investigate the contribution of $PKR^{-/-}$ in the interferon resistance to VSV, both wild type and $PKR^{-/-}$ mice were pre-treated with $2 x10^4$ or $2 x10^5$ units of mouse IFN-α/β 18 hours prior to intranasal infection with VSV (Figure 3, panels A &B, respectively). Wild type animals showed complete alleviation of the aforementioned symptoms at both doses of IFN. $PKR^{-/-}$ mice however, showed no increase in survival (Figure 4, panels A&B) and no alleviation of symptoms in the IFN treated population, even at the highest dose.

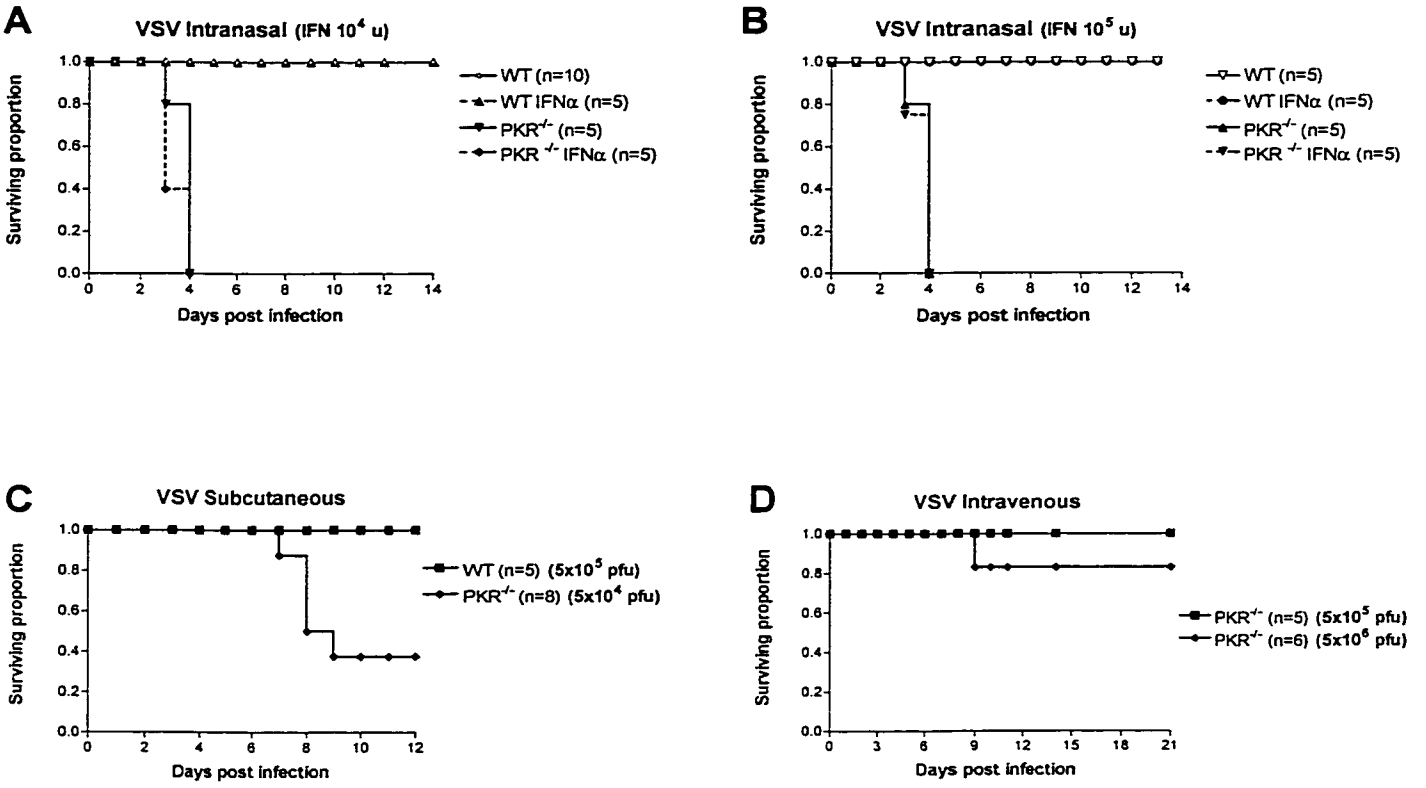
Route of infection determines pathology for $PKR^{-/-}$ animals

The observation that $PKR^{-/-}$ mice succumb to respiratory pathology rather than neuropathology was unexpected. To investigate this further, we infected both wild type and $PKR^{-/-}$ animals through various routes and monitored their survival and symptoms. As expected wild type animals were completely resistant to subcutaneous VSV infection at a dose of $5x10^5$ pfu (Figure 3, panel C). These mice never showed any signs of infection or distress. In contrast $PKR^{-/-}$ animals infected at a 10 fold lower dose demonstrated a marked

Figure 3. PKR$^{-/-}$ mice are acutely sensitive to intranasal VSV infection and demonstrate a deficiency in IFN mediated resistance.

(A & B) PKR$^{-/-}$ and control mice were infected intranasally with $5x10^4$ pfu of VSV and monitored for morbidity and survival over the course of 14 days, after which remaining animals were deemed to have survived the infection. PKR$^{-/-}$ mice showed a severe decrease in survival compared to wild-type mice (WT), succumbing by day 3 or 4, while all control mice survived the infection. IFN-α/β pretreatment (18 h prior to infection) with either $2 x10^4$ IU (A) or $2 x10^5$ IU (B) had no protective effect in PKR$^{-/-}$ animals. (C) PKR$^{-/-}$ mice infected subcutaneously ($5x10^4$ pfu) showed increased survival when compared with intranasally infected PKR$^{-/-}$ animals (A&B), but were still more susceptible when compared with subcutaneously infected control mice which appeared unaffected even at a 10 fold higher dose ($5x10^5$ pfu). All PKR$^{-/-}$ animals scored here as fatalities displayed hind limb paralysis and were euthanized. (D) PKR$^{-/-}$ mice demonstrate resistance to intravenous VSV infection ($5x10^5$ & $5x10^6$ pfu) with all animals except one surviving (1 of 6 in $5x10^6$ cohort). This animal displayed signs of hind limb paralysis and was euthanized.

Figure 3


A
VSV Intranasal (IFN 10^4 u)
Surviving proportion
Days post infection
WT (n=10)
WT IFNα (n=5)
PKR-/- (n=5)
PKR -/- IFNα (n=5)
B
VSV Intranasal (IFN 10^5 u)
Surviving proportion
Days post infection
WT (n=5)
WT IFNα (n=5)
PKR-/- (n=5)
PKR -/- IFNα (n=5)
C
VSV Subcutaneous
Surviving proportion
Days post infection
WT (n=5) (5x10^5 pfu)
PKR-/- (n=8) (5x10^4 pfu)
D
VSV Intravenous
Surviving proportion
Days post infection
PKR-/- (n=5) (5x10^5 pfu)
PKR-/- (n=6) (5x10^6 pfu)

decrease in survival, with less than 40% of the animals remaining after 12 days pi. Furthermore, these animals did not present with severe respiratory distress, as was the case with intranasal infection, but instead developed hind limb paralysis.

$PKR^{-/-}$ animals infected intravenously showed good resistance to VSV infection at doses as high as $5x10^6$ pfu (Figure 3, panel D). At this dose, only one of 6 animals showed any signs of sickness and was eventually euthanized at day 9 pi after developing hind limb paralysis. Tissues assayed for VSV from this animal showed virus present only in the brain at levels similar to intranasally infected animals (data not shown).

$PKR^{-/-}$ mice show high lung titres following intranasal infection

To determine the extent of viral infection, assorted tissues were titrated for virus following infection through various routes. $PKR^{-/-}$ and control animals ($Sty^{-/-}$) were infected intranasally, and on day 4 when $PKR^{-/-}$ animals appeared quite sick, blood, brain, lung, liver, kidney and spleen tissues were removed aseptically and analyzed to determine the titre of virus present. Wild type animals showed significant titres in the brain (Figure 4) while all other organs had no detectable virus (data not shown). $PKR^{-/-}$ mice also showed high titres in the brain, but in contrast, had significantly higher titres (3 logs higher) of VSV in the lungs when compared to control mice (Figure 4). As was the case with wild type mice, all other tissues from $PKR^{-/-}$ animals showed no detectable infection with VSV, by this method. Furthermore, pre-treatment with increasing doses of IFN-α/β was not able to decrease viral titres in the lungs of $PKR^{-/-}$ animals infected intranasally with VSV (Figure 4).

