Heterogeneous Ribonucleoprotein A1 Is Part of an Oncogenic Signaling Pathways

Nathalie Matter‡§, Manuela Marx‡§, Susanne Weg-Remers‡¶, Helmut Ponta‡, Peter Herrlich‡¶, and Harald König‡**

From the ‡Forschungszentrum Karlsruhe, Institut für Toxikologie und Genetik, and Universität Karlsruhe, Institut für Genetik, Postfach 3640, 76021 Karlsruhe, Germany

Regulation of alternative pre-mRNA splicing, recognized as increasingly important in causing human disease, was studied using the CD44 gene, whose splice variants have been implicated in tumor progression. We identified heterogeneous ribonucleoprotein (hnRNP) A1 as a protein interacting in vitro and in vivo with regulatory splice elements in CD44 variant exon v5. Transient overexpression of hnRNP A1 prevented v5 exon inclusion, dependent on the exonic elements. HnRNP A1-dependent repression was exon-specific and could be relieved by coexpression of oncogenic forms of Ras and Cdc42. The results define hnRNP A1 as a decisive part of an oncogene-regulated splice-silencing complex, which can select between multiple alternatively spliced exons.

Alternative pre-mRNA splicing, an important mechanism of differential gene expression in higher eukaryotes, gives rise to functionally distinct proteins encoded by a single gene. The generation of alternatively spliced mRNAs can be regulated, e.g. according to the developmental or physiological state of cells in an organism (for recent reviews see Refs. 1 and 2). Pre-mRNA splicing is accomplished by large ribonucleoprotein complexes called spliceosomes, which are assembled on pre-mRNA, directed by small conserved sequences localized at the intron ends, the splice sites (3). Several alternatively spliced pre-mRNAs have been shown to contain regulatory sequences, in addition to the splice sites, that can affect splice site selection dependent on developmental cues. Such sequences have been found in exons and introns, both in Droso phila and mammalian pre-mRNAs (reviewed in Refs. 2–4). In Droso phila, sex-specific splice regulators have been cloned by genetic approaches (4–6). In contrast, in mammalian cells, factors that govern alternative splicing during differentiation processes or in pathological conditions are largely unknown. Likewise, the mechanisms underlying the regulated generation of complex splice patterns due to selection between multiple alternatively spliced exons, a hallmark of many genes encoding highly variable proteins, are still elusive. Selection of splice sites in mammalian cells has been studied primarily by biochemical means. Two groups of proteins have been found that can affect splice site choice: serine/arginine-rich (SR) proteins (for reviews on SR proteins and splicing, see Refs. 7–10) and heterogeneous nuclear ribonucleoproteins (hnRNP) (for reviews, see Refs. 11–13).

Splice site selection plays an important role in human diseases (14–18), and alternative splicing of several genes has been implicated in tumorigenesis and tumor progression (15, 19 and references therein). The best known example of these genes encodes the cell surface molecule CD44 (for reviews on CD44 splicing and cancer, see Refs. 20 and 21). Alternative pre-mRNA splicing generates variant CD44 isoforms by the inclusion of up to 10 variant exons (v1–v10) during embryonic development, upon activation of lymphocytes (22–25) and of dendritic cells (26) and during tumor progression (20, 21, 27). We showed previously by cell fusion experiments the existence of trans-acting factors regulating the expression of CD44 isoforms in different cell types and in tumor cells (28). In addition, using a minigene containing CD44 exon v5, an exon sequence frequently included in mRNA upon activation of immune cells and during tumor progression, we could identify splice regulatory RNA elements for this exon. These elements are located within the v5 exon coding sequence and are necessary for cell type-specific exon inclusion as well as for inclusion of the exon in response to phorbol-ester tumor promoters or oncogenic Ras (29).

Here we show that the splice regulatory elements of exon v5 are recognized by hnRNP A1 and that hnRNP A1, dependent on these elements, represses inclusion of the exon in vivo. Silencing of exon inclusion by hnRNP A1 is exon-specific and is relieved by oncogenic forms of the small G proteins p21Ras and Cdc42. These findings define hnRNP A1 as a decisive component of an oncogene-controlled splice-silencing complex, which can select between multiple alternatively spliced exons.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

CB3 erythroleukemia cells (30; kindly provided by Yaacov Ben-David) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM t-glutamine, and 10 μM β-mercaptoethanol. S194 mouse plasmacytoma cells (obtained from Thomas Wirth), KLN205 mouse carcinoma cells

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‡ Both authors contributed equally to this work.

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** To whom correspondence should be addressed. Tel.: 49-7247-82-3293; Fax: 49-7247-82-3354; E-mail: harald.koenig@itg.fzk.de.

