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| <b>13. ABSTRACT (Maximum 200 Words)</b><br><br>The primary goal of the Idea Award research is to develop methods for the identification of factors that influence the regulation of splicing in breast cancers. During the 1 <sup>st</sup> year of research funded by this grant, we developed assays for the isolation and characterization of serine-arginine-(SR)-repeat proteins, and associated factors. SR proteins are important for both constitutive and regulated pre-mRNA splicing. Our work has resulted in the identification of the oncoprotein DEK as an SR-associated protein that assembles into splicing complexes. Although our studies did not reveal a function for DEK in splicing, we made the interesting observation that this protein forms a splicing-dependent interaction with mRNA. In conjunction with concurrent studies on SRm160 (the SR-related nuclear matrix protein of 160 kDa) splicing coactivator, which associates with DEK, our results suggest that DEK may be involved in the splicing-dependent promotion of mRNA export from the nucleus. These results are summarized in the first part of this report.<br><br>We have also initiated the development of an exon microarray for the large-scale analysis of alternative splicing profiles. A major application of this new technology will be the identification of aberrant alternative splicing events that are specifically associated with breast cancers. The development of an exon microarray will ultimately provide new information on the factors that influence the "global" regulation and coordination of alternative splicing events, and may lead to the identification of breast cancer-specific alternative splicing events. This approach may, therefore, provide a valuable route towards the identification of new factors that facilitate the diagnosis, typing and, ultimately, the treatment of breast cancers. |                                                                 |                                                                |                                                                         |                               |
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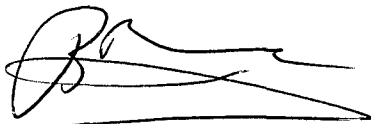
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## INRODUCTION

Deregulation of alternative splicing as been linked to malignant transformation and the formation of metastases in breast cancers. For example, specific alternatively spliced forms of the pre-mRNA encoding the cell surface adhesion glycoprotein CD44 have been correlated with invasive tumor formation (Taneba and Saya, 1994; Cooper and Dougherty, 1995; Cooper and Mattox, 1997). Moreover, it has been demonstrated that expression of specific alternative spliced forms of CD44 mRNAs in non-metastatic cell lines result in the transition to full metastatic potential (Günthert et al., 1991). We are interested in understanding the mechanisms underlying the regulation of alternative splicing with the goal of identifying and targeting trans-acting splicing factors that are involved in the formation of breast and other types of cancer. To this end, we have undertaken two different but complementary strategies towards the identification of factors that are important for the regulation of splicing.

The first strategy involves biochemical purification of proteins that contain domains rich in alternating serine and arginine residues (RS domains), and associated factors. Proteins that contain RS domains, which are most often referred to as serine/arginine-repeat (SR) proteins, are known to be important for the regulation of splicing (Blencowe et al., 1999; Graveley, 2000; Smith and Valcarcel, 2000). We have developed methods for the isolation of new RS domain proteins and associated factors with the aim of extending our knowledge and understanding of the role of these proteins in the splicing process (Blencowe et al., 1995; McGarvey et al., 2000). During the 1<sup>st</sup> year of the Idea Award research, this work led to the identification of the oncoprotein DEK as

an SR protein-associated factor that may function to coordinate splicing with downstream steps in gene expression (McGarvey et al., 2000).

In the second strategy, we have initiated the development of an "exon microarray" in collaboration with the Ontario Cancer Institute Microarray Facility. This new technology will be tailored for the broad analysis of splicing patterns in different tissues, including breast tumors, so that we can begin to investigate the possible relationships between breast cancer and alternative splicing. The exon chip should facilitate the identification of alternative splicing events that correlate with the onset and development of breast tumors and may, therefore, provide an alternative strategy for the detection, diagnosis, and possible identification of future targets for therapeutic intervention in this life-threatening disease.

This report describes our progress during the past year in the development and exploitation of the two strategies described above. In Part I of the report, our work on the characterization of the DEK oncoprotein is summarized. Part II of the report summarizes our work on the development of the exon microarray.

## **BACKGROUND (FOR PART I)**

Increasing evidence suggests that different processes in gene expression communicate and are functionally coordinated with each other. For example, it has been demonstrated that transcription factors can influence the processing of a pre-mRNA. In this case, splicing and selection of polyadenylation sites can be influenced by promoter sequences that drive transcription by RNA polymerase II (pol II) (Leroy et al., 1991; Cramer et al., 1997; Cramer et



al., 1999). Moreover, the carboxyl-terminal domain (CTD) of pol II is important for efficient capping, splicing and 3' end cleavage of nascent pre-mRNA (Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b). Splicing and 3'-end formation also influence each other, and interactions between these processes are important for the definition of terminal exons in pre-mRNA (Niwa and Berget, 1991; Wassarman and Steitz, 1993; Nesic and Maquat, 1994; Gunderson et al., 1997; Bauren et al., 1998; Lou et al., 1998; Vagner et al., 2000). Recently, it was demonstrated that the transport of mRNA from the nucleus to the cytoplasm is promoted by prior splicing, since mRNAs that had not been generated by splicing were inefficiently transported (Luo and Reed, 1999). Currently, it is not understood how individual steps in the gene expression pathway communicate with each other, although several lines of evidence indicate that proteins containing domains rich in alternating arginine and serine residues (RS domains), including several splicing factors (see below), may be important for the coupling of splicing to other steps in pre-mRNA metabolism (reviewed in: (Fu, 1995; Corden and Patturajan, 1997; Blencowe et al., 1999).

Pre-mRNA splicing involves the step-wise assembly of large RNA-protein complexes, termed spliceosomes (reviewed in: (Kramer, 1996; Reed and Palandjian, 1997; Burge et al., 1999)). The major spliceosome contains four small nuclear ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) and many non-snRNP splicing factors. A large number of non-snRNP splicing factors contain RS domains. Among these are members of the "SR-family", which share a common domain structure consisting of one or two N-terminal RNA recognition motifs and a phosphorylated C-terminal RS domain (Fu, 1995; Manley and Tacke, 1996; Valcarcel and Green, 1996). Other RS domain-containing non-snRNP splicing factors have a distinct structural

organization and are referred to as “SR-related” proteins (Fu, 1995; Blencowe et al., 1999).

These include factors such as the alternative splicing regulators Tra, Tra2, and SWAP, as well as the subunits of the SRm160/300 splicing coactivator. The RS domains of SR family and SR-related proteins form interactions with each other and with snRNP-associated proteins that contain RS domains, and increasing evidence indicates that these interactions are critical for the formation of networks of interactions that promote splice site recognition across both intron and exon sequences.

Several SR-related proteins and interacting factors have been identified recently that are associated with transcription and 3' end processing. These include the E2 protein of the epidermodysplasia verruciformis (EV)- associated human papillomavirus (HPV), which binds to promoter sequences and is important for the transcriptional regulation of viral genes. The EV-HPV E2 protein contains an RS “hinge” domain that interacts with SR proteins involved in splicing and appears to promote specifically the splicing of pre-mRNAs transcribed from promoters activated by the EV-HPV E2 protein (Lai et al., 1999). A class of SR-related proteins referred to as SCAFs (SR-like CTD-associated factors) has been identified that preferentially bind to the hyperphosphorylated CTD of RNA pol II, which is the form of the CTD associated with elongating transcription complexes (Yuryev et al., 1996; Bourquin et al., 1997; Tanner et al., 1997; Patturajan et al., 1998). An SR-related protein (PCG-1) has been identified that functions as a transcriptional coactivator of nuclear receptors, which is implicated in adaptive thermogenesis in mice (Puigserver et al., 1998). Another new transcriptional coactivator (p52) has been identified that lacks an RS domain but interacts with the SR family protein ASF/SF2, as well as with several transcription factors (Ge et al., 1998). The 68kDa subunit of the mammalian

cleavage factor (CFIm), which functions in the 3' end processing of transcripts, was recently isolated and found to contain an RS domain (Ruegsegger et al., 1998). These studies indicate that a variety of proteins containing RS domains are candidates for factors that coordinate different steps during the synthesis and processing of pre-mRNA.

SR family and SR-related proteins have been implicated in steps downstream of pre-mRNA splicing. Several SR proteins, including SRm160/300 and ASF/SF2, remain bound preferentially to the ligated-exon product, but not the intron-lariat product of the splicing reaction, which associates with snRNPs (Blencowe et al., 1998; Hanamura et al., 1998; Blencowe et al., 2000). A subset of SR family proteins shuttle between the nucleus and the cytoplasm, although it is not known if they shuttle in association with mRNA (Caceres et al., 1998). Recently, it was observed that complexes formed on exon product RNAs generated by splicing are larger and more efficiently transported to the cytoplasm than complexes formed on the same RNAs that have not been through splicing (Luo and Reed, 1999). This observation suggests there are factors that are targeted for association with mRNA by the splicing machinery and may relate to numerous earlier observations that introns are important for the efficient expression of genes.

In the present study we have identified the oncoprotein DEK as a splicing complex component that remains associated with spliced exons dependent on prior splicing of pre-mRNA. DEK is a ~45 kDa phosphoprotein that was previously identified in the context of several human diseases. It was first isolated as part of a fusion that arises in a subtype of acute myeloid leukemias (AMLs) involving (6;9) chromosomal translocations (von Lindern et al., 1992). These translocations occur within intron sequences of the genes encoding *dek* and the nucleoporin *can*

and result in the expression of an in-frame chimera encoding the majority of DEK fused N-terminally to the C-terminal two-thirds of CAN. DEK was later identified as an autoimmune antigen in patients with pauciarticular onset juvenile rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases (Szer et al., 1994; Dong et al., 1998; Wichmann et al., 2000). In another study, it was reported that DEK binds to a TG-rich element, the “pets” (peri-ets) site, which is adjacent to two binding sites of the ets family member Elf-1 in the HIV-2 enhancer, which mediates enhancer activation by a number of signaling processes (Fu et al., 1997). However, the specificity and functional significance of the binding of DEK to the pets site is not known. The results in the present study indicate that DEK may have one or more functions in association with pre-mRNA processing. In particular, the results identified DEK as one of the first factors that associates with mRNA in a splicing-dependent manner, indicating that it could function to coordinate splicing with one or more subsequent steps in gene expression.

## **BODY: (PART I)**

In last year's report, we demonstrated that DEK co-purifies with SR proteins and that it associates with splicing complexes. In continuation of this project during the past year, we have found that DEK associates with SR proteins *in vivo*, and that it forms a splicing dependent interaction with mRNP complexes. The results are described in detail below:

### **DEK associates with SR proteins *in vivo***

In order to investigate whether DEK associates with splicing components *in vivo*, we double-immunolabeled HeLa cells with rAb-DEK and the anti-SR protein antibody mAb-NM4 (Blencowe et al., 1995) (Figure 1, A-F) (corresponds to Figure 6 in the attached publication). Similar to many antibodies that recognize SR proteins, mAb-NM4 specifically immunolabels nucleoplasmic interchromatin granule clusters or "speckles" that are highly enriched in splicing factors (B,E and data not shown). In the majority of cells within a field, rAb-DEK-labeling reveals that DEK is specifically localized in the nucleus in a "diffuse-granular" pattern that largely, but not completely, excludes nucleoli (A). In these cells, DEK overlaps with speckle domains, although it does not appear significantly more concentrated in these structures than in other regions of the nucleoplasm (C). However, in approximately 15% of cells within a field, DEK is significantly more enriched in speckle structures compared to the surrounding nucleoplasm (compare D and F with A and C). The detection of DEK by immunostaining with rAb-DEK is specific since pre-immune serum did not immunolabel nuclei (Fornerod et al.,

1996). These results indicate that in a subset of cells, DEK is concentrated with SR proteins and other splicing factors in nuclear speckle structures.