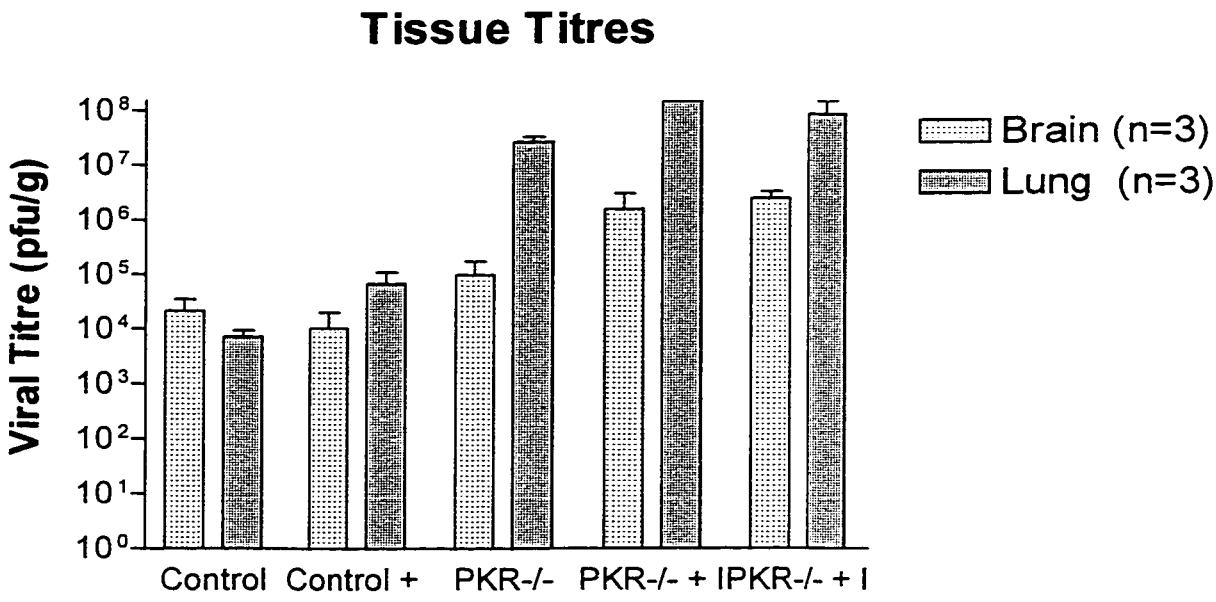
When $PKR^{-/-}$ animals were infected subcutaneously and assayed on day 8 pi, we could

only find virus in the brain while the lungs had very low or undetectable levels of VSV. Wild type animals, in contrast, had low titres in the brain and no detectable virus in any other organs. Intravenous infection with VSV again demonstrated no virus in any other organs except the brain (data not shown).

Figure 4. VSV infected $PKR^{-/-}$ mice show high lung titres compared with control mice.

IFN-α treated and untreated $PKR^{-/-}$ and control mice were infected intranasally with $5x10^4$ pfu and sacrificed for their organs on day 4 pi. Blood, spleen, kidney, liver, brain and lung tissues were titrated of which only brain and lung showed any detectable virus. Although brain titres from both $PKR^{-/-}$ and control mice were similar ($<10^5$ pfu/g), lung titres in $PKR^{-/-}$ animals were 2-3 logs higher than those seen in control mice. IFN-α/β treatment of $PKR^{-/-}$ mice did not reduce viral titres, and perhaps even resulted a slight increase in viral titre in both brain and lung tissues. Viral titres expressed as pfu per gram of tissue (pfu/g). Data represents means +/- SEM of triplicate experiments.

Figure 4


Tissue Titres
Viral Titre (pfu/g)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Control
Control +
PKR-/-
PKR-/- + I
PKR-/- + I
Brain (n=3)
Lung (n=3)

Discussion

Biochemical data from many laboratories have clearly defined PKR as an interferon inducible gene product whose enzymatic activity is stimulated by dsRNA (13, 57, 98, 121). Because of these properties, PKR has been predicted to play a major role in interferon mediated antiviral defence. Indeed PKR demonstrated an antiviral role in cultured cells following various means of over expression of the wild type or catalytically inactive mutants (100, 101, 122). However, previous studies from our lab and others have failed to demonstrate a definitive role for PKR on an organismal level using genetic ablation of PKR in mice (3, 206). Yang *et al.* (206) challenged their $PKR^{-/-}$ mice with EMCV (IV ~1000 pfu) but found no difference in survival as compared with wild type animals but did show a diminished protective effect from pretreatment with either IFN-γ or the dsRNA analogue (p(I).p(C)). In a recent report Zhou *et al.* have also shown only a very slight difference in survival between wild-type animals and the Yang $PKR^{-/-}$ mice following EMCV infection (IV 100 pfu) (217). These data indicate that during the course of infection by intravenously injected EMVC, PKR does not play an important role in antiviral defense, but can be called upon to bolster defenses if the animal is pretreated with dsRNA or IFN-γ prior to infection. Abraham et al. (3) also found no deficiency in resistance to either influenza or vaccinia virus in their $PKR^{-/-}$ mice. As mentioned above, these results were unexpected as the biochemical evidence predicted a substantial role for PKR in antiviral defense.

We continued to investigate the role of PKR in viral resistance and have observed in this study that MEFs derived from the Abraham mouse (3) were more permissive to VSV

infection than MEFs from wild type animals (Figure 1). We further demonstrated that although IFN-γ treatment was equally protective in both $PKR^{-/-}$ MEFs and control MEFs, IFN-α/β was much less effective in protecting MEFs devoid of PKR than in PKR+/+ control cells (Figure 1). This deficiency in type I IFN mediated response in $PKR^{-/-}$ MEFs advocates the importance of PKR in the IFN mediated resistance to VSV in fibroblasts. This is consistent with a recent report by Zhou *et al.* describing similar results with MEFs derived from the Yang mouse (217). They showed a decrease in type I interferon mediated protection in $PKR^{-/-}$ MEFs as compared to wild type derived MEFs (217); and further showed the interferon mediated resistance to VSV in cultured cells can be attributed almost exclusively to PKR, as MEFs from mice triply deficient in Mx1, RNAseL and PKR showed no increase in susceptibility to VSV as compared to MEFs deficient for PKR alone (217). The differences in susceptibility between wild type and $PKR^{-/-}$ cultured cells only became apparent in this study, with increased IFN pretreatment; that is to say that untreated MEFs from both $PKR^{-/-}$ cells were only 2-5 fold more susceptible to VSV than were control MEFs. This is consistent with our *in vitro* data that in cultured primary embryo fibroblasts at least, PKR on its own is only slightly protective against VSV infection. Type I IFN pretreatment however, can provide complete resistance to VSV infection, only if PKR is present.

To examine this in the whole mouse, we intranasally infected $PKR^{-/-}$ mice with various doses of VSV and observed a significant increase in susceptibility compared to control mice. At doses as low as 50 pfu, all of the $PKR^{-/-}$ mice succumbed to the infection, while all control mice survived. This was surprising considering the modest difference in susceptibility observed in cultured cells from these mice. Firstly, this demonstrates

unequivocally, that PKR plays a major role in host defense against VSV infection. Without PKR, a mouse cannot survive intranasal infection with even a minute inoculum of VSV (Table 1). Furthermore, since pretreatment with IFN-α/β could not rescue these animals (Figure 3, panels A and B) nor could it ameliorate their symptoms, PKR may in fact be the major component for the IFN pathway mediating resistance to intranasal VSV infection. This is in good agreement with the data mentioned above in cultured cells, but highlights a critical limitation for studies with tissue culture cells. Although a modest correlation was found between PKR and VSV susceptibility in MEFs, in the mouse model presented here, PKR is essential for survival. This could perhaps be explained by different tissue sensitivities to the virus. In fact, intranasal infection of $PKR^{-/-}$ mice resulted in an acute respiratory pathology leading to the death of the animal. Tissue titres from $PKR^{-/-}$ mice showed high levels of virus in the lungs and brains, while control animals had equally high titres in the brain but greatly reduced titres in the lungs compared to $PKR^{-/-}$ mice (Figure 4). This is also supported by histological analysis of tissues harvested from infected mice. The lungs of $PKR^{-/-}$ mice showed considerable inflammation of the alveolar ducts while control mice showed no such pathology (data not shown). IFN-α/β treatment again showed no protective effect in $PKR^{-/-}$ mice, as VSV tissue titres did not decrease with increasing doses of IFN (Figure 4). In fact viral tissue titres appeared to increase following IFN pretreament; although we are not convinced that this represents a true exacerbation of the infection, as increasing doses of IFN-α/β did not seem to result in consistently decreased survival (Figure 3, panels A & B). Taken together with the observed symptoms of respiratory distress displayed by $PKR^{-/-}$ during the infection, which were not evident in the control mice, it would

appear that tissues in the lungs have an exquisite sensitivity to VSV infection in the absence of the *pkr* gene product. Interestingly, in a study by Ronni et al. (160) human lung epithelium was shown to have only a modest type I IFN response and a slow MxA protein accumulation following infection with influenza A virus. It maybe therefore that lung epithelium relies heavily on PKR to dampen viral infection until humoral host defences can be mounted.