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The abbreviations used are: SR, serine/arginine-rich protein; hnRNP, heterogeneous ribonucleoprotein; RT-PCR, reverse transcriptase-polymerase chain reaction; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK1, MEK kinase 1; bp, base pair(s); kb, kilobase(s); RNAP, RNA-affinity precipitation; TrT, tropolone T; JNK, c-Jun NH2-terminal kinase; PKCζ, protein kinase Cζ; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ERK, extracellular signal-regulated kinase.
hnRNP A1 in Regulated Alternative Splicing of CD44

(ATCC CRL-1453), and NIH-3T3 cells (ECACC CB2435) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All cells were grown at 37 °C and 6% CO₂.

Transfections were performed using the polycationic reagent SuperFect (Qiagen) according to the instructions of the manufacturer. Unless stated otherwise, in the cotransfection experiments analyzed by RT-PCR, 2 μg of CD44 v5 minigene-plasmid DNA were cotransfected with 8 μg of hnRNP A1 expression plasmid or empty expression plasmid. For analysis of hnRNP A1 expression levels in cotransfections, 8 μg of expression plasmid for myc-tagged hnRNP A1 was used. Where indicated, 3 μg of expression constructs for activated Ras, Cdc42, MEKK1, or corresponding control constructs were transfected. Transfection efficiency of NIH-3T3 cells was assessed by transfecting cells with pCMVSPORT-βgal (Life Technologies, Inc.) followed by X-gal staining.

Plasmid Constructions

CD44 v5 Minigene Constructs—The CD44 v5 minigene pETv5 and the mutants ΔBlue, ΔBluev, and v5β87α have been described previously (29). The v5ΔR(TnT2), construct was generated by cloning a dimer of the oligonucleotides CGCGGAGAGAGAA (upper strand) and CGCGTTGTCTCTCTC (lower strand), spanning a 9-bp exonic enhancer element of the cardiac troponin T exon 5 (31), into the MluI site of pETv5ΔR (29). The minigene containing expression constructs for activated Ras, Cdc42, MEKK1, or corresponding control constructs were transfected. Transfection efficiency of NIH-3T3 cells was assessed by transfecting cells with pcMVSPORT-βgal (Life Technologies, Inc.) followed by X-gal staining.

RNA-affinity Precipitation and Western Blotting

RNA oligonucleotides were synthesized with two 5’-biotin residues (Eurogentec Bel S.A.) and had the following sequences: L, AUAAGCA- GAAGACCGACAGCGUCAUGAGAAAAUGGACC; M, CCAGCC- CAGCCGGCUGCUUUAACAAACGAGUACGG; R, GAGCCA- UAGAGGAAAGAGGAGAACCCAUCAUGACAGCAA; Blue, GGAGUUGAUUGGUUAUGAUGGUCGCAAGCCCGCGCUGG.

For RNA precipitation assays, nuclear extracts (120 μg for silver staining, 60 μg for Western blotting) were prepared with 0.5 μl of UltraLink-NeutrAvidin beads (Pierce) per μg of extract at 4 °C for 45 min. Precleared extracts were incubated in binding buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 0.025% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, 2 mM aprotonin, 2 mM leupeptin) with 1.2 μg of yeast tRNA and 4 units of RNase inhibitor (RNasin, Promega) per μg of extract at 30 °C for 10 min. 6.5 ng of biotinylated RNA oligonucleotide per μg of extract was added, and the reactions were further incubated at 30 °C for 15 min. After washing the beads four times with binding buffer without glycerol, precipitated complexes were resolved by SDS-polyacrylamide gel electrophoresis and either subjected to silver staining or transferred to a polyvinylidene difluoride membrane (Millipore) followed by Western blotting. Western blot analysis was performed using standard procedures involving the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) using either 1:1-diluted tissue culture supernatant of mAB104 hybridoma (ATCC CRL-2067), to detect SR proteins, or 1:1000-diluted ascites fluid of monoclonal antibodies 4B10 and 4F4 (kindly provided by Gideon Dreyfuss) to detect hnRNP A1 and hnRNP C proteins, respectively.

For analysis of transiently expressed myc-tagged hnRNP A1 (in Fig. 6B) the monoclonal antibody 9E10 (Santa Cruz Biotechnologies) recognizing the human c-myc-epitope EQKILISEEDL was used, followed by ECL detection.