In order to extend these observations, we next tested whether the localization of DEK is influenced by the intranuclear distribution of SR proteins. Recently, we have observed that SRm160 containing an N-terminal Flag epitope (Flag-SRm160), when over-expressed by transient transfection, concentrates with endogenous SR proteins in enlarged speckle structures (Figure 1H and data not shown). To determine whether the elevated levels of SRm160 in speckles results in a change in the nucleoplasmic distribution of DEK, cells transiently transfected with a flag-SRm160 construct were double-immunolabeled with anti-flag and rAb-DEK antibodies. Significantly, the presence of flag-SRm160 in speckles (H) correlated with an accumulation of DEK in these structures (G). This effect was specific since the distribution of DEK was not altered significantly by the over-expression of a flag-B23 protein, which concentrates in nucleoli (compare J-L). Although every speckle containing flag-SRm160 also contained an increased level of DEK, in some cases the size of the speckle domain detected with the anti-flag antibody was significantly smaller than the size of the corresponding speckle domain detected with rAb-DEK. This may reflect differences in the relative concentrations of these proteins between speckles, and/or the differential accessibility of these proteins to antibodies in different speckles. In either case, the results demonstrate that the distribution of DEK within nuclei can be specifically influenced by the localization of SRm160, and that, consistent with the *in vitro* interactions with SR proteins including SRm160/300 described above, DEK probably concentrates in speckles by associating with one or more of these splicing factors.

### **Depletion of DEK does not prevent pre-mRNA splicing**

The results so far suggest that DEK could function as a splicing factor, perhaps by associating with one or more SR proteins and modulating their activity. To investigate this possibility, we have prepared nuclear extracts that are specifically and efficiently depleted of DEK (Figure 2A, compare lanes 1 and 2). Surprisingly, efficient immunodepletion of DEK did not affect the level of splicing of several pre-mRNAs tested, including the PIP85A (Figure 2B, compare lanes 1 and 2) and the dsx(GAA)<sub>6</sub> substrate (data not shown). Likewise, in contrast to SR proteins and hnRNP A1, which promote the selection of intron-proximal and -distal 5' splice sites, respectively (Ge and Manley, 1990; Krainer et al., 1990; Fu et al., 1992; Mayeda and Krainer, 1992; Cáceres et al., 1994), altering the level of DEK by specific immunodepletion *in vitro*, or by over-expression *in vivo*, did not significantly alter the ratio of intron-distal to -proximal splice sites in different pre-mRNAs containing cis-competing splice sites (data not shown). While it cannot be excluded that a minor amount of DEK remaining in the immunodepleted extract is important for splicing activity, or that DEK is important for the splicing of specific substrates not assayed, the results so far suggest that DEK may provide a function that is not directly related to splicing activity.

### **The association of DEK with the exon-product RNA requires prior splicing**

The experiments in Figure 3 reveal that DEK, like SRm160/300, remains bound preferentially to spliced exons after splicing. This observation, and the finding that DEK does not associate

efficiently with RNAs that cannot assemble functional splicing complexes, raises the possibility that its association with spliced exons requires the prior formation of a spliceosome. To determine whether the association of DEK with spliced exons is facilitated by splicing, we compared the ability of rAb-DEK to immunoprecipitate complexes formed on the exon-product RNA generated by splicing of PIP85A pre-mRNA, versus complexes formed on the identical exon-product RNA that has been derived by transcription from a PIP85A cDNA construct (Figure 3). In this assay, splicing reactions were incubated with equal amounts of PIP85A pre-mRNA and a second RNA that is not a splicing substrate (a fragment of the HIV-1 Rev-response-element), as an internal control RNA for recovery (lanes 1 and 3). Although this latter RNA does not assemble into functional splicing complexes, like the *dsx* antisense and *dsx* $\Delta$ E transcripts, it associates weakly with SRm160/300 and DEK, permitting its use for monitoring immunoprecipitation levels (see below; data not shown). In a parallel reaction, an equivalent amount of the internal control RNA was mixed with an exon-product RNA transcribed from the PIP85 cDNA construct (lanes 2 and 4). Both reactions were incubated for 1 hour under splicing conditions prior to immunoprecipitation with rAb-DEK. Significantly, although a higher level of cDNA-derived exon-product RNA than spliceosome-derived exon-product RNA is present in the total splicing reactions (compare lanes 1 and 3 with lanes 2 and 4), rAb-DEK only immunoprecipitated efficiently the spliceosome-derived exon-product RNA (compare lanes 7 and 8). This difference was not due to a non-specific loss of the cDNA-derived exon-product RNA, since approximately equivalent levels of the internal control RNA were immunoprecipitated from both reactions (compare lanes 7 and 8). In contrast to the efficient immunoprecipitation of the spliceosome-derived exon-product complex, rAb-DEK immunoprecipitated a significantly lower level of the lariat-product complex of the splicing



reaction. This is consistent with the observation that the majority (80-95%) of exon-product RNAs generated by splicing are released from the spliceosome during *in vitro* splicing reactions (H. Le Hir, M. Moore and L. Maquat, personal communication), and also that a relatively low level of DEK remains bound to the lariat-product complex after splicing. Preferential immunoprecipitation of the PIP85A exon-product complex that had been generated by splicing, over the complex formed on the cDNA-derived exon-product RNA, was also observed with antibodies to both subunits of the SRm160/300 splicing coactivator (unpublished observations). This is in agreement with very recent results showing that SRm160 specifically cross-links to spliced exons dependent on prior splicing (Le Hir et al., 2000a; Le Hir et al., 2000b) (see Discussion), and also our recent finding that DEK and SRm160/300 associate (McGarvey et al., 2000). Thus, DEK forms a component of an exon-product complex, the assembly of which requires prior splicing. These results suggest that DEK could function to "tag" spliced mRNAs as a means to facilitate one or more post-splicing processes.

## DISCUSSION (PART I)

We have identified the acute myeloid leukemia (AML)-associated protein DEK as a factor that interacts *in vitro* and *in vivo* with SR proteins involved in pre-mRNA splicing (McGarvey et al., 2000). DEK associates efficiently with splicing complexes through interactions mediated by SR proteins. Its association with a pre-mRNA is stimulated by the presence of an exonic splicing enhancer (ESE) which binds to SR family and SR-related proteins. Moreover, its association with a pre-mRNA is strongly stimulated by the addition of purified SR proteins to an SR protein-depleted reaction. After splicing, DEK remains associated preferentially with exon-product complexes. Importantly, this interaction requires prior splicing since DEK does not associate with an exon-product RNA that has not been generated by splicing. The results indicate that interactions involving DEK may distinguish a transcript that has been through splicing from a transcript that has not. Moreover, they suggest that DEK could function to communicate between splicing and one or more steps downstream in gene expression.

Increasing evidence indicates that the nuclear history of a transcript can influence its subsequent metabolism. For example, it has been demonstrated in many studies that the presence of an intron is important for efficient gene expression. These intron-mediated effects have been attributed to influences on mRNA stability, transport and translation. For example, prior splicing is important for translation-dependent degradation of mRNA by the nonsense-mediated decay (NMD) pathway (reviewed by: (Maquat, 1995; Maquat, 1996; Hentze and Kulozik, 1999)). In a recent study, it was shown that an exon-product RNA generated by splicing assembles into a complex that is significantly larger and more efficiently exported to the cytoplasm than the

complex formed on the equivalent RNA synthesized from an intron-less transcript (Luo and Reed, 1999). The presence and location of an intron, whether at the 5' or 3' end of a transcript, can also influence its translation in the cytoplasm. For example, *Xenopus* maternal mRNAs transcribed *in vivo* are normally translationally masked but can be relieved from translational repression by the insertion of an intron at the 5', but not at the 3' end, of a transcript (Matsumoto et al., 1998; references within). All of the above observations indicate the existence of factors that package an mRNP dependent on its passage through the spliceosome and that modulate downstream steps in gene expression. However the factors that specifically "tag" mRNPs dependent on prior splicing are not known.

Heteronucleoriboproteins (hnRNPs) such as hnRNP-A1 are candidate factors for influencing the composition and activity of an mRNP dependent on prior splicing. HnRNPA1 associates with nascent pre-mRNA, influences splicing activity, and associates with spliced mRNA in the nucleus and cytoplasm; it has also been detected in association with translationally-engaged mRNA (Mayeda and Krainer, 1992; (Visa et al., 1996). However, neither hnRNP-A1 or other hnRNP proteins have been detected in purified spliceosomes and it is also not known whether their association with mRNPs is dependent on prior splicing. In contrast, it has been demonstrated that specific SR proteins, including the SRm160/300 splicing coactivator subunits and ASF/SF2, associate with splicing complexes through both steps of the splicing reaction and remain preferentially bound to the exon-product RNA (Blencowe et al., 1998; Hanamura et al., 1998; Blencowe et al., 2000). Moreover, a *Chironomus tentans* SR family protein, hrp45, which is related to mammalian ASF/SF2, associates with intron-containing Balbiani ring transcripts and remains bound to spliced mRNP until the time the mRNP is translocated through the nuclear

pore complex, upon which it appears to be released (Alzhanova-Ericsson et al., 1996). Very recently, Le Hir and colleagues have used an elegant cross-linking strategy to detect proteins that cross-link at or near spliced exon junctions dependent on prior splicing. In this study, splicing-dependent cross-links were identified for SRm160, the U5 snRNP protein hPRP8/p220, and several unidentified proteins (Le Hir et al., 2000). The results in the present study are consistent with these findings. In particular, since the association of DEK with pre-mRNA is promoted by interactions with SR proteins, it is possible that its association with spliced exons is mediated by SRm160. The results further suggest that interactions involving factors that define mRNP composition occur as early as the commitment stage of splicing complex formation, when U1 snRNP and SR family proteins bind to the pre-mRNA and facilitate the association of factors including SRm160/300 and DEK with the substrate (Blencowe et al., 1998; present study).

The results of the present study, taken together with a previous finding indicating that DEK associates with the *pets* site within the HIV-II promoter (Fu et al., 1997) (see Introduction), reveals an interesting relationship with a growing number of factors that are associated with both RNA processing and malignant transformation. For example, it has been reported that certain isoforms of the Wilm's tumor protein (WT-1) bind to DNA at specific promoter sequences and concentrate within transcription factor domains *in vivo*, whereas other isoforms specifically associate with splicing components, including snRNPs and the 65kDa subunit of the U2 snRNP auxiliary factor (U2AF-65), and concentrate within splicing factor domains in the nucleus (Larsson et al., 1995; Davies et al., 1998). In another case, the oncoprotein TLS/FUS, which becomes fused to the CHOP protein in liposarcomas, or to the ERG protein in a subset of myeloid leukemias, binds to both DNA and RNA and has recently been identified as hnRNP-P2,

a component of hnRNP complexes (Calvio et al., 1995; and references within). Intriguingly, also similar to DEK, it has been reported that TLS/FUS interacts with SR protein splicing factors (Yang et al., 1998). Another factor with dual RNA and DNA binding properties is the Ets-related transcription factor Spi-1/PU.1. This factor, besides its role as a hematopoietic-specific transcription factor, has been reported to interact with both TLS/FUS and the polypyrimidine tract-binding protein NonO/p54<sup>nrb</sup>, and to influence splicing activity (Hallier et al., 1998). NonO/p54<sup>nrb</sup>, which also has the ability to bind to both DNA and RNA, and a closely related protein PSF (the polypyrimidine binding protein-associated splicing factor), were both identified recently in fusions with the transcription factor TFE in papillary renal cell carcinomas (Clark et al., 1997; and references within). Thus, all of the RNA processing-associated factors implicated in different human malignancies to date, like DEK, have associations with transcription components as well as with pre-mRNA processing. While the significance of these similar features is not known, it is tempting to speculate that malignancies involving alterations to DEK and these other proteins could affect functions in transcription, RNA processing, or perhaps interactions that coordinate these steps in gene expression (see Introduction). However, it is also possible that AMLs involving the DEK/CAN fusion involve an alteration to the normal function of CAN (Fornerod et al., 1995). Further work will be required to determine the normal function of DEK and whether its function is altered by fusion to CAN in AML.