The route of inoculation also plays a role in the course of the infection. Subcutaneous infection of $PKR^{-/-}$ mice with $5x10^4$ pfu VSV resulted in 40% survival as compared to Balb-C animals which were unaffected even at a 10 fold higher dose (Figure 3, panel C). This survival rate is significantly better than that seen when $PKR^{-/-}$ animals were infected intranasally. Interestingly, $PKR^{-/-}$ mice did not present with severe respiratory distress, as was the case during intranasal infection, but instead showed neuropathology manifested as hind limb paralysis. As well, $PKR^{-/-}$ animals demonstrated relatively high resistance to VSV infection when delivered intravenously (Figure 3, panel D). Doses as high as $5x10^6$ pfu were well tolerated by these mice with only one animal affected, and subsequently euthanized due to signs of hind limb paralysis. When the organs of this animal were titred for the presence of VSV, it showed virus in the brain only. Therefore, route of infection determines not only the severity but also the type of pathology seen. This again suggests that $PKR^{-/-}$ mice may be acutely sensitive to infection of the respiratory system, as routes of infection which bypass the respiratory tract, show increased survival of these animals.

It is also formally possible that $PKR^{-/-}$ mice show a deficiency in resistance at the initial sites of infection thereby determining the tropism of the virus. During the intranasal

infection in wild type animals, VSV initially infects the olfactory receptor neurons, and then spreads quickly to the rest of the central nervous system (CNS)(146). The respiratory epithelium appears to be relatively resistant to VSV(110). Perhaps in $PKR^{-/-}$ mice however, the infection cannot be dampened by the innate immune system at the respiratory epithelium because of the lack of PKR and therefore allows the virus to spread to and eventually inundate the respiratory system, leading to the death of the animal. Although the exact mechanisms remain to be determined, it is clear that mice deficient in PKR do not succumb to VSV via the standard pathology of the CNS, but die first from a massive infection and inflammation of the respiratory system.

The IFN pathway has many tools by which it protects the host from viral infections. It is becoming clear now that the host relies on each of these mechanisms to various degrees depending on the pathogen at hand. The genetic ablation of PKR in mice has allowed us to determine the contribution of this gene product to interferon mediated defence. We have shown here that PKR appears to be a critical facet of the innate immune response protecting the host against VSV infection. Other viruses such as EMCV, influenza and vaccinia have evolved mechanisms to defeat PKR (27, 39, 102), and therefore have perhaps diminished the role of PKR in resisting infection from these viruses, forcing the host instead to rely on other effectors of the IFN pathway to counter these invaders.

Chapter 4 Exploiting tumour specific defects in the interferon pathway with a novel oncolytic virus

Summary

Interferons are potent cytokines which have both antiviral and anti-cell proliferative properties. We have exploited defects in the interferon pathway found in many tumour cells as a means to selectively target neoplastic cells. We found that vesicular stomatitis virus (VSV) rapidly replicates in and kills a variety of human tumour cell lines even in the presence of doses of interferon which completely protect normal human primary fibroblasts from infection. When added to mixed cultures of normal and malignant cells, VSV showed selective killing of the tumour component leaving normal primary fibroblasts and bone marrow stem cells unaffected. Finally, a single intratumoural injection of VSV was effective in reducing the tumour burden of nude mice bearing subcutaneous human melanoma xenografts. Our results support the use of VSV as a replication-competent oncolytic virus and demonstrate a novel strategy for the treatment of interferon non-responsive tumours.

Introduction

Despite significant improvements in early detection and advances in the use of surgery, chemotherapy and radiation protocols over the last several decades, cancer remains one of the leading causes of death in North America. In particular, the cure of widely disseminated metastatic cancers is a highly desirable but elusive goal of the oncologist. A new modality of therapy which holds promise is the use of self-replicating bacterial or viral strains with the ability to specifically kill tumour but not normal cells. Indeed certain strains of Clostridia (54) and Salmonella (109) have been reported to grow in the milieu of a tumour

due to its hypoxic or other characteristics.

As discussed earlier, IFNs are circulating factors that bind to cell surface receptors, activating a signaling cascade that ultimately leads to a number of biological responses (185). Type I IFNs signal through the IFN-α/β receptor-activating the receptor associated tyrosine kinases Jak1 and Tyk2, which in turn phosphorylate the interferon stimulated gene factor 3 complex (ISFG3). ISFG3 complexes mediate transcription of a variety of genes via interferon stimulated regulatory elements (ISRE) in the promoters of type I responsive genes. Type II IFNs (IFN-γ) bind to their own type II receptor and activate Jaks 1 and 2. Once activated, Jaks 1 and 2 phosphorylate STAT1 initiating its homodimerization and nuclear translocation to gamma activation sequence (GAS) elements which drive the transcription of IFN-γ stimulated genes (185). Two outcomes of interferon signalling are tightly linked: (1) an antiviral response which includes the Mx proteins, 2-5' Adenylate synthase/RNaseL system, inducible nitric oxide synthetase, and double-stranded RNA activated protein kinase PKR; and (2) induction of growth inhibitory and/or apoptotic signals in normal and malignant cells. In the latter case, attempts to exploit the ability of IFNs to limit the growth of tumours in patients has met with limited results. Clinical trials using Type I interferons showed only 10-40 % of patients with melanomas, myelomas and renal cell cancers demonstrating appreciable responses, while the more common solid tumours such as breast, prostate, stomach, colon, lung and uterus, appear to be essentially refractory (94). This lack of interferon responsiveness is likely due to the cancer specific mutation of gene products in the interferon signalling cascade that have been found in a multitude of cancers (15, 31, 42, 65, 76, 85, 123, 138, 144, 145, 155, 172, 204). We hypothesize that if cancer cells gain a

survival advantage over their normal counterparts by acquiring mutations in the interferon pathway, they would do so at the expense of critical antiviral responses. To test this idea, we used vesicular stomatitis virus (VSV), a member of the Rhabdoviridae family of RNA viruses. VSV is an enveloped virus with a single-stranded, negative-sense RNA genome of approximately 11 kb in length encoding five genes(103). The virus reportedly causes a disease symptomatically similar to foot-and-mouth disease in cattle, horses, and pigs and may be transmitted to other species, including humans, causing mild influenza-like symptoms. While VSV is a rapidly growing cytolytic virus, it is also known to be exquisitely sensitive to interferon treatment (9). We believe that VSV may be an ideal tumouricidal virus as its growth in normal tissues can be attenuated by interferon treatment while remaining oncolytic in interferon non-responsive tumour cells.

Materials and Methods

Cell lines and virus

The Indiana serotype of VSV was used throughout this study and was propagated in L929 cells. The following cell lines and corresponding culture media were used throughout: AG 1522 human foreskin fibroblasts (αMEM with 10% FBS)(153); (OSF7, OSF12 and OSF16 primary normal human fibroblasts, established at the Ottawa Regional Cancer Centre from forearm biopsies, (αMEM with 10% FCS); MN11 mouse fibrosarcoma (αMEM with 10% FBS) (201); A2780 human ovarian (DMEM/F12 with 10% FBS) (97). OVCA 420 and OVCA 432 human ovarian carcinomas (αMEM with 10%FBS, 2mM L-gultamine, 1% NaPyruvate, 1% non-essential amino acids) (11); C13 human ovarian carcinoma (RPMI 1640 with 10 % FBS) (5); SK-MEL3 human melanoma (DMEM/F12 HAM with 10% FBS) (202);

LC80 human lung carcinoma (αMEM with 10% FBS): HCT116 human colon carcinoma (DMEM with 10% FBS) (ref); and LNCAP human prostate carcinoma (αMEM with 10% FBS) (124).

Viral production and CPE in tumour cells and primary fibroblasts

Cell lines and primary fibroblasts were seeded to 80% confluence in 35 mm dishes containing 2 ml of α-MEM (Gibco/BRL; Canada) supplemented with 10% FBS and either not treated, or supplemented with various doses of IFN; then incubated for 16 hours. Virus diluted in medium to an appropriate multiplicity of infection (MOI) was added to the dishes and allowed to adsorb for 30 min. at 37° C. The dishes were subsequently rinsed 3 times with PBS overlaid with 2 ml of medium and incubated for 8 hours. Virus laden media from these dishes were then titred as described previously (26) with minor modifications. Briefly, virus containing media were serially diluted in α-MEM medium supplemented with 10% FBS. Each virus inoculum was allowed to adsorb to a monolayer of L cells for 30 min. at 37° C and then overlaid with 0.5% agarose in media. Following an overnight incubation at 37° C the agar was removed, the monolayers were fixed in 4% paraformaldehyde and stained with 0.5% methylene blue. Plaques were counted visually.

CPE was monitored in infected, live monolayers grown on coverslips and infected as described above. At 12 hours post infection phase contrast micrographs of monolayers were taken using a Zeiss Axiophot light microscope.

Kinetics of cytolytic VSV

Various cell lines were grown in 12-well plates to approximately 75% confluency.