Yeast Three-hybrid Analysis

The yeast three-hybrid analysis was performed in Saccharomyces cerevisiae strain L40uraMS2 (Mat a, ura3-52, leu2-3112, his32200, trp1A1, ade2, LYS2::LexA op) 4-HIS3, ura3::(LexA-op)8-LacZ) carrying pLexA/MS2/Zeo (Zeocin®). This strain is derived from L40 coat described previously (35). All plasmids used were amplified in Escherichia coli strain DH5α. The hybrid-RNA expression constructs and the constructs expressing the transcription-activation domain proteins, or the transcription domain only, were cotransformed into S. cerevisiae L40uraMS2 cells. Transformants were grown on synthetic complete medium lacking uracil and tryptophan, and his3 auxotrophic screening using the URA3, TRP1, and HIS3 marker genes. Growing colonies were analyzed further for LacZ expression using a filter lift assay to detect β-galactosidase activity by X-gal staining (36).

RESULTS

hnRNP A1 Binds to CD44 v5 Exon Sequences in Vitro and in Vivo—We have previously identified exon sequences in CD44 variant exon 5 that account for exon inclusion or exon skipping in different cell lines and upon Ras signaling (29). The sequences form an exonic composite splice regulatory unit encompassing an exon recognition element and splice silencer elements in the 3’ (or right) part of the exon, R, and splice silencer domains in the 5’ (left) part, L, and in the middle portion, M, of the exon (29; see Fig. 1A). Both types of elements are necessary for regulated cell type-specific and -inducible inclusion of the exon. In a T-cell line equipped with a v5 exon-carrying mini-gene, concanavalin A, phorbol-ester tumor promoters, or expression of oncogenic p21Ras induced exon inclusion (29).

To identify and characterize the nuclear protein complexes related to the exonic elements, we set up RNA-affinity precipitations (RAP) using biotinylated RNA oligonucleotides corresponding to the subdomains L, M, and R of the CD44 v5 regulatory splice unit (see Fig. 1A). As a control for nonspecific binding to the matrix or for unspecific RNA binding, the oligonucleotide was omitted from the precipitation reaction, or precipitates were performed.
with a control oligonucleotide, "blue." The blue oligonucleotide equals the L, M, and R oligonucleotides in length (42-mer) but is derived from an unrelated polylinker sequence (from the pBluescript vector, Stratagene). The blue sequence in place of either one of the regulatory subdomains in the CD44 v5 exon abolishes their regulatory effects in vivo (29). Upon silver staining of the RNA-affinity precipitates separated by SDS-polyacrylamide gel electrophoresis (Fig. 1B), several protein bands were detectable that were specifically bound to the CD44 v5 exon sequences. Of these proteins, some bind to all three exon sequences (L, M, and R), whereas others bind only to one sequence (Fig. 1B).

In an approach to identify the proteins in the RNA-affinity precipitates, we screened the separated proteins by immunoblotting for the presence of two groups of proteins, which can affect splice site selection in mammalian cells: SR proteins and hnRNPs. Using an antibody recognizing a conserved phosphoepitope on all SR protein family members (mAb104 [37, 38]), we could not detect SR proteins in the precipitates (data not shown). The screen for hnRNP proteins, however, was successful. Although there were no hnRNP C proteins detectable in the precipitates (not shown), each of the three regulatory subdomains of the v5 exon, L, M, and R, were bound to hnRNP A1 (Fig. 1C, lanes 3–5). No hnRNP A1 binding was detectable with the matrix alone (lane 1) or with the unrelated blue oligonucleotide (lane 2), suggesting a specific association of hnRNP A1 with the CD44 v5 exon sequences.

To exclude that the interaction of hnRNP A1 with v5 sequences was merely an in vitro artifact, we employed a yeast three-hybrid system in which the binding of a bifunctional hybrid-RNA to each of two hybrid proteins activates transcription of reporter genes in yeast [35; see Fig. 2A]. The yeast strain employed, L40uraMS2, contains a HIS3 gene and a lacZ gene under the control of lexA operator sites. In addition, it expresses the first hybrid protein in which the bacterial LexA DNA-binding domain is fused to the coat protein of the bacteriophage MS2 that recognizes an RNA-stem loop structure in MS2 RNA. For the expression of the second hybrid protein we generated a construct (pYESTrp2-A1) that gives rise to an hnRNP A1 protein fused to the B42 transcripational activation domain. As hybrid-RNA supposed to bridge the two fusion proteins, we used the entire CD44 v5 exon sequence plus approximately 30 bp of upstream and downstream intron sequences fused to two tandemly repeated bacteriophage-MS2 RNA recognition sites (pRH5'-v5). A construct in which the v5 exon sequence (except for the four 5' most and the three 3' most nucleotides) had been replaced for a pBluescript-polylinker sequence, served as a negative control (pRH5'-blue).

Co-transformation of the MS2-v5 hybrid-RNA-encoding plasmid and the B42-hnRNP A1 expression construct (pRH5'-v5+pYESTrp2-A1) resulted in L40uraMS2 yeast cells, which could grow on histidine-deficient plates (Fig. 2