## **BACKGROUND (PART II)**

It is not known to what extent the normal splicing events are altered during tumorigenesis or play a causative role in cancers. Clearly, in order to address these questions, it will be essential to develop an understanding of how splicing is regulated on a global level, and in particular, how cellular changes associated with human diseases including breast cancers impact on this regulation. To this end, new technologies will be needed that permit the rapid and simultaneous analysis of thousands of regulated splicing events in response to both normal as well as disease-associated changes in cell growth and differentiation.

In collaboration with Dr. Chris Burge, a bioinformatics expert (Dept. of Biology, MIT), and Ontario Cancer Institute Microarray Facility, we are developing an exon-microarray for the large-scale analysis of alternative splicing profiles in normal and malignant human cells. DNA fragments corresponding to specific regulated and constitutive exon sequences are being arrayed on glass slides in a manner that permits a measure of the level of inclusion/exclusion of specific regulated exons.

## **BODY (PART II)**

### **RESULTS AND DISCUSSION**

To date, a proof-of-principle experiment has been established, which has permitted the accurate detection of alternative exon inclusion in a small number of genes. In future work, we aim to scale-up the exon array such that tens of thousands of alternative splicing events can be analyzed.

Our immediate goals for exploiting the exon-array technology include the following: (1) To identify alternative exons that respond to changes in the expression levels and activities of specific alternative splicing factors (eg. SR proteins and hnRNP proteins). We will establish stable cell lines in which the expression of wild-type and mutated alternative splicing factors are placed under inducible control, such that differential exon selection can be monitored in a dose- and time-responsive manner. (2) To monitor changes in alternative splicing profiles associated with normal cell growth and division, cell death (apoptosis), and differentiation. (3) To monitor alternative splicing profiles in tissues at different stages of tumorigenesis in breast cancers, in order to identify aberrant alternative splicing events linked to cancer progression.

The data sets generated from these experiments will provide valuable insights into the “global” functions of individual splicing factors in the selection of alternative splice sites among thousands of pre-mRNA transcripts. Moreover, it will permit the large-scale identification of alternative splicing events linked to both normal cell growth and differentiation, as well as aberrant alternative splicing events linked to tumorigenesis. Clustering methods will be used to identify exons which display common patterns of alternative splicing, and sensitive sequence comparison methods will be performed on the clustered sets in order to identify common sequence features in exons and introns that might account for the coordinated splicing patterns. The role of these sequences in the regulation of splice site selection will be verified using *in vitro* and *in vivo* splicing assays that are established in my lab (see recent publication attached). Thus, the development of a large-scale exon microarray will facilitate the identification of splicing regulatory sequences that respond to changes in the levels and activities of specific splicing factors, as well as other specific cellular changes. This information will be valuable for

developing improved algorithms for the prediction of splicing signals as well as the prediction of regulatory properties of specific exons in normal and disease situations. Importantly, the exon array experiments will facilitate the identification of new aberrant splicing events linked to the progression of different cancers. This information will provide new insights into the cellular changes that contribute to the altered properties of cancers cells. It will also provide new avenues for the development of diagnostic and typing methods, and the identification of potential new targets for therapeutic intervention in cancers.



## **KEY RESEARCH ACCOMPLISHMENTS**

- **Identification of the oncoprotein DEK as an SR protein-associated factor**
- **Demonstration that DEK associates with splicing complexes in vitro**
- **Demonstration that DEK forms a splicing-dependent association with mRNA (this represented one of the first discoveries of such a factor)**
- **Development of a proof-of-principle for an exon microarray for the analysis of alternative splicing events.**

## REPORTABLE OUTCOMES

### Publications 1999-2000

- Blencowe, B.J. and Lamond, A.I. (1999). Purification and depletion of RNP particles by antisense affinity chromatography. *Methods in Molecular Biology*. 118: 275-287.
- Blencowe, B.J., Bowman, J.A.L., McCracken, S. and Rosonina, E. (1999). SR-related proteins and the processing of mRNA precursors. *Biochem. and Cell Biol.* 77: 1-15.
- Li, Y. and Blencowe, B.J. (1999). Distinct factor requirements for exonic splicing enhancer function and binding of the U2 snRNP auxiliary factor to the polypyrimidine tract. *J. Biol. Chem.* 274: 35074-35079
- Blencowe, B.J. (2000) Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends in Bioch. Sci.* 25: 106-110.
- McGarvey T., Rosonina, E. McCracken, S., Li, Q. Arnaout, R., Nickerson, J.A., Awrey, D., Greenblatt, J. Grosveld, J. and Blencowe, B.J. (2000). The acute myeloid leukemia-associated protein DEK forms a splicing-dependent interaction with exon-product complexes. *J. Cell Biol.* 150: 309-320.
- Rodrigues, J.P., Gatfield, D., Rode, M., Blencowe, B.J., Fonseca M.-C., and Izaurralde, E. (2000). REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus. *Proc. Natl. Acad. Sci. USA.* In press.

### Meeting Abstracts 1999-2000

A.E. Eldridge, Y. Li, , Sharp, P.A. and Blencowe, B.J.\*. "Role of the SRm160/300 splicing coactivator in exon-enhancer function". Winternational Symposium, Lac Delage, Quebec. 2/99

McGarvey, T., Rosonina, E., McCracken, S., Li, Q., Nickerson, J.A., Awrey, D., Greenblatt, J. Grosveld, G. and Blencowe, B.J.\* The acute myeloid leukemia-associated protein DEK is a component of a spliceosome-derived mRNP. RNA Society Meeting 2000. Madison Wisconsin.

\* Platform presentation/invited speaker

## CONCLUSIONS

The results described in Part I of the Body section of this proposal indicate that DEK probably has a function in association with pre-mRNA metabolism. In particular, its association with spliced exons dependent on prior splicing represents one of the first examples of a protein with this property. It is possible that this splicing-dependent association is important for facilitating one or more steps downstream in gene expression that are influenced by the prior removal of an intron in pre-mRNA. Thus, based on the previous and present findings, DEK is a candidate for mediating interactions between transcription and pre-mRNA processing as well as between splicing and one or more subsequent splicing-dependent steps in the gene expression pathway. These studies therefore provide a foundation for investigating how different steps in gene expression communicate and are coordinated. This information will ultimately be important for understanding how gene expression becomes deregulated in life-threatening diseases including breast cancers.

The results described in Part II of the Body section outline preliminary experiments that we have performed towards establishing an exon-microarray for the analysis of alternative splicing. Once established, this system will allow the large-scale screening of alternative splicing patterns in normal and malignant cells. One application of this technology is the monitoring of changes in splicing events associated with breast cancer progression. As such, exon microarrays represent a potentially valuable platform from which to identify new targets for diagnosis and treatment of breast cancers.

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## APPENDIX 1

### FIGURE LEGENDS

**Figure 1.** (see Fig. 6 in attached pub)

#### **Association of DEK with splicing components in vivo**

A-F. HeLa cells were immunolabeled with rAb-DEK (red; A,D) and the anti-SR protein antibody mAb-NM4 (green; B,E). In the majority of cells, DEK is diffusely distributed throughout the nucleoplasm, partially excluding nucleoli (A). In a subset of cells (~15%), DEK is detected at higher levels in interchromatin granule clusters containing SR proteins compared to

the surrounding nucleoplasm (compare D and A and the corresponding overlays between D+E and A+B in panels F and C, respectively).

G-J. Cells were transfected with constructs expressing N-terminal flag-tagged SRm160 (flag-SRm160; G-I) or N-terminal flag-tagged B23 (flag-B23; J-L) and the effects on over-expression of these proteins on the distribution of DEK were examined. The transfected cells were double-immunolabeled with rAb-DEK (red; G,J) and anti-flag (H,K) antibodies; the sites of overlap between the signals in G+H and J+K are shown in the overlays (I and L, respectively). All of the images were scanned using a Leica SP confocal microscope. Scale bar= 5 $\mu$ m)

**Figure 2. Immunodepletion of DEK does not prevent pre-mRNA splicing**

*(see Fig. 7 in attached pub)*

A. HeLa nuclear extracts immunodepleted of DEK with rAb-DEK (lane 2) or mock-depleted with pre-immune serum (lane 1) were separated on a 12% SDS polyacrylamide gel and immunoblotted with rAb-DEK. Sizes markers are indicated in kDa.

B. Splicing reactions containing rAb-DEK or pre-immune-depleted nuclear extract were incubated with PIP85A pre-mRNA for 1 hr. RNA recovered from the splicing reactions was separated on a 15% denaturing polyacrylamide gel.

**Figure 3. Association of DEK with spliced exons requires prior splicing**

*(see Fig. 8 in attached pub)*

Immunoprecipitations were performed with rAb-DEK from splicing reactions incubated for 1hr containing PIP85A pre-mRNA or a PIP85A exon-product RNA transcribed from a PIP85A

cDNA construct. Both reactions also contained an internal control RNA for recovery (indicated by a wavy line; see text). RNA recovered after immunoprecipitation with rAb-DEK (lanes 7,8) or control serum (lanes 6,6) was separated, along with the corresponding total samples (lanes 3-4 and 1-2, respectively), on a 15% denaturing polyacrylamide gel.

# The Acute Myeloid Leukemia-associated Protein, DEK, Forms a Splicing-dependent Interaction with Exon-product Complexes

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**Abstract.** DEK is an ~45-kD phosphoprotein that is fused to the nucleoporin CAN as a result of a (6;9) chromosomal translocation in a subset of acute myeloid leukemias (AMLs). It has also been identified as an autoimmune antigen in juvenile rheumatoid arthritis and other rheumatic diseases. Despite the association of DEK with several human diseases, its function is not known. In this study, we demonstrate that DEK, together with SR proteins, associates with the SRm160 splicing coactivator in vitro. DEK is recruited to splicing factor-containing nuclear speckles upon concentration of SRm160 in these structures, indicating that DEK

and SRm160 associate in vivo. We further demonstrate that DEK associates with splicing complexes through interactions mediated by SR proteins. Significantly, DEK remains bound to the exon-product RNA after splicing, and this association requires the prior formation of a spliceosome. Thus, DEK is a candidate factor for controlling postsplicing steps in gene expression that are influenced by the prior removal of an intron from pre-mRNA.