Medium was replaced with fresh medium with or without interferon alpha (100 units/ml; INTRON-A, Shering, USA) and incubated for another 12 hours prior to infection. The cells were infected with VSV in 20 μl of medium at a M.O.I. of 0.1 pfu per cell for 30 minutes at 37 C, after which each well was topped up with fresh medium. At twelve hour time points, medium was removed and cells were fixed with methanol and stained with methylene blue and eosin.

Mixed Cell Cultures

Equal numbers of normal human fibroblasts and 293T cells were co-cultured on gelatin-coated coverslips in αMEM (10% FBS plus/minus interferon-α at 100U/ml) and were infected with VSV at an MOI of 0.1 pfu/cell. Eighteen hours post-infection the supernatants were collected and the cells were fixed with methanol. A polyclonal anti-TAg antibody was hybridized to the fixed cells and detected with a goat anti-rabbit CY3-conjugated secondary antibody (Sigma). Nuclei were stained with DAPI. The coverslips were mounted in antifade and viewed under a fluorescent microscope.

Hematopoietic Cell Culture

White blood cells were isolated from normal human marrow and exposed to virus at various multiplicities of infection for 30 minutes. The cells were then washed 3 times with PBS and incubated in IMDM (20% FBS, 10% 5637 conditioned medium) overnight. Medium was then collected and titred as above.

The leukemic cell line OCI/AML3 was cultured in IMDM (10% FBS) while OCI/AML4 and OCI/AML5 were cultured in IMDM (10% FBS, 10% 5637 conditioned

media). The dose of virus which caused 50% killing at both 24 and 48 hours was determined using the MTS which measures the bioreduction of a tetrazoleum compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) into a formazan which can be measured colourimetrically (34).

The growth factor-independent cell line OCI/AML3 was mixed 1:9 with normal human marrow and co-cultured in IMDM (20% FBS, 10% 5637 conditioned medium). Test cultures were infected at an MOI of 1.0 pfu/cell or 5 pfu/cell while controls were left uninfected. 24 hours post-infection cells were plated in methylcellulose containing 12% FBS plus or minus growth factors (10% 5637 conditioned medium, kit ligand and erythropoietin). Colonies were counted 14 days later.

VSV treatment of xenograph tumours

Xenographs were initiated in 8-10 week old female Balb-C nude mice by injecting 5 $x10^6$ SK-MEL 3 human melanoma cells subcutaneously in each of two sites per mouse. When tumours reached 4-5mm in diameter, they were either left untreated or treated with a single intratumoral injection with either 10^8 pfu VSV in culture medium or $2.5x10^6$ VSV infected SK-MEL 3 cells (infected at an MOI of >10 pfu/ml for 2 hours). The health of the mice was monitored and tumour sizes were measured daily.

Statisical Analysis

For single comparisons (Figure 4; day 6), the tumour volumes were analysed by Student's *t* test. For multiple comparisons (Figure 4; day 3, 4 or 5), the data were first analyzed by one-way analysis of variance, and statistical significance was determined by

Bonferroni's multiple comparison test using Prism 3.0 software (Graphpad Software Inc; CA, USA).

Results

VSV rapidly kills interferon treated tumour cells

As a first test we used the Indiana strain of VSV to infect a series of normal and tumour cell lines in the presence and absence of interferon. As can be seen in Table 1, infection of primary human fibroblasts (AG 1522), in general, produced lower virus yields following an overnight infection than did parallel infections of a range of tumour cells. More importantly, pre-treatment of the normal cell cultures with interferon completely abrogated the growth of VSV while tumour cell lines produced copious amounts of virus particles. Some tumour cell lines (MN11, C13) showed reduction in virus titre following interferon priming suggesting that they have an impaired but not completely defective interferon response. The differences between the various cell types was reflected not only in production of virus particles, but also in the cytopathic effect (cpe) observed at the microscopic level. As shown in Figure 1, at 12 hours post infection primary human fibroblasts show little or no cpe and the interferon treated cultures are indistinguishable from uninfected cells. On the other hand, tumour cells (SK-MEL3) infected with VSV show extensive cpe and are not protected by interferon. It is important to note that in these experiments, target cells were infected at a

Table 1. Virus yield after overnight infection of various cell lines either untreated or treated with IFN

A variety of normal and transformed cell lines were either untreated or pre-treated with 100 units of IFN-α, infected at an MOI of 0.1 pfu/ml and incubated for 18 hours at 37 °C. Culture medium from each sample was titred for VSV production.

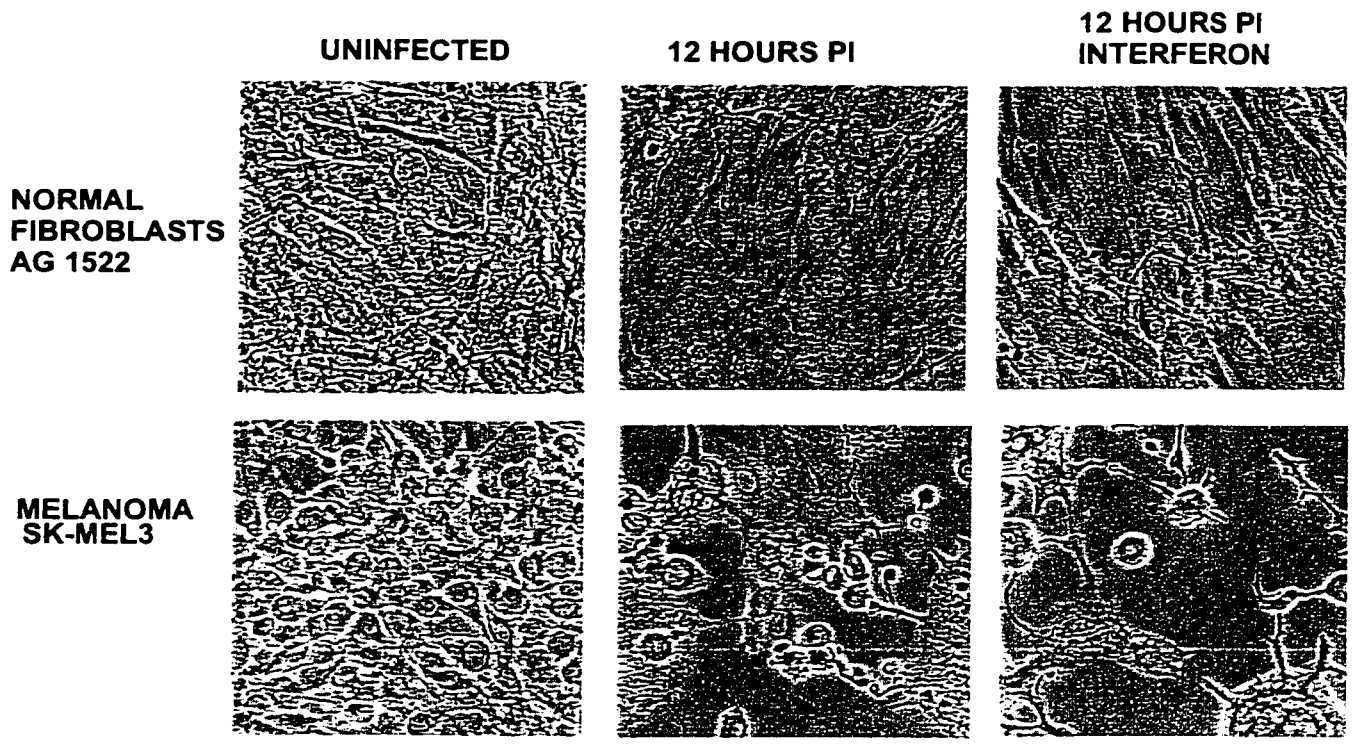
Table I

Cell Line	*Viral Titre (pfu/ml)*	
	Untreated	*IFN-α*
OSF7 (normal human fibroblast)	1×10^6	0
OSF12 (normal human fibroblast)	2×10^5	0
OSF16 (normal human fibroblast)	1×10^5	0
MN11 (murine fibrosarcoma)	1×10^8	1×10^4
A2780 (human ovarian carcinoma)	2×10^8	1×10^7
OVCA 420 (human ovarian carcinoma)	1×10^8	3×10^6
C13 (human ovarian carcinoma)	1×10^8	1×10^5
LC80 (human lung carcinoma)	2×10^9	6×10^7
SK-MEL3 (human melanoma)	1×10^9	1×10^9
LNCAP (human prostate carcinoma)	4×10^9	5×10^9
HCT116 (human colon carcinoma)	1×10^9	2×10^9
293T (HEK cells transformed with T antigen and E1A and E1B)	1×10^8	8×10^7

Figure 1. VSV induced cytopathic effect visible in human melanoma cells but not in normal fibroblasts with or without IFN-α.