**Key words:** SR proteins • spliceosome • mRNP • mRNA • transport

## Introduction

Increasing evidence suggests that different processes in gene expression communicate and are functionally coordinated with each other. For example, it has been demonstrated that transcription factors can influence the processing of a pre-mRNA. In this case, splicing and selection of polyadenylation sites can be influenced by promoter sequences that drive transcription by RNA polymerase II (pol II)<sup>1</sup> (Leroy et al., 1991; Cramer et al., 1997, 1999). Moreover, the COOH-terminal domain (CTD) of pol II is important for efficient capping, splicing, and 3' end cleavage of nascent pre-mRNA (Cho et al., 1997; McCracken et al., 1997a,b). Splicing and 3' end formation also influence each other, and interactions between these processes

are important for the definition of terminal exons in pre-mRNA (Niwa and Berget, 1991; Wassarman and Steitz, 1993; Nesic and Maquat, 1994; Gunderson et al., 1997; Bauren et al., 1998; Lou et al., 1998; Vagner et al., 2000). Recently, it was demonstrated that the transport of mRNA from the nucleus to the cytoplasm is promoted by prior splicing, since mRNAs that had not been generated by splicing were inefficiently transported (Luo and Reed, 1999). Currently, it is not understood how individual steps in the gene expression pathway communicate with each other, although several lines of evidence indicate that proteins containing domains rich in alternating arginine and serine residues (RS domains), including several splicing factors, may be important for the coupling of splicing to other steps in pre-mRNA metabolism (reviewed in Fu, 1995; Corden and Patturajan, 1997; Blencowe et al., 1999).

Pre-mRNA splicing involves the step-wise assembly of large RNA-protein complexes, termed spliceosomes (reviewed in Kramer, 1996; Reed and Palandjian, 1997; Burge et al., 1999). The major spliceosome contains four small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4/U6, and U5) and many non-snRNP splicing factors. A large number of non-snRNP splicing factors con-

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<sup>1</sup>Abbreviations used in this paper: AML, acute myeloid leukemia; CTD, COOH-terminal domain; ESE, exonic splicing enhancer; EV, epidermodysplasia verruciformis; hnRNP, heteronucleoriboprotein; HPV, human papillomavirus; pets, peri-ets; rAb, rabbit polyclonal antibody; RNA pol II, RNA polymerase II; RS domain, domain rich in alternating arginine and serine residues; snRNPs, small nuclear ribonucleoprotein particles; SR protein, serine/arginine-repeat protein.

tain RS domains. Among these are members of the SR family, which share a common domain structure consisting of one or two NH<sub>2</sub>-terminal RNA recognition motifs and a phosphorylated COOH-terminal RS domain (Fu, 1995; Manley and Tacke, 1996; Valcarcel and Green, 1996). Other RS domain-containing non-snRNP splicing factors have a distinct structural organization and are referred to as SR-related proteins (Fu, 1995; Blencowe et al., 1999). These include factors such as the alternative splicing regulators Tra, Tra2, and SWAP, as well as the subunits of the SRm160/300 splicing coactivator. The RS domains of SR family and SR-related proteins form interactions with each other and with snRNP-associated proteins that contain RS domains, and increasing evidence indicates that these interactions are critical for the formation of networks of interactions that promote splice site recognition across both intron and exon sequences.

Several SR-related proteins and interacting factors have been identified recently that are associated with transcription and 3' end processing. These include the E2 protein of the epidermodysplasia verruciformis (EV)-associated human papillomavirus (HPV), which binds to promoter sequences and is important for the transcriptional regulation of viral genes. The EV-HPV E2 protein contains an RS hinge domain that interacts with serine/arginine-repeat proteins (SR proteins) involved in splicing, and appears to promote the splicing of pre-mRNAs transcribed from promoters activated specifically by the EV-HPV E2 protein (Lai et al., 1999). A class of SR-related proteins, referred to as SCAFs (SR-like CTD-associated factors), has been identified that preferentially bind to the hyperphosphorylated CTD of RNA pol II, which is the form of the CTD associated with elongating transcription complexes (Yuryev et al., 1996; Bourquin et al., 1997; Tanner et al., 1997; Patturajan et al., 1998). An SR-related protein (PCG-1) has been identified that functions as a transcriptional coactivator of nuclear receptors, which is implicated in adaptive thermogenesis in mice (Puigserver et al., 1998). Another new transcriptional coactivator (p52) has been identified that lacks an RS domain, but interacts with the SR family protein ASF/SF2, as well as with several transcription factors (Ge et al., 1998). The 68-kD subunit of the mammalian cleavage factor (CFIm), which functions in the 3' end of processing transcripts, was recently isolated and found to contain an RS domain (Rueggsegger et al., 1998). These studies indicate that a variety of proteins containing RS domains are candidates for factors that coordinate different steps during the synthesis and processing of pre-mRNA.

SR family and SR-related proteins have been implicated in steps downstream of pre-mRNA splicing. Several SR proteins, including SRm160/300 and ASF/SF2, remain bound preferentially to the ligated exon-product, but not the intron-lariat product of the splicing reaction, which associates with snRNPs (Blencowe et al., 1998, 2000; Hanamura et al., 1998). A subset of SR family proteins shuttle between the nucleus and the cytoplasm, suggesting that they could function in the nuclear-cytoplasmic transport of mRNA (Cáceres et al., 1998). Recently, it was observed that complexes formed on exon-product RNAs generated by splicing are larger and more efficiently transported to the cytoplasm than complexes formed on the same RNAs

that have not been through splicing (Luo and Reed, 1999). This observation suggests that there are factors that are targeted for association with mRNA by the splicing machinery, and may relate to numerous earlier observations that introns are important for the efficient expression of genes.

In the present study, we have identified the oncoprotein DEK as a splicing complex component that remains associated with spliced exons dependent on prior splicing of pre-mRNA. Furthermore, we show that the association of DEK with splicing complexes is mediated by specific interactions involving SR proteins. DEK is an ~45-kD phosphoprotein that was previously identified in the context of several human diseases. It was first isolated as part of a fusion that arises in a subtype of acute myeloid leukemias (AMLs) involving (6;9) chromosomal translocations (von Lindern et al., 1992). These translocations occur within intron sequences of the genes encoding *dek* and the nucleoporin *can*, and result in the expression of an in-frame chimera encoding the majority of DEK fused NH<sub>2</sub>-terminally to the COOH-terminal two-thirds of CAN. DEK was later identified as an autoimmune antigen in patients with pauciarticular onset juvenile rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases (Szer et al., 1994; Dong et al., 1998; Wichmann et al., 2000). In another study, it was reported that DEK binds to a TG-rich element, the pets (peri-ets) site, which is adjacent to two binding sites of the ets family member, Elf-1, in the HIV-2 enhancer, which mediates enhancer activation by a number of signaling processes (Fu et al., 1997). However, the specificity and functional significance of the binding of DEK to the pets site is not known. The results in the present study indicate that DEK may have one or more functions in association with pre-mRNA processing. In particular, the results identify DEK as one of the first factors that associates with mRNA in a splicing-dependent manner, indicating that it could function to coordinate splicing with one or more subsequent steps in gene expression.

## Materials and Methods

### Protein Purification

**Fractionation of p130.** Fractionation was initiated with ~200 ml of HeLa nuclear extract (~10 mg/ml) that was prepared as described by Dignam et al. (1983), except that the final dialysis was against a modified buffer D containing 10% glycerol (20 mM Hepes, pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM DTT). The dialyzed nuclear extract was chromatographed over a poly-U Sepharose column as described by Zamore and Green (1989). The flow-through fraction was chromatographed over a phosphocellulose column as described by Samuels et al. (1982). p130 and other SR proteins were detected entirely in the 1.0 M salt elution. The 1.0 M salt elution from the phosphocellulose column was dialyzed against SR-dialysis buffer (SRDB; 65 mM KCl, 15 mM NaCl, 10 mM Hepes, pH 7.6, 1 mM EDTA, 2 mM DTT, 5 mM KF, 5 mM  $\beta$ -glycerophosphate, 0.2 mM Pefabloc [Boehringer]; modified from Zahler et al., 1992). Precipitate formed during dialysis, which contained the majority of SRm160, but not p130, was removed by centrifugation. The cleared fraction was incubated for 15 min at 30°C in the presence of 1.5 mM ATP and 5 mM creatine-PO<sub>4</sub> to regenerate phosphoepitopes detected by mAb-104, and was then recentrifuged to remove further precipitate. The supernatant fraction was mixed with an equal volume of SRDB containing 6 M urea and then chromatographed over a Q-Sepharose column pre-equilibrated with 3 M urea-SRDB. Proteins were step-eluted with increasing concentrations of KCl in the 3 M urea-SRDB buffer. A sample of the

0.3 M step elution (Q-Sepharose-0.3 M, Q-0.3M), which contained the majority of p130 and other SR proteins detected by immunoblotting with mAb-104, was precipitated with TCA in the presence of deoxycholate. The TCA pellet was washed with ice-cold acetone, dried briefly, and resuspended in Laemmli loading buffer. The sample was separated on a 12% SDS polyacrylamide gel and detected by Coomassie staining (Fig. 1 C). The p130 doublet and other visible bands were excised, digested in situ with trypsin, and analyzed by MALDI-TOF mass spectroscopy using a PerSeptive Biosystems (model DE-STR) mass spectrometer. Details of sample preparation can be obtained from borealis biosciences, inc.

**Purification of SR Family Proteins.** Purification of the set of 6 defined SR proteins was performed essentially as described by Zahler et al. (1992).

## Antibodies

The following antibodies were used in this study: murine mAb-104 (Roth et al., 1990); mAb-B1C8 (Blencowe et al., 1994; Wan et al., 1994; Matritsch); mAb-B3 (Mortillaro et al., 1996); mAb-NM4 (Blencowe et al., 1995; Matritsch); mAb anti-Flag (VWR-Scientific); rabbit polyclonal antibody (rAb)-DEK (Fornerod et al., 1995); and rAb-SRm300 (Blencowe et al., 2000).

## Nuclear and Cytoplasmic Extracts

HeLa nuclear and cytoplasmic S100 extracts were prepared essentially as described by Dignam et al. (1983). Nuclear extract depleted of DEK protein was prepared by the same procedure as described for SRm160/300 in Blencowe et al. (1998).

## Splicing and Immunoprecipitation Assays

Immunoprecipitation of SR protein complexes in the presence and absence of ribonuclease (Fig. 2) was performed as described by Eldridge et al. (1999). Immunoprecipitation of splicing complexes was performed as described in Blencowe et al. (1994). Splicing assays using the PIP85A pre-mRNA were carried out as described by Blencowe et al. (1998). Splicing assays using the dsx(GAA)<sub>6</sub> pre-mRNA were performed as described by Eldridge et al. (1999). The splicing reactions containing S100 extract (see Fig. 5) were performed in a total volume of 15  $\mu$ l and were supplemented with  $\sim$ 3  $\mu$ g of purified SR family proteins where indicated. The splicing reactions containing PIP85A pre-mRNA were analyzed on 15% denaturing polyacrylamide gels; doublesex (dsx) splicing reactions were analyzed on 7 or 10% denaturing polyacrylamide gels.

## Affinity Selection of Splicing Complexes

Affinity selection of splicing complexes assembled on biotinylated dsx pre-mRNAs was performed essentially as described in Li and Blencowe (1999).

## SDS-PAGE and Immunoblot Analysis

SDS-PAGE and immunoblots were performed essentially as described by Harlow and Lane (1988). Immunoblots in Figs. 1, 4, and 5 were developed using a secondary antibody conjugated to HRP and chemiluminescence detection (NEN Life Science Products), as per the manufacturer's instructions.