Gelatin-coated coverslips with normal fibroblasts and SK-MEL3 cells untreated or pretreated with IFN-α (100 units) were infected with VSV at an MOI of 0.1 pfu/ml. Twelve hours post infection, micrographs were taken demonstrating cytopathic effect (cpe) in the melanoma cells and not in normal fibroblasts. IFN-α pretreatment was not shown to protect SK-MEL3 cells from VSV induced cpe.

Figure 1


UNINFECTED
12 HOURS PI
12 HOURS PI
INTERFERON
NORMAL
FIBROBLASTS
AG 1522
MELANOMA
SK-MEL3

multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) per cell. Thus the extensive cpe observed in the tumour cell population reflects the rapid growth and spread of VSV through the tumour cell culture. To examine the kinetics of the cytolytic properties of VSV in a number of cell lines we adapted an assay previously used to study the oncolytic properties of the Onyx-015 adenovirus (Figure 2) (75). Cells were seeded into multiwell plates and again infected at a MOI of 0.1 pfu per cell. At the indicated times, the infection was stopped and the monolayers were fixed and stained. As can be seen, at this MOI up to 48 hours later the majority of the normal infected cells, interferon treated or untreated, appeared viable and remained attached to the plastic plate (compare Figure 2, normal fibroblast 0-48h with CTRL). In the case of the tumour cells tested however, as early as 12 hours post infection, the monolayer is essentially destroyed. Consistent with our earlier observations (Table 1), the cytopathic effect of VSV in these cell lines is unaffected by the presence of interferon.

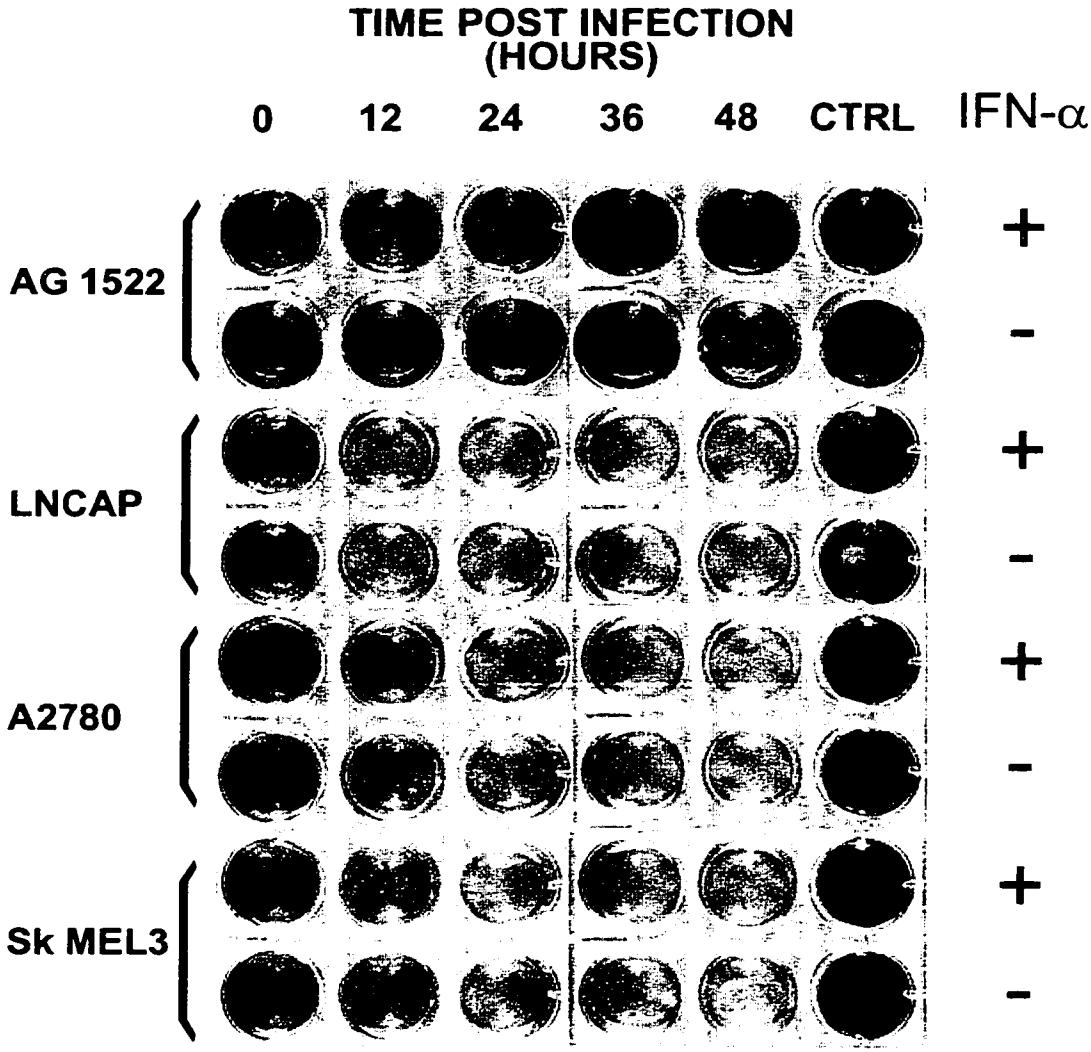
VSV selectively kills tumour cells in co-cultures

We tested the ability of VSV to infect and kill tumour cells co-cultured with normal cells. In this experiment, we used the human 293T transformed cell line which expresses SV40 large T antigen (TAg) and normal human foreskin fibroblasts (HFFs). Using antibodies to large T antigen we are able to distinguish 293T cells (Figure 3, red nuclei) from HFFs (Figure 3, blue nuclei). Equal numbers of each cell type were cultured on coverslips and infected at a MOI of 0.1 pfu/cell both in the presence and absence of IFN. Initially both cell types displayed a spindle-like morphology with large oval nuclei. After 18 hours, the number of 293T cells was decreased, and by 24 hours post-infection, the few remaining large T

Figure 2. **Malignant cells are rapidly killed following VSV infection and are not protected by IFN-α.**

Monolayers of normal fibroblasts and several tumour cell lines were either untreated or pretreated with IFN-α (100 units) and then infected with VSV at an MOI of 0.1 pfu/ml. At 12 hours increments the infections were terminated by cell fixation and staining to determine the kinetics of cell killing. Control (CNTL) monolayers were left to grow, uninfected, over the course of the experiment and therefore stain more intensely.

Figure 2


TIME POST INFECTION
(HOURS)
0
12
24
36
48
CTRL
IFN-α
AG 1522
LNCAP
A2780
Sk MEL3
+
-
+
-
+
-
+
-

antigen expressing cells displayed severely condensed or fragmented nuclei (Figure 3). The viral titre from the culture medium at 24 hours post infection was assayed to be 8 $x10^7$ pfu/ml, but as expected, the HFFs were largely unaffected. This selective destruction of the transformed cells was seen in both the presence and absence of interferon demonstrating that the 293T cells could not be protected by IFN-α.

Human bone marrow but not leukemic cells are VSV resistant

A key rate-limiting site of conventional cancer therapies is bone marrow toxicity. We tested the susceptibility of human marrow to VSV. Marrows from two separate healthy donors were acutely infected with VSV at a range of multiplicities of infection up to 10 pfu/cell. The following day, cell culture supernatants were titred and methylcellulose assays were performed. We found that even when infected at a MOI of 10 pfu/cell, bone marrow cultures produced no infectious VSV particles. Moreover, the infected bone marrow cultures were indistinguishable from mock-infected cultures in their ability to form the normal spectrum of hematopoietic cell types following *in vitro* culture in methylcellulose. These results were obtained in the absence of interferon and indicate that normal hematopoietic progenitor cells have a strong innate resistance to infection by VSV. In contrast, we found leukemic cells were highly susceptible to infection and killing by VSV. In these experiments, leukemic cell lines were inoculated with decreasing amounts of VSV, cultured for 24 or 48 hours, and the percentage of viable cells remaining in the culture determined using an MTS assay as described in the materials and methods. The AML cell lines OCI/AML3, OCI/AML4 and OCI/AML5 were highly susceptible to VSV infection with an average MOI of 0.05 pfu/cell killing 50% of the cells at 24 hours while as little as 0.0003

pfu/cell resulted in 50%

Table 2. Selective Killing of AML Cells Co-cultured with Normal Bone Marrow.

The growth factor independent cell line OCI/AML3 was mixed 1:9 with normal bone marrow and infected for 24 hours with VSV. Various dilutions of cells were then plated in methylcellulose plus and minus growth factors and colony counts were performed 14 days later. Data shown are for dishes receiving 10^4 cells. * No leukemic colonies were detected on the growth factor minus dishes even when 10^5 cells were plated per dish.

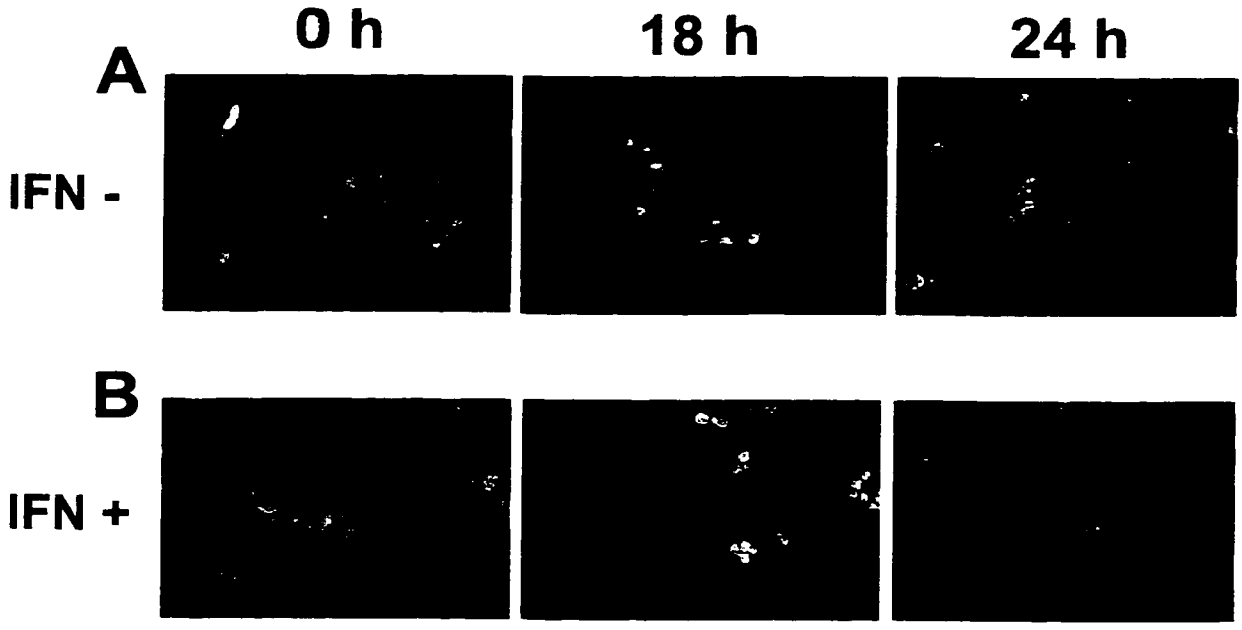
Table II

	Multiplicity of Infection		
Colony Type	*0.0*	*1.0*	*5.0*
Leukemic	172	0*	0*
Neutrophil	12	7	5
Mixed	6	3	4
Monocyte	10	7	5

Figure 3. VSV selectively kills transformed cells co-cultured with normal fibroblasts.

Equal numbers of 293T cells and normal human fibroblasts were plated on gelatin-coated coverslips and infected at an MOI of 0.1 both in the presence (A) and absence (B) of interferon. Cells were fixed at 18 and 24 hours post-infection. Fixed cells were stained with an anti-TAg antibody and DAPI. The red staining 293T cells were quickly killed, regardless of interferon treatment, with those few remaining cells displaying condensed or fragmented nuclei while the normal fibroblasts had intact, oval nuclei throughout.

Figure 3



killing at 48 hours. Clearly, VSV was able to infect, replicate in and rapidly spread throughout these leukemic cell cultures.

To further test the relative susceptibility of these AML cell lines to VSV infection, bone marrow purging experiments were performed. A 1:9 mixture of OCI/AML3 cells and bone marrow from a normal volunteer was exposed to VSV at an MOI of 1 pfu/cell or 5 pfu/cell for 24 hours and then plated in methylcellulose with or without growth factors. In the presence of growth factors, both normal marrow and tumour cells will grow while only OCI/AML3 cells can form colonies in the absence of growth factors. Colony counts were performed after 14 days and demonstrated a complete ablation of growth factor-independent leukemic cells and sparing of normal bone marrow progenitors (Table 2). Identical results were observed when a 1:3 mixture of OCI/AML3 cells and normal marrow were used (data not shown). These data show the selective destruction of leukemic cells in a mixed population of normal marrow, and when taken together with the data above, advocates the potential utility of VSV in bone marrow purging.

VSV inhibits tumour growth in a nude mouse model

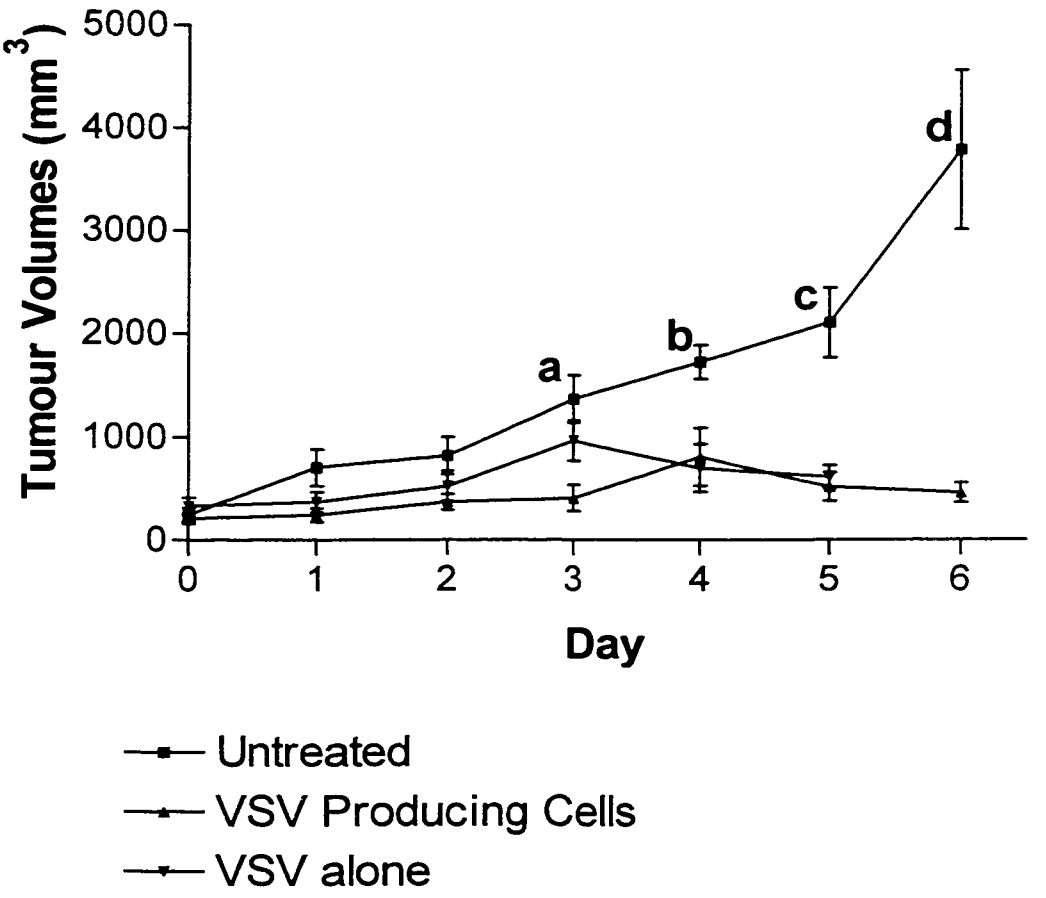
Human tumour xenografts in nude mice provide a convenient model for studying potential anti-cancer therapeutics including certain oncolytic viruses (19). In the case of VSV however, this system is somewhat limited since it is known that the immunological impairment of this mouse strain ultimately renders it susceptible to killing by VSV (33, 80, 196). The rapid killing of tumour cells with VSV suggested that it might be possible, however, to assess the *in vivo* oncolytic properties of the virus in an acute short term

experiment. To this end, SK-MEL3 cells were implanted subcutaneously on both flanks of mice, and allowed to form palpable tumours before direct infection with VSV. In light of the recent demonstration by Coukos *et al.* of improved delivery of HSV in the treatment of ovarian cancer using virus producing cells as opposed to virus alone(35), we also tested VSV producing cells in this melanoma xenograft model. The size of tumours and the well being of the animals were assessed on a daily basis. Tumours which were not injected with VSV showed unabated growth and ultimately the animals were sacrificed on day 6 because of their tumour burden (Figure 4). In contrast, tumours injected with a single dose of VSV or cells producing VSV, stopped growing and in some cases decreased in size (Figure 4).

Figure 4. VSV inhibits growth of human melanoma xenografts in nude mice.

SK-MEL3 derived tumours were developed in 8-10 week old female athymic mice. On day 0, tumours were either left untreated or were infected with 10^8 pfu VSV in culture medium or with $2.5x10^6$ VSV infected SK-MEL 3 cells (VSV producing cells). Tumours were measured daily for 6 days at which time infected animals were euthanized due to complications associated with VSV infection of nude mice (see text). Statistical differences were calculated between treated and untreated groups at each data point with the following confidence values (b: $p<0.01$; c: $p<0.001$; d: $p=0.007$). On day 3, only tumours treated with VSV producing cells were significantly smaller than untreated tumours (a: $p<0.001$). No statistically significant differences in tumour volumes between groups were apparent from day 0 to day 2. Data points represent means +/- SEM from multiple tumours (untreated n=8; VSV producing cells n=8; VSV alone n=4).