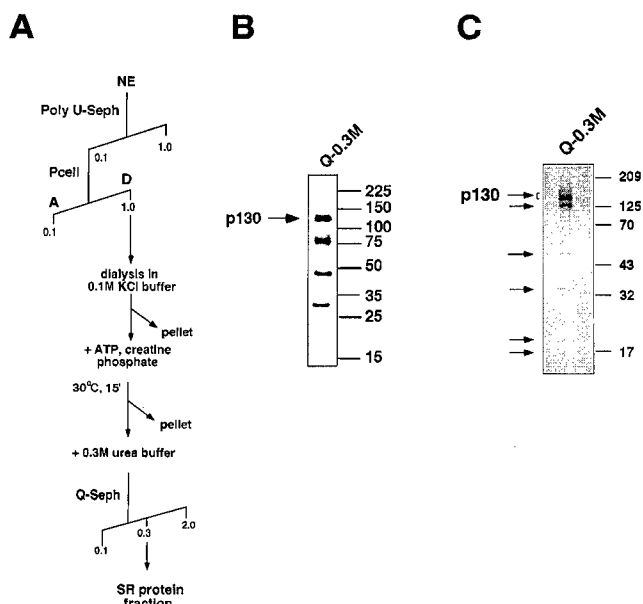
## Immunocytochemistry

HeLa cells were immunolabeled as described by Blencowe et al. (1998). The samples were examined and recorded using a Leica-SP confocal microscope.

## Results

### DEK Copurifies with SR Proteins

To identify new SR-related proteins and associated factors that function in pre-mRNA processing, we have fractionated HeLa cell nuclear extract for proteins that react with mAb-104, which binds to a phosphopeptide located within the RS domains of many SR proteins (Roth et al., 1990).



**Figure 1.** DEK copurifies with SR proteins. A, Fractionation scheme for a 130-kD antigen recognized by mAb-104 (p130; see Materials and Methods). B, mAb-104 immunoblot of the Q-Sepharose 0.3-M (Q-0.3M) fraction obtained by the scheme in A. SR proteins of  $\sim$ 75, 50, and 30 kD detected by mAb-104 copurify with p130. Size markers are indicated in kD. C, Coomassie-stained SDS polyacrylamide gel of the Q-0.3M fraction. The identities of the abundant polypeptides, migrating at 130, 50, and 34 kD, were identified by MALDI-TOF mass spectroscopy as Hel117, DEK, and SC35, respectively. Size markers are indicated in kD.

We initially focused our attention on an antigen of 130 kD (p130), since an SR protein of this size recognized by mAb-104 had not been isolated previously. Using immunoblotting with mAb-104 to monitor purification, we fractionated HeLa nuclear extract for p130 by the scheme shown in Fig. 1 A (see Materials and Methods). The 0.3 M KCl elution step from the final (Q-Sepharose) column yielded subfractions that were highly enriched in a doublet of mAb-104 antigens that migrated at 130 kD, as well as antigens detected by this antibody corresponding in size to previously identified SR family proteins of  $\sim$ 75, 55, and 30 kD. The levels of the p130 doublet detected by immunoblotting with mAb-104 correlated precisely with the levels of a doublet of polypeptides of 130 kD detected in the same subfractions by Coomassie staining, indicating that one or both of the latter species correspond to p130 (data not shown). Fig. 1 B shows an immunoblot of the peak Q-0.3M fraction probed with mAb-104, and Fig. 1 C shows a corresponding Coomassie-stained gel. Besides the 130-kD doublet, relatively abundant polypeptides of 120, 50, 35, 20, and 17 kD were detected in the fraction by Coomassie staining (indicated by arrows in Fig. 1 C).

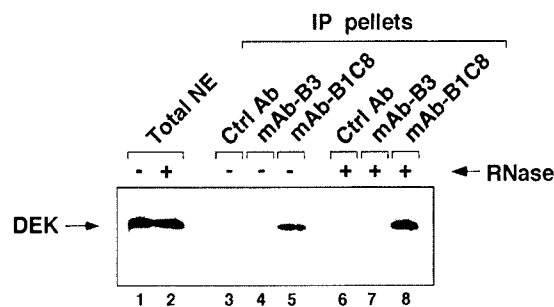
To determine the identity of the p130 proteins, as well as the other proteins in the Q-0.3M fraction, the bands indicated in Fig. 1 C were excised, digested with trypsin in situ, and the released peptides were analyzed by MALDI-TOF mass spectroscopy. The masses of peptides from each protein were used to search for corresponding peptide masses in conceptually translated sequences from the databases.

Close matches were obtained between peptides from both polypeptides in the p130 doublet, identifying these as Hel117. The ~50- and 35-kD proteins were identified as DEK and SC35, respectively (data not shown). Consistent with its reactivity with mAb-104, Hel117 is a previously reported protein of unknown function that contains an RS domain (Sukegawa and Blobel, 1995), and SC35 is a previously characterized member of the SR family of splicing factors (Fu and Maniatis, 1992). Hel117 contains other domain features found in splicing factors, including a DEAD-box and associated motifs shared between proteins with dsRNA unwinding and/or nucleic acid-dependent ATPase activity. It also contains a separate domain that is homologous to a region within the yeast splicing factor, PRP5. Consistent with a possible role in splicing, Hel117 was previously localized to nuclear speckle domains enriched in pre-mRNA splicing factors (Sukegawa and Blobel, 1995), and we have found that it associates with other SR proteins and splicing complexes in vitro (unpublished observations). Interestingly, DEK is a multidisease-associated phosphoprotein of unknown function that was originally identified as an in-frame fusion partner with the nucleoporin CAN, generated by a specific (6;9) chromosomal translocation in a subset of AML (von Lindern et al., 1992; see Introduction). It contains several regions rich in charged residues, including an NH<sub>2</sub>-terminal acidic domain similar to those found in transcriptional activator proteins. A comparison of the DEK amino acid sequence against sequences in the databases identified homologous sequences within predicted open reading frames of zebrafish, fly, and plant expressed sequence tag cDNAs, but did not reveal a significant homology with any characterized protein (data not shown).

The 55- and 75-kD SR proteins that were detected in the Q-0.3M fraction by immunoblotting with mAb-104 were not detected in the Coomassie stained gel in Fig. 1 C because these proteins are lower in abundance than the polypeptides that were analyzed. The detection of these antigens and the identification by mass spectroscopy of two RS domain proteins in the Q-0.3M fraction, Hel117 and SC35, demonstrates that the fractionation scheme in Fig. 1 A enriches for SR proteins associated with pre-mRNA processing. The cofractionation of DEK with several SR proteins suggested that it either has similar chromatographic properties as these factors, or, more interestingly, that it may be associated with one or more SR proteins in a complex. To distinguish between these possibilities, we next addressed whether DEK interacts with SR proteins.

### DEK Associates with SR Proteins In Vitro

Immunoprecipitates were collected from HeLa nuclear extract using an mAb specific for the SRm160 subunit of the SRm160/300 splicing coactivator (mAb-B1C8; Blencowe et al., 1994; Wan et al., 1994). This antibody immunoprecipitates SRm160/300 efficiently and, in the presence of low salt concentrations, coimmunoprecipitates several proteins that contain RS domains, including Hel117, hTra2beta (a human homologue of the *Drosophila melanogaster* alternative splicing factor, Tra2) and SR family proteins (Eldridge et al., 1999; and our unpublished data).



**Figure 2.** DEK associates with SR proteins. Immunoprecipitates were collected from HeLa nuclear extract preincubated with (lanes 6–8) or without (lanes 3–5) RNase using the following antibodies: an mAb specific for the SRm160 subunit of the SRm160/300 splicing coactivator (mAb-B1C8, lanes 5 and 8); an mAb specific for hyperphosphorylated RNA pol II large subunit (mAb-B3, lanes 4 and 7); and a control antiserum (rAb anti-mouse, lanes 3 and 6). The immunoprecipitates were separated on an SDS polyacrylamide gel and immunoblotted with rAb-DEK. The corresponding total HeLa nuclear extracts treated without or with RNase, corresponding to 10% of the amount of nuclear extract used in each immunoprecipitation, are separated in lanes 1 and 2, respectively.

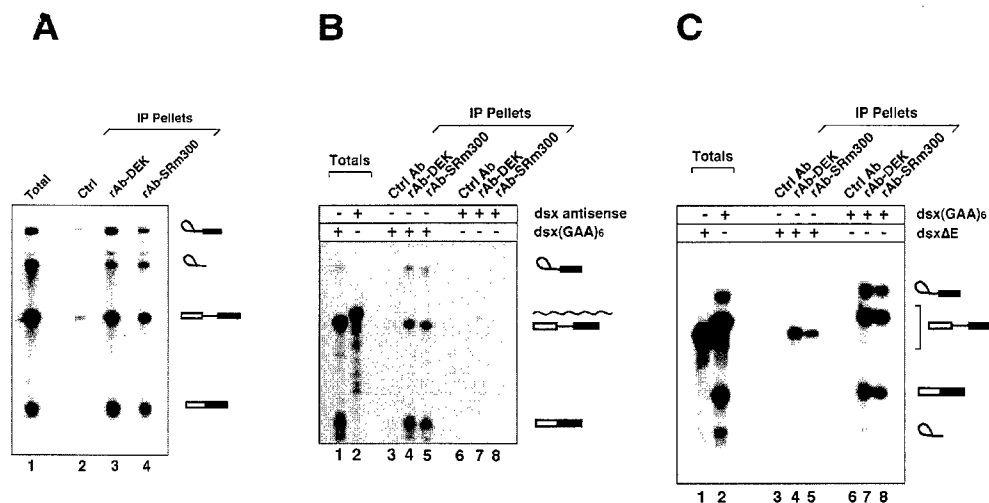
To determine whether DEK associates with one or more of these SR proteins, the immunoprecipitates were immunoblotted with an antibody that is highly specific for DEK (rAb-DEK; Fig. 2; Fornerod et al., 1995). In total HeLa nuclear extract, rAb-DEK detected a major band of ~50–55 kD and a minor band of ~40 kD. The major band corresponds in size to a form of DEK that is posttranslationally modified by phosphorylation, whereas the minor band is thought to correspond to a cleavage product of DEK (Fig. 2, lanes 1 and 2; Fornerod et al., 1995). The specificity of rAb-DEK for DEK has been confirmed by immunoblotting whole cell extracts from DEK<sup>+/+</sup> and DEK<sup>-/-</sup> mice. Both bands are detected by rAb-DEK in <sup>+/+</sup> extracts, but neither band, nor any other antigen, is detected in the <sup>-/-</sup> extracts (data not shown).

mAb-B1C8, but not a control mAb of the same isotype that is specific for a hyperphosphorylated form of the RNA pol II large subunit (mAb-B3; Mortillaro et al., 1996), specifically coimmunoprecipitated DEK (Fig. 2, compare lane 5 with 4). This coimmunoprecipitation was not reduced by extensive pretreatment of nuclear extract with RNase (Fig. 2, compare lanes 5 and 8), indicating that DEK associates with one or more SR proteins through protein–protein interactions and is not tethered to these proteins by RNA. Thus, consistent with the cofractionation of DEK with SR proteins (Fig. 1), the results indicate that DEK is associated with one or more SR proteins that function in pre-mRNA splicing. To extend these observations, we next determined whether or not DEK associates with SR proteins during the formation of pre-mRNA splicing complexes (Fig. 3).

### Association of DEK with Splicing Complexes

Remarkably, rAb-DEK immunoprecipitated splicing complexes assembled on a constitutively spliced pre-mRNA derived from adenovirus PIP85A (Fig. 3 A). Similar to a





**Figure 3.** Association of DEK with splicing complexes. **A**, Immunoprecipitation of splicing complexes with rAb-DEK from reactions incubated for 1 h containing PIP85A pre-mRNA. RNA recovered after immunoprecipitation (lanes 2–4) and RNA recovered directly from a parallel splicing reaction (lane 1), was loaded on a 15% denaturing polyacrylamide urea gel. RNA loaded in lane 1 represents 25% of the total amount recovered, whereas RNA loaded in lanes 2–4 from each immunoprecipitation represents 50% of the total amounts recovered.

ered. Immunoprecipitations were performed with preimmune serum (lane 2), rAb-DEK (lane 3), and rAb-SRm300 as a positive control (lane 4). **B**, Complexes were immunoprecipitated from splicing reactions incubated for 45 min containing either a dsx(GAA)<sub>6</sub> pre-mRNA (lanes 1, and 3–5) or the corresponding antisense RNA (lanes 2, and 6–8). RNA recovered after immunoprecipitation (lanes 3–8) and RNA recovered directly from parallel splicing reactions (lanes 1 and 2) was loaded on a 10% denaturing polyacrylamide urea gel. The antibodies used for immunoprecipitation and the relative amounts of RNA loaded from the total and immunoprecipitation samples are as described in **A**. **C**, Association of DEK with a two-exon dsx pre-mRNA is promoted by a 6xGAA ESE sequence in the 3' exon. Immunoprecipitation was performed from splicing reactions incubated for 45 min containing dsx pre-mRNA lacking an ESE (dsxΔE; lanes 1, and 3–5) or from reactions containing dsx pre-mRNA with the 6xGAA ESE (dsx(GAA)<sub>6</sub>; lanes 2, and 6–8). RNA recovered after immunoprecipitation (lanes 3–8) and RNA recovered directly from a parallel splicing reactions (lanes 1 and 2) was loaded on a 10% denaturing polyacrylamide urea gel. Antibodies used for immunoprecipitation and the relative amounts of RNA loaded from the total and immunoprecipitation samples are as described in **A**.

polyclonal antibody specific for SRm300 (rAb-SRm300; Blencowe et al., 2000), which was used as a positive control for immunoprecipitation, rAb-DEK efficiently immunoprecipitated splicing complexes containing exon sequences (pre-mRNA, splicing intermediates, and exon-product RNA) and, to a lesser degree, the complex containing the lariat product of the splicing reaction (Fig. 3 **A**, lanes 3 and 4). The immunoprecipitation of splicing complexes by rAb-DEK was specific since neither preimmune serum, nor a saturating amount of a control immune serum bound to protein A beads, immunoprecipitated splicing complexes (Fig. 3 **A**, lane 2, and data not shown; see also Eldridge et al., 1999). These results provide evidence that DEK associates with splicing complexes through both steps of the splicing reaction. Moreover, the similar profile of immunoprecipitation of splicing complexes observed between rAb-DEK and rAb-SRm300 is consistent with the data in Fig. 2, indicating that DEK and SRm160/300 associate with each other (see below).

To investigate the specificity of the interaction of DEK with splicing complexes in more detail, we next determined whether it can associate with a distinct pre-mRNA, and also whether it can associate with an RNA that is not a splicing substrate. To test for these properties, rAb-DEK was assayed for its ability to immunoprecipitate splicing complexes assembled on a pre-mRNA derived from exons 3 and 4 of the *dsx* gene of *Drosophila*, and also the corresponding antisense RNA, which does not assemble functional splicing complexes (Fig. 3 **B**). Wild-type *dsx* pre-mRNA contains a suboptimal 3' splice site upstream of exon 4. In *Drosophila*, efficient recognition of this splice

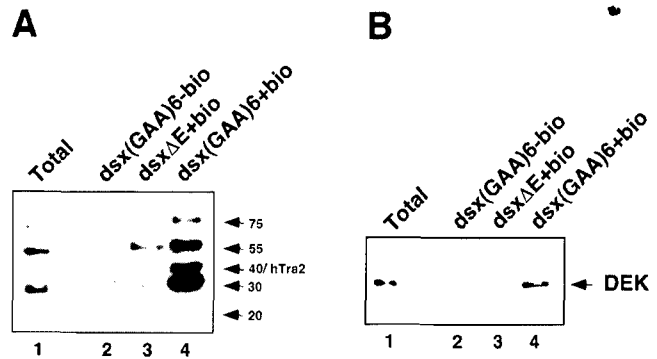
site and subsequent intron removal requires the assembly on a specialized exonic splicing enhancer (ESE) within exon 4 of a multisubunit complex that contains the RS domain splicing factors Tra, Tra2, and the SR family protein RBP-1 (SRp20; Inoue et al., 1992; Tian and Maniatis, 1993, 1994). The substrate used in the present study contains a typical purine-rich mammalian ESE consisting of six GAA repeats in place of the *dsx* ESE (Yeakley et al., 1996). This 6xGAA ESE promotes the stable association of hTra2β, several SR family proteins, and SRm160/300 with the *dsx* pre-mRNA, and is required for efficient splicing of this substrate in HeLa nuclear extracts (see below; Yeakley et al., 1996; Eldridge et al., 1999; Li and Blencowe, 1999). The results in Fig. 3 **B** demonstrate that rAb-DEK, like rAb-SRm300, efficiently immunoprecipitated splicing complexes assembled on the dsx(GAA)<sub>6</sub> pre-mRNA (lanes 4 and 5), but only weakly immunoprecipitated the antisense RNA (lanes 7 and 8). These results indicate that DEK may be a general component of splicing complexes, but that it does not associate stably with an RNA that does not form functional splicing complexes.

Given the association of DEK with SR proteins, including SRm160/300 observed in Fig. 2, and the similar profiles of immunoprecipitation of splicing complexes observed between antibodies to both DEK and SRm300 (Fig. 3, **A** and **B**), we next determined whether or not SR proteins mediate the association of DEK with splicing complexes. To test if this is the case, we initially compared the ability of DEK to associate with splicing complexes assembled on the dsx(GAA)<sub>6</sub> pre-mRNA versus the same substrate lacking an ESE (dsxΔE; Fig. 3 **C**). Previously, we have

shown that U1 snRNP and U2AF-65 kD bind equally to these substrates but, as mentioned above, SRm160/300, Tra2beta, and SR family proteins only associate stably with the dsx(GAA)<sub>6</sub> pre-mRNA (Eldridge et al., 1999; Li and Blencowe, 1999). Similar to rAb-SRm300, rAb-DEK efficiently immunoprecipitated splicing complexes assembled on the dsx(GAA)<sub>6</sub> substrate, but inefficiently immunoprecipitated complexes assembled on the dsxΔE substrate (Fig. 3 C, compare lanes 7 and 8 with 4 and 5). The data demonstrate that a 6xGAA ESE results in a significant increase in the association of both DEK and SRm300 with the dsx pre-mRNA, again consistent with the data in Fig. 2 indicating that these components interact. As before, the ability of rAb-DEK and rAb-SRm300 to immunoprecipitate splicing complexes on the dsx substrates was not due to nonspecific interactions since neither preimmune serum, nor high levels of control immune sera, immunoprecipitated significant levels of these complexes (Fig. 3 C, lanes 3 and 6; see also Eldridge et al., 1999).

To confirm that the different levels of immunoprecipitation of the dsxΔE and dsx(GAA)<sub>6</sub> splicing complexes by rAb-DEK reflect the differential association of DEK with these substrates and are not due to the differential accessibility to antibody in these complexes, we next probed directly for the presence of DEK in splicing complexes affinity-selected on the dsx substrates (Fig. 4). Splicing complexes were assembled on biotinylated derivatives of the dsx pre-mRNAs and affinity-selected using streptavidin agarose. Bound proteins were eluted in high salt and immunoblotted first with mAb-104 to confirm the recovery of splicing complex proteins (Fig. 4 A). In agreement with our previous results (Li and Blencowe, 1999), low levels of SR proteins of 30, 40, 55, and 75 kD were detected by mAb-104 in splicing complexes affinity-selected on the dsxΔE pre-mRNA (Fig. 4 A, lane 3), whereas the levels of these proteins increased significantly with the dsx(GAA)<sub>6</sub> pre-mRNA (Fig. 4 A, lane 4). Consistent with the immunoprecipitation results in Fig. 3, DEK was detected in splicing complexes assembled on the dsx(GAA)<sub>6</sub> substrate, but not in complexes on the dsxΔE pre-mRNA (Fig. 4 B, compare lanes 3 and 4). The detection of DEK in association with the dsx(GAA)<sub>6</sub> substrate was specific, since essentially no DEK was selected from a splicing reaction containing complexes assembled on a dsx(GAA)<sub>6</sub> pre-mRNA lacking biotinylated residues (Fig. 4 B, lane 2). The absence of a level of DEK above background in the affinity-selected dsxΔE complexes (Fig. 4 B, lane 3), in comparison with the low level detected in association with the dsxΔE pre-mRNA by immunoprecipitation (Fig. 3 C, lane 4), is probably due to the more stringent conditions used in the affinity-selection assay (300 mM salt) compared with the conditions in the immunoprecipitation assay (100 mM salt; see Materials and Methods). Moreover, the higher levels of SR proteins detected in the affinity-selected splicing complexes compared with DEK indicates that they may be more stably associated with the dsx pre-mRNA (Fig. 4, A and B, compare lanes 1, 3, and 4). These results confirm that DEK is associated with splicing complexes on the dsx(GAA)<sub>6</sub> pre-mRNA and that its association with this substrate, like SRm160/300, hTra2beta, and SR family proteins, is promoted by a purine-rich ESE.

To confirm that SR proteins are required for the stable

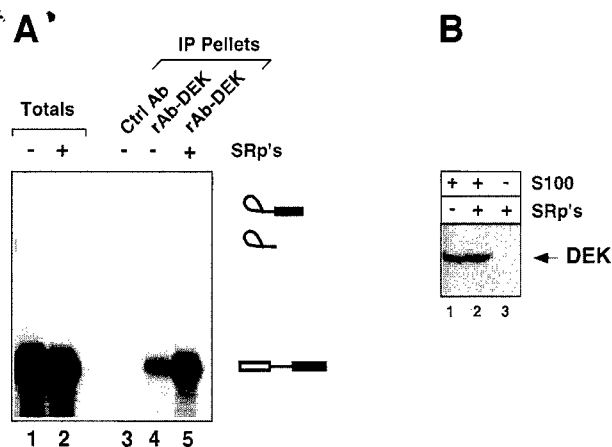


**Figure 4.** Detection of DEK in affinity-purified splicing complexes. A and B, Biotinylated dsx pre-mRNAs were transcribed cold and incubated in splicing reactions for 15 min before selection on streptavidin-agarose. Selections were performed from splicing reactions incubated with a control, non-biotinylated, dsx pre-mRNA (dsx(GAA)<sub>6</sub>-bio, lane 2), with a biotinylated dsx pre-mRNA lacking an ESE (dsxΔE+bio, lane 3), and with a biotinylated dsx pre-mRNA containing a 6xGAA ESE (dsx(GAA)<sub>6</sub>+bio, lane 4). Proteins recovered from the affinity-selected splicing complexes were separated on 12% SDS polyacrylamide gels and analyzed by immunoblotting with mAb-104 (A) and rAb-DEK (B). Total nuclear extract (lane 1), corresponding to ~3% of the amount of extract used in each affinity selection, was separated as a marker for the eluted SR and DEK proteins. Sizes of the SR proteins (in kD) detected by mAb-104 are indicated to the right of A.

association of DEK with pre-mRNA, we next assayed the ability of rAb-DEK to immunoprecipitate splicing complexes from a cytoplasmic S100 extract (Fig. 5). S100 extracts contain all of the factors required for splicing, except SR proteins (Krainer et al., 1990b). These extracts contain a lower level of DEK than in the same volume of HeLa nuclear extract (data not shown). Addition of purified SR family proteins to an S100 reaction resulted in a partial restoration of splicing activity to the PIP85A pre-mRNA (Fig. 5, compare lanes 1 and 2). The rAb-DEK antibody immunoprecipitated a low level of PIP85A pre-mRNA from an S100 reaction in the absence of SR family proteins (Fig. 5, lane 4). However, upon addition of purified SR family proteins, immunoprecipitation of PIP85A pre-mRNA by rAb-DEK was restored to a level comparable to that observed in reactions containing total nuclear extract (Fig. 5, lane 5, and data not shown). This promotion of immunoprecipitation by SR proteins was not due to an increase in the level of DEK in the splicing reaction, since no DEK was detected in the purified SR protein preparation by immunoblotting with rAb-DEK (Fig. 5 B, lane 3). Moreover, no difference in the level of DEK was observed in the S100 splicing reactions after incubation with or without SR proteins (Fig. 5 B, compare lanes 1 and 2). Together with the data in Figs. 3 and 4, these results demonstrate that the association of DEK with pre-mRNA requires interactions mediated by SR proteins.