Figure 4


Tumour Volumes (mm3)
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
Day
a
b
c
d
Untreated
VSV Producing Cells
VSV alone

Discussion

Over the last several decades there have been numerous reports correlating virus infections with tumour regression (179, 181). With an increasing understanding of the molecular events leading to the generation and evolution of malignancies, it is now possible to begin tailoring and/or selecting viruses for their ability to replicate preferentially in tumour cells. The Onyx-015 adenovirus mutant is believed to have enhanced replication in tumour cells lacking a functional p53 protein although recent reports suggest that other cellular gene products could be influencing the growth of this virus (166). Another promising approach is to alter virus tropism by modifying viral surface antigens or by conditionally expressing toxic gene products with tissue specific gene promoter elements. Alternatively, as we suggest here, the ability of a virus to selectively kill tumour cells may be determined by cancer specific defects in innate anti-viral responses. For example, the tumour specific growth of reovirus type III is thought to depend upon an activated Ras pathway and subsequent down regulation of the PKR gene product (187). Oncolytic herpes virus mutants contain an altered ICP34.5 gene product which enhances eIF2a phosphorylation by PKR (23). As with VSV, we speculate that the oncolytic properties of this virus may be due in part to the defective components of the interferon response of many tumour cells. Certain isolates of Newcastle Disease virus (NDV) have a selective ability to lyse tumour cells (107, 108). Lorence et al. have demonstrated the shrinkage of human neurofibroblastoma and fibrosarcoma xenografts in athymic mice following intratumoural injection of NDV 73-T. Furthermore, human fibroblasts became up to 1000 fold more permissive to NDV following transfection with N-ras(107). Since the human fibrosarcoma used in these studies (HT-1080) are known to

express the N-ras oncogene the authors suggest that, as with reovirus, the ability of NDV to selectively replicate and lyse tumour cells is in part dependent on activated ras (107).

The widespread application of VSV as a cancer therapeutic may be limited by the frequency with which tumour cells contain interferon response defects. However, from our study and work published elsewhere we argue that defects/down-regulation of components of the interferon pathway may be a common feature of a broad range of malignancies. For instance the PKR kinase, a key effector of the interferon response, has been reported to be reduced in expression and/or activity in head and neck cancers (67), hepatocarcinomas (175) and mutant ras expressing tumours (30, 128, 129, 187). Stat1, an essential mediator of the interferon response, is deficient in certain melanoma cell lines (202), myeloid leukemias, carcinomas of the lung, ovary and cervix (143), a gastric adenocarcinoma (4) and a cutaneous T cell lymphoma cell line (188). A recent report indicates that the HPV-18 E7 gene product interrupts the interferon alpha pathway and if this proves to be true would suggest that the 95% of cervical carcinomas caused by HPV infection should be interferon alpha non-responsive(8).

Clearly some malignant cells retain an intact interferon response as this cytokine has been used to inhibit the growth of some cancers. Frequently, however, the benefits of interferon are short-lived. Perhaps as interferon resistant cells arise they could become targets for viral therapy. This might open the door to possible combination treatments whereby patients receive the benefits of interferon treatment until resistance is detected; after which the patient's tumour(s) will have become sensitive to treatment with oncolytic VSV or other viruses.

Resistance to interferon treatment has also become a confounding factor in the treatment of chronic Hepatitis Virus C (HCV) infections. Recently, the hepatitis gene products NS5A and E2 have been implicated in HCV resistance to interferon via their ability to inactivate PKR(58-62, 149, 176, 193). Similarily, some HIV gene products are able to ablate PKR activity. Perhaps VSV or related viruses could be used therapeutically to target and eliminate reservoirs of chronically HIV or HCV infected cells.

In this study we have investigated the potential use of VSV as an oncolytic agent. We were initially directed towards this virus by the discovery that our PKR null mouse line was very sensitive to VSV infection (Stojdl, unpublished observations). VSV has many of the properties that make it particularly suited as an oncolytic agent. Unlike adenovirus, reovirus and herpes virus, the incidence of VSV infection in the North American population is very low so it is unlikely that many individuals will have a pre-existing anti-VSV immune response. VSV grows very rapidly (cytopathic effects are detected in some tumour cell lines within 1-2 hours) and to high titers. While the growth of VSV is limited in normal cells, it can initially propagate in many tissue types perhaps allowing a widespread viremia which could facilitate infection of distant metastasis. Perhaps most importantly, we show here that while growth of VSV in a wide range of tumour cells is refractory to, or only moderately affected by interferon treatment, replication in normal cells can be controlled by administration of interferon.

Conclusions

These studies began, several students ago, with the cloning of two proteins using a screening strategy to identify protein tyrosine kinases. After careful biochemical study, it was determined that these protein kinases could phosphorylate on tyrosine residues and serine and threonine residues, and hence became founding members of a new subclass of kinases known as dual-specificity protein kinases. The work in this thesis represents ongoing efforts to understand the role of these proteins and the mechanisms by which they function. Our studies have led us on two divergent paths as our understanding of each of these proteins unfolded: one towards RNA splicing and the other towards protein translation. Common to both however, was their emerging roles as regulatory molecules involved in controlling the flow of genetic information from DNA towards proteins. In the first section of this thesis, we present evidence that the Clk family of kinases are able to influence the regulator process known as alternative splicing. In the second portion, we discuss PKR, a regulator of protein translation, and demonstrate it to be an essential mediator of antiviral defence. In the final chapter, we consider a possible application of the data from our PKR studies, and propose an oncolytic therapy based on putative defects in regulatory pathways controlling PKR and other members of the IFN pathway.

Clk kinases and Alternative Splicing

Alternative splicing is critical to the control of cellular and viral gene expression (178). SR proteins have been implicated as essential components of both constitutive and alternative splicing programs (recently reviewed in 114, 197). One of the important questions

that still remains to be answered is: how is the splicing machinery regulated? It has been shown that the level of expression of SR proteins affects splicing decisions such as exon exclusion/inclusion(21, 115). Tissue specific variations in SR protein expression could account for developmental regulation of mRNA splicing (211), however it could be argued that post-translational modification of SR proteins would be a more dynamic regulatory mechanism. SR proteins are known to be phosphorylated (164, 210)and studies previous to the initiation of this work demonstrated that the SRPK1 and Clk1 kinases can phosphorylate and initiate the re-distribution of SR proteins in vivo (32, 211). The results presented here indicate that the three related but distinct kinases, Clk1, Clk2 and Clk3, are able to affect SR protein distribution and pre-mRNA splicing *in vivo*. This study represents the first demonstration that a protein kinase can influence alternate splice site selection *in vivo*. Taken together these results support the idea that protein kinases are able to influence pre-mRNA splicing and thus provide a possible connection between signal transduction pathways and splicing.

What is the relationship between SR protein re-localization and pre-mRNA splicing? Splicing factors, including SR proteins, have been localized to the sites of transcription, as well as nuclear speckles (79, 127, 213). However, while it does appear that splicing occurs at sites of transcription, elegant studies by Zhang *et al.* (214) suggest splicing does not take place at or within speckles. It has been suggested that the speckles represent stores of inactive splicing factors and that splicing occurs in the nucleoplasm at or near sites of transcription (49, 182, 214). In support of this model, Misteli *et al.*(127) show the dynamic nature of splicing factor distribution in the nucleus where upon activation of transcription at a

single gene, splicing factors appear to leave speckles and accumulate at the new sites of transcription. The ability of Clk kinases therefore, to redistribute SR proteins from speckles may represent an increase in nucleoplasmic concentrations of splicing factors, thereby changing the equilibrium of factors available to modify splice-site selection at a particular pre-mRNA. As mentioned above, the concentration of SR proteins has been shown to affect alternative splicing patterns(21). We suggest that the Clk kinases regulate SR protein release from speckles thus altering their effective nucleoplasmic concentration. Alternatively phosphorylation of SR proteins could directly affect their activity in the splicing reaction. In support of this idea, recent work from Manley's group has demonstrated that *in vitro* phosphorylation of the SR protein, ASF, by the mClk1 kinase, affects its ability to interact with other splicing factors and RNA molecules (203). Perhaps each of the Clk kinases phosphorylates a discrete subset of SR proteins thereby regulating the splicing of a distinct cohort of pre-mRNAs. This may be consistent with our data demonstrating the somewhat decreased ability of hClk3 to redistribute SR proteins. If Clk3 affects a smaller subset of SR proteins, we may expect to detect less "de-specklization" and would perhaps require higher levels of expression to overcome this limitation; which is what we consistently observe. Alternatively, it is possible that the three kinases phosphorylate the same SR proteins but interface with distinct signal transduction pathways. It remains to be determined which of these models is correct, although both are not mutually exclusive.