#### DEK Associates with SR Proteins In Vivo

To investigate whether DEK associates with splicing components in vivo, we double-immunolabeled HeLa cells with rAb-DEK and the anti-SR protein antibody, mAb-



**Figure 5.** SR proteins promote the association of DEK with pre-mRNA in S100 splicing reactions. **A**, Immunoprecipitations with rAb-DEK were performed from S100 splicing reactions incubated for 1 h containing PIP85A pre-mRNA, with (lanes 2 and 5) or without (lanes 1, 3, and 4) purified SR family proteins. RNA recovered after immunoprecipitation (lanes 3–5) and RNA recovered directly from a parallel splicing reaction (lanes 1 and 2) was loaded on a 15% denaturing polyacrylamide urea gel. RNA loaded in lane 1 represents 25% of the total amount recovered, whereas RNA loaded in lanes 2–4 from each immunoprecipitation represents 50% of the total amounts recovered. Immunoprecipitations were performed with a preimmune serum (lane 3) and rAb-DEK (lanes 4 and 5). **B**, Proteins recovered from parallel S100 splicing reactions as shown in **A** were separated on a 12% SDS polyacrylamide gel and immunoblotted with rAb-DEK. Lanes 1 and 2 correspond to lanes 1 and 2 in **A**. Lane 3 contains  $\sim 3 \mu\text{g}$  of the SR protein preparation used in **A**.

NM4 (Blencowe et al., 1995; Fig. 6, A–F). Similar to many antibodies that recognize SR proteins, mAb-NM4 specifically immunolabels nucleoplasmic interchromatin granule clusters or speckles that are highly enriched in splicing factors (B and E, and data not shown). In the majority of cells within a field, rAb-DEK labeling reveals that DEK is specifically localized in the nucleus in a diffuse-granular pattern that largely, but not completely, excludes nucleoli (Fig. 6 A). In these cells, DEK overlaps with speckle domains, although it does not appear significantly more concentrated in these structures than in other regions of the nucleoplasm (Fig. 6 C). However, in  $\sim 15\%$  of cells within a field, DEK is significantly more enriched in speckle structures compared with the surrounding nucleoplasm (Fig. 6, compare D–F with A–C). The detection of DEK by immunostaining with rAb-DEK is specific since preimmune serum did not immunolabel nuclei (Fornerod et al., 1996; data not shown). These results indicate that in a subset of cells, DEK is concentrated with SR proteins and other splicing factors in nuclear speckle structures.

To extend these observations, we next tested whether the localization of DEK is influenced by the intranuclear distribution of SR proteins. Recently, we have observed that SRm160 containing an  $\text{NH}_2$ -terminal Flag epitope (Flag-SRm160), when overexpressed by transient transfection, concentrates with endogenous SR proteins in enlarged speckle structures (Fig. 6 H, and data not shown). To determine whether the elevated levels of SRm160 in

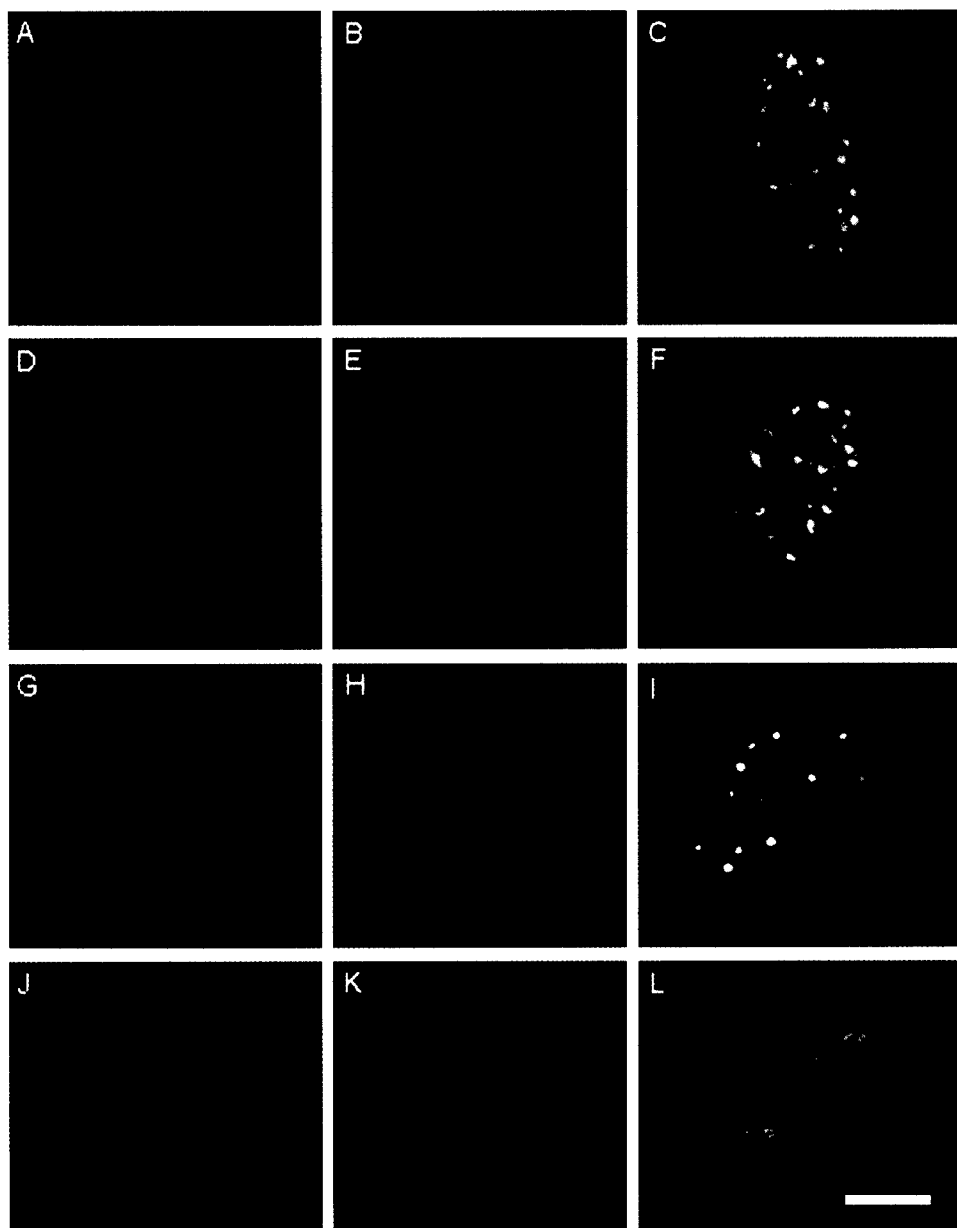
speckles results in a change in the nucleoplasmic distribution of DEK, cells transiently transfected with a flag-SRm160 construct were double-immunolabeled with anti-flag and rAb-DEK antibodies. Significantly, the presence of flag-SRm160 in speckles (Fig. 6 H) correlated with an accumulation of DEK in these structures (Fig. 6 G and I). This effect was specific since the distribution of DEK was not altered significantly by the overexpression of a flag-B23 protein, which concentrates in nucleoli (Fig. 6, compare J–L). Although every speckle containing flag-SRm160 also contained an increased level of DEK, in some cases the size of the speckle domain detected with the anti-flag antibody was significantly smaller than the size of the corresponding speckle domain detected with rAb-DEK. This may reflect differences in the relative concentrations of these proteins between speckles, and/or the differential accessibility of these proteins to antibodies in different speckles. In either case, the results demonstrate that the distribution of DEK within nuclei can be specifically influenced by the localization of SRm160, and that, consistent with the *in vitro* interactions with SR proteins, including SRm160/300 described above, DEK probably concentrates in speckles by associating with one or more of these splicing factors.

#### Depletion of DEK Does Not Prevent Pre-mRNA Splicing

The results so far suggest that DEK could function as a splicing factor, perhaps by associating with one or more SR proteins and modulating their activity. To investigate this possibility, we have prepared nuclear extracts that are specifically and efficiently depleted of DEK (Fig. 7 A, compare lanes 1 and 2). Surprisingly, efficient immunodepletion of DEK did not affect the level of splicing of several pre-mRNAs tested, including the PIP85A (Fig. 7 B, compare lanes 1 and 2) and the *dsx*(GAA)<sub>6</sub> substrate (data not shown). Likewise, in contrast to SR proteins and heteronucleoriboprotein (hnRNP) A1, which promote the selection of intron proximal and distal 5' splice sites, respectively (Ge and Manley, 1990; Krainer et al., 1990a; Fu et al., 1992; Mayeda and Krainer, 1992; Cáceres et al., 1994), altering the level of DEK by specific immunodepletion *in vitro*, or by overexpression *in vivo*, did not significantly alter the ratio of intron distal to proximal splice sites in different pre-mRNAs containing cis-competing splice sites (data not shown). Whereas it cannot be excluded that a minor amount of DEK remaining in the immunodepleted extract is important for splicing activity, or that DEK is important for the splicing of specific substrates not assayed, the results so far suggest that DEK may provide a function that is not directly related to splicing activity.

#### The Association of DEK with the Exon-product RNA Requires Prior Splicing

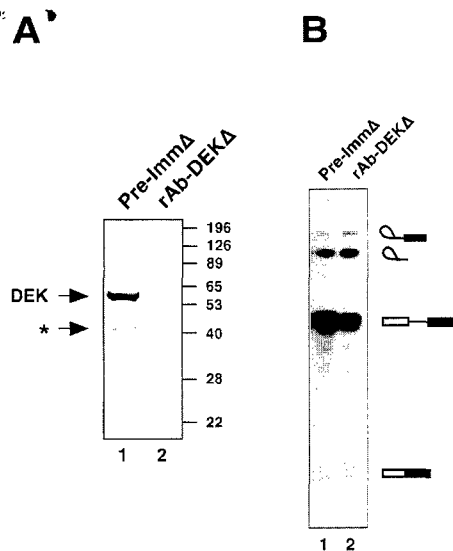
The experiments in Fig. 3 reveal that DEK, like SRm160/300, remains bound preferentially to spliced exons after splicing. This observation, and the finding that DEK does not associate efficiently with RNAs that cannot assemble functional splicing complexes, raises the possibility that its association with spliced exons requires the prior formation of a spliceosome. To determine whether the association of



**Figure 6.** Association of DEK with splicing components in vivo. A–F, HeLa cells were immunolabeled with rAb-DEK (red; A and D) and the anti-SR protein antibody, mAb-NM4 (green; B and E). In the majority of cells, DEK is diffusely distributed throughout the nucleoplasm, partially excluding nucleoli (A). In a subset of cells (~15%), DEK is detected at higher levels in interchromatin granule clusters containing SR proteins compared with the surrounding nucleoplasm (compare D and A, and the corresponding overlays between D and E, and A and B in F and C, respectively). G–L, Cells were transfected with constructs expressing NH<sub>2</sub>-terminal flag-tagged SRm160 (flag-SRm160; G–I) or NH<sub>2</sub>-terminal flag-tagged B23 (flag-B23; J–L), and the effects on overexpression of these proteins on the distribution of DEK were examined. The transfected cells were double-immunolabeled with rAb-DEK (red; G and J) and anti-flag (H and K) antibodies; the sites of overlap between the signals in G and H, and J and K, are shown in the overlays (I and L, respectively). All of the images were scanned using a Leica SP confocal microscope. Bar, 5  $\mu$ m).

DEK with spliced exons is facilitated by splicing, we compared the ability of rAb-DEK to immunoprecipitate complexes formed on the exon-product RNA generated by splicing of PIP85A pre-mRNA, versus complexes formed on the identical exon-product RNA that has been derived by transcription from a PIP85A cDNA construct (Fig. 8). In this assay, splicing reactions were incubated with equal amounts of PIP85A pre-mRNA and a second RNA that is not a splicing substrate (a fragment of the HIV-1 Rev-response element), as an internal control RNA for recovery (Fig. 8, lanes 1 and 3). Although this latter RNA does not assemble into functional splicing complexes, like the *dsx* antisense and *dsx* $\Delta$ E transcripts, it associates weakly with SRm160/300 and DEK, permitting its use for monitoring immunoprecipitation levels (see below; data not shown). In a parallel reaction, an equivalent amount of the internal control RNA was mixed with an exon-product RNA transcribed from the PIP85 cDNA construct (Fig. 8,

lanes 2 and 4). Both reactions were incubated for one hour under splicing conditions before immunoprecipitation with rAb-DEK. Significantly, although a higher level of cDNA-derived exon-product RNA than spliceosome-derived exon-product RNA is present in the total splicing reactions (Fig. 8, compare lanes 1 and 3 with lanes 2 and 4), rAb-DEK only immunoprecipitated efficiently the spliceosome-derived exon-product RNA (compare lanes 7 and 8). This difference was not due to a nonspecific loss of the cDNA-derived exon-product RNA, since approximately equivalent levels of the internal control RNA were immunoprecipitated from both reactions (Fig. 8, compare lanes 7 and 8). As observed earlier (Fig. 3), in contrast to the efficient immunoprecipitation of the spliceosome-derived exon-product complex, rAb-DEK immunoprecipitated a significantly lower level of the lariat-product complex of the splicing reaction. This is consistent with the observation that the majority (80–95%) of exon-product



**Figure 7.** Immunodepletion of DEK does not prevent pre-mRNA splicing. **A**, HeLa nuclear extracts immunodepleted of DEK with rAb-DEK (lane 2) or mock-depleted with preimmune serum (lane 1) were separated on a 12% SDS polyacrylamide gel and immunoblotted with rAb-DEK. Size markers are indicated in kD. **B**, Splicing reactions containing rAb-DEK or preimmune-depleted nuclear extract were incubated with PIP85A pre-mRNA for 1 h. RNA recovered from the splicing reactions was separated on a 15% denaturing polyacrylamide gel.

RNAs generated by splicing are released from the spliceosome during *in vitro* splicing reactions (Le Hir et al., 2000), and also that a relatively low level of DEK remains bound to the lariat-product complex after splicing. Preferential immunoprecipitation of the PIP85A exon-product complex that had been generated by splicing, over the complex formed on the cDNA-derived exon-product RNA, was also observed with antibodies to both subunits of the SRm160/300 splicing coactivator (unpublished observa-

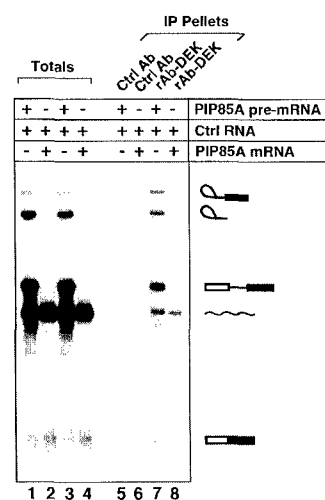
tions). This is in agreement with very recent results showing that SRm160 specifically cross-links to spliced exons dependent on prior splicing (Le Hir et al., 2000; see Discussion), and also our finding in the present study that DEK and SRm160/300 associate. Thus, DEK forms a component of an exon-product complex, the assembly of which requires prior splicing. These results suggest that DEK could function to tag spliced mRNAs as a means to facilitate one or more postsplicing processes.

## Discussion

We have identified the AML-associated protein DEK as a factor that interacts *in vitro* and *in vivo* with SR proteins involved in pre-mRNA splicing. DEK associates efficiently with splicing complexes through interactions mediated by SR proteins. Its association with a pre-mRNA is stimulated by the presence of an ESE that binds to SR family and SR-related proteins. Moreover, its association with a pre-mRNA is stimulated by the addition of purified SR proteins to an SR protein-depleted reaction. After splicing, DEK remains associated preferentially with exon-product complexes. Importantly, this interaction requires prior splicing since DEK does not associate with an exon-product RNA that has not been generated by splicing. The results indicate that interactions involving DEK may distinguish a transcript that has been through splicing from a transcript that has not. Moreover, they suggest that DEK could function to communicate between splicing and one or more steps downstream in gene expression.

Increasing evidence indicates that the nuclear history of a transcript can influence its subsequent metabolism. For example, it has been demonstrated in many studies that the presence of an intron is important for efficient gene expression. These intron-mediated effects have been attributed to influences on mRNA stability, transport and translation. For example, prior splicing is important for translation-dependent degradation of mRNA by the nonsense-mediated decay (NMD) pathway (reviewed by Maquat, 1995, 1996; Hentze and Kulozik, 1999). In a recent study, it was shown that an exon-product RNA generated by splicing assembles into a complex that is significantly larger and more efficiently exported to the cytoplasm than the complex formed on the equivalent RNA synthesized from an intron-less transcript (Luo and Reed, 1999). The presence and location of an intron, whether at the 5' or 3' end of a transcript, can also influence its translation in the cytoplasm. For example, *Xenopus* maternal mRNAs transcribed *in vivo* are normally translationally masked, but can be relieved from translational repression by the insertion of an intron at the 5', but not at the 3' end, of a transcript (Matsumoto et al., 1998). All of the above observations indicate the existence of factors that package an mRNP dependent on its passage through the spliceosome and that modulate downstream steps in gene expression. However, the factors that specifically tag mRNPs dependent on prior splicing are not known.

hnRNPs such as hnRNP-A1 are candidate factors for influencing the composition and activity of an mRNP dependent on prior splicing. HnRNP-A1 associates with nascent pre-mRNA, influences splicing activity, and associates



**Figure 8.** Association of DEK with spliced exons requires prior splicing. Immunoprecipitations were performed with rAb-DEK from splicing reactions incubated for 1 h containing PIP85A pre-mRNA or a PIP85A exon-product RNA transcribed from a PIP85A cDNA construct. Both reactions also contained an internal control RNA for recovery (indicated by a wavy line; see Results). RNA recovered after immunoprecipitation with rAb-DEK (lanes 7 and 8) or control serum (lanes 5 and 6) was separated, along with the corresponding total samples (lanes 3 and 4, and 1 and 2, respectively), on a 15% denaturing polyacrylamide gel.

splicing activity, and associates

with spliced mRNA in the nucleus and cytoplasm; it has also been detected in association with translationally engaged mRNA (Mayeda and Krainer, 1992; Visa et al., 1996). However, neither hnRNP-A1 or other hnRNP proteins have been detected in purified spliceosomes and it is also not known whether their association with mRNPs is dependent on prior splicing. In contrast, it has been demonstrated that specific SR proteins, including the SRm160/300 splicing coactivator subunits and ASF/SF2, associate with splicing complexes through both steps of the splicing reaction and remain preferentially bound to the exon-product RNA (Blencowe et al., 1998, 2000; Hanamura et al., 1998). Moreover, a *Chironomus tentans* SR family protein, hrp45, which is related to mammalian ASF/SF2, associates with intron-containing Balbiani ring transcripts and remains bound to spliced mRNP until the time the mRNP is translocated through the nuclear pore complex, upon which it appears to be released (Alzhanova-Ericsson et al., 1996). Very recently, Le Hir and colleagues have used an elegant cross-linking strategy to detect proteins that cross-link at or near spliced exon junctions, dependent on prior splicing. In this study, splicing-dependent cross-links were identified for SRm160, the U5 snRNP protein hPRP8/p220, and several unidentified proteins (Le Hir et al., 2000). The results in the present study are consistent with these findings. In particular, since the association of DEK with pre-mRNA is promoted by interactions with SR proteins, it is possible that its association with spliced exons is mediated by SRm160. The results further suggest that interactions involving factors that define mRNP composition occur as early as the commitment stage of splicing complex formation, when U1 snRNP and SR family proteins bind to the pre-mRNA and facilitate the association of factors including SRm160/300 and DEK with the substrate (Blencowe et al., 1998; present study).

The results of the present study, taken together with a previous finding indicating that DEK associates with the *pets* site within the HIV-II promoter (Fu et al., 1997; see Introduction), reveals an interesting relationship with a growing number of factors that are associated with both RNA processing and malignant transformation. For example, it has been reported that certain isoforms of the Wilm's tumor protein (WT-1) bind to DNA at specific promoter sequences and concentrate within transcription factor domains in vivo, whereas other isoforms specifically associate with splicing components, including snRNPs and the 65-kD subunit of the U2 snRNP auxiliary factor (U2AF-65), and concentrate within splicing factor domains in the nucleus (Larsson et al., 1995; Davies et al., 1998). In another case, the oncoprotein TLS/FUS, which becomes fused to the CHOP protein in liposarcomas, or to the ERG protein in a subset of myeloid leukemias, binds to both DNA and RNA and has recently been identified as hnRNP-P2, a component of hnRNP complexes (Calvio et al., 1995). Intriguingly, also similar to DEK, it has been reported that TLS/FUS interacts with SR protein splicing factors (Yang et al., 1998). Another factor with dual RNA and DNA binding properties is the Ets-related transcription factor Spi-1/PU.1. This factor, besides its role as a hematopoietic-specific transcription factor, has been reported to interact with both TLS/FUS and the polypyrimidine tract-binding protein NonO/p54nrb, and to influence

splicing activity (Hallier et al., 1996, 1998). NonO/p54nrb, which also has the ability to bind to both DNA and RNA, and a closely related protein, PSF (the polypyrimidine binding protein-associated splicing factor), were both identified recently in fusions with the transcription factor TFE in papillary renal cell carcinomas (Clark et al., 1997). Thus, all of the RNA processing-associated factors implicated in different human malignancies to date, like DEK, have associations with transcription components, as well as with pre-mRNA processing. While the significance of these similar features is not known, it is tempting to speculate that malignancies involving alterations to DEK and these other proteins could affect functions in transcription, RNA processing, or perhaps interactions that coordinate these steps in gene expression (see Introduction). However, it is also possible that AMLs involving the DEK/CAN fusion involve an alteration to the normal function of CAN (Fornerod et al., 1995). Further work will be required to determine the normal function of DEK and whether its function is altered by fusion to CAN in AML.

The results in the present study suggest that DEK has a function in association with pre-mRNA metabolism. In particular, its association with spliced exons dependent on prior splicing represents one of the first examples of a protein with this property. It is possible that this splicing-dependent association is important for facilitating one or more steps downstream in gene expression that are influenced by the prior removal of an intron in pre-mRNA. Thus, based on the previous and present findings, DEK is a candidate for mediating interactions between transcription and pre-mRNA processing, as well as between splicing and one or more subsequent splicing-dependent steps in the gene expression pathway.

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REPLY TO  
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26 Nov 02

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