One of the unique properties of the Clk kinase family is the common mode of differential splicing (45, 70) which results in the co-expression of catalytically inactive and catalytically active isoforms. We have found that overexpression of the $Clk1^{T}$ protein

promotes exon inclusion while the Clk1 kinase promotes exon exclusion. These observations coupled with the demonstration that Clk1 can form heterodimers with $Clk1^{T}$ (45)suggest that the alternatively spliced Clk isoforms may be self regulatory.

In further support of our results that Clk kinases regulate splicing, a recent report demonstrating the role of the Doa kinase in Drosophila sex determination. Doa is an SR protein kinase in *Drosophila melanogaster,* and the sole homologue to the mammalian Clk kinase family. In Drosophila, sex determination is regulated by a series of events beginning with the autosomal/X dosage-dependent expression of the *sxl* splicing regulator, triggering a cascade of alternative splicing events leading eventually to the sex specific expression of a variety of transcription factors (reviewed in7). During one of those key splicing events, splicing factors Tra1 and Tra2 work together with the SR protein Rbp1 to modulate the splicing of the *dsx* pre-mRNA in a sex specific manner. Female-specific splicing of the *dsx* pre-mRNA requires the formation of a complex of these factors bound at the exon enhancer sequences within the *dsx* female-specific exon. Protein-protein interactions among these factors are necessary for their cooperative binding to the enhancer (111) and subsequent attraction of the splicing machinery to the female-specific exon, thereby promoting its inclusion in the fully-spliced mRNA (218). Recent evidence reported by Du *et al.* (43) indicates that the Doa kinase plays a role in sex specific splicing of *dsx*. $Doa^{-/-}$ mutations are almost always lethal, demonstrating a critical role for this kinase during development (209). However, female flies expressing partial-loss-of-function alleles of *doa* demonstrated partial male differentiation, indicating reduction in the activity of this kinase causes defects in female sex determination. This group went on to demonstrate a defect at the level of *dsx* sex

specific splicing. This defect in alternative splice site choice was not a global one, as the splicing of only 2 of 3 sex specific genes were affected by reductions in Doa activity. Doa was found to interact with Rbp1, Tra1 and Tra2 in yeast two hybrid experiments and found to phosphorylate these proteins *in vitro*. Furthermore, Rbp1 proteins were found to be hypophosphorylated in pupae extracts from Doa mutant flies and Tra2 was found to be localized to enlarged speckles reminisent of phosphatase overexpressing cells, in contrast to its normally more nucleoplasmic staining. This data when taken together, strongly argues that phosphorylation of one or more splicing factors by Doa is a crucial event in the splicing of more than one gene during the splicing regulation that controls sexual development in Drosophila. This type of evidence is sorely lacking in mammalian models and would go a long way in determining what is the true significance of splicing factor phosphorylation in vivo.

Phosphorylation has been implicated in assembly of the spliceosome, regulation of splice site selection, modulation of SR protein activity and subnuclear localization. In understanding phosphorylation and splicing, many of the individual components have been characterized, however, many experiments remain to be done before we comprehend this putative regulatory mechanism. In the future, it will be important to determine which kinases phosphorylate which splicing component(s) *in vivo*, and how these kinases are regulated. We are undertaking definitive genetic experiments using homologous recombination to establish the exact role(s) of the Clk kinases in splicing regulation.

PKR and VSV

As mentioned above, we used a functional screening assay to clone tyrosine kinases and in doing so we cloned the murine orthologue of PKR. At the time, the biochemistry of PKR had been actively studied and implicated this protein as a potential antiviral mediator. Our lab initiated a project to understand the relevance of this protein in the context of the whole animal by using homologous recombination mediated gene targeting to ablate this gene in the mouse. During this process, another group reported the generation of a $PKR^{-/-}$ mouse(207). Their work, together with our initial studies, contrary to the early biochemical data, indicated no requirement for PKR as an antiviral defense mediator, at least against such pathogens as influenza, vaccinia and EMCV(3, 207). The work in this thesis re-examines the hypothesis that PKR is an essential antiviral effector.

IFN induced anti viral responses are mediated through a variety of proteins, including the double-stranded RNA dependent protein kinase, PKR. Here we show that fibroblasts derived from $PKR^{-/-}$ mice are more permissive to VSV infection than wild type fibroblasts, and demonstrate a deficiency in IFNα/β mediated protection. We further show that mice lacking PKR are extremely susceptible to intranasal VSV infection, succumbing within days after instillation with as few as 50 infectious viral particles. Again type I IFNs were unable to rescue $PKR^{-/-}$ from VSV infection. We present evidence that the lungs in particular are dependent on PKR. These results confirm the role of PKR as the major component of IFN mediated resistance to VSV infection. In light of early findings that PKR is dispensable when challenged with influenza, vaccinia or EMCV, our findings underscore the premise that the various IFN-mediated antiviral defenses are required differentially to counter different

pathogens.

VSV as an oncolytic agent

In the course of our PKR studies we began to appreciate that many tumour cell lines had defects in PKR and/or other members of the IFN pathway. Having established VSV as a potent lytic agent in PKR defective cells and animals, we hypothesized that VSV could be used as an antitumour agent, targeting neoplastic cells with defects in PKR or other constituents of the IFN pathway. This work documents the acute susceptibility of an array of various tumour cell lines to VSV relative to normal human fibroblasts. We further demonstrate the selective killing of tumour cells in a mixed population of normal cells or bone marrow. In each case, IFN was not protective in tumour cells. Since the antiviral and anti-proliferative properties of IFN require overlapping signaling pathways, we propose that antiviral defenses may be compromised as the tumour acquires mutations compatible with uncontrolled growth. We conclude these studies demonstrating the oncolytic effects of VSV in human melanoma xenograft bearing nude mice.

Curriculum Vitae

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David F. Stojdl, N. Abraham, S Knowles, R. Marius, A. Brasey, E. G. Brown, N. Sonenberg, John C. Bell. 2000 The murine double-stranded RNA dependent protein kinase, PKR is required for resistance to VSV. *Journal of Virology* in press

David F. Stojdl[*], B. Lichty[*], S. Knowles, R. Marius H. Atkins, N. Sonenberg, J. C. Bell. 2000 Exploiting tumour specific defects in the interferon pathway with a novel oncolytic virus. *Nature Medicine* 6:821-825. [*]contributed equally

David F. Stojdl and John C. Bell. 1999 *SR protein kinases: the splice of life* Biochemistry and Cell Biology 77:293-298

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Stojdl, D.F. and Clarke, M.W. 1996 Trypanosoma brucei: Analysis of cytoplasmic calcium during differentiation of bloodstream stages in vitro. *Experimental Parasitology* 83:134-146.

ABSTRACTS AND MEETINGS:

University of Ottawa 150th Anniversary Cross Disciplinary Poster Competition (Sept 98)
Represented Dept. of Biochemistry after winning the Departmental competition for best PhD presentation (May 98).
Abstract and Poster: *Protein Kinases Regulate Gene Expression through Modulation of Alternative Splicing*

RNA 97 (May 1997) Banff Canada
Abstract and Poster: *Alternative splicing regulated by the Clk family of dual-specificity kinases*

Great Lakes Mammalian Development Meeting (April, 1997) Toronto, Canada
Abstract and Oral presentation: *The Clk family of kinases regulate alternative splicing*

The 11th Annual Meeting on Oncogenes (June, 1995) Bethesda, USA
Abstract and Poster presentation: *Strategy for the identification of transforming mutants of murine dual specificity kinases.*

Contributions of Collaborators

The work presented in this thesis was modified from manuscripts published or submitted for publication as described below:

Chapter 1 & 2

David F. Stojdl and John C. Bell. 1999 *SR protein kinases: the splice of life* Biochemistry and Cell Biology 77:293-298

Chapter 2

David F. Stojdl*, Peter I. Duncan*, Ricardo. M. Marius and John C. Bell 1997 In vivo regulation of alternative splicing by the Clk1 protein kinase. *Molecular and Cellular Biology* 17:5996-6001. *contributed equally

Chapter 3

David F. Stojdl, N. Abraham, S Knowles, R. Marius, A. Brasey, E. G. Brown, N. Sonenberg, John C. Bell. The murine double-stranded RNA dependent protein kinase, PKR is required for resistance to VSV. *Journal of Virology* in press

Chapter 4

David F. Stojdl*, B. Lichty*, S. Knowles, R. Marius H. Atkins, N. Sonenberg, J. C. Bell. Exploiting tumour specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature Medicine* 6:821-825. *contributed equally

The following people have contributed to the experimental work presented in this thesis:

Peter Duncan performed indirect immunofluorescence experiments co-localizing SR proteins with Clks. Ricardo Marius performed western blots for the Clk/splicing experiments. Shane Knowles and Ricardo Marius performed all viral titrations. Brian Lichty performed experiments resulting in figures 3 and 4 in Chapter 4. Mike Markidis helped in the creation of several GFP/BFP tagged fusion constructs.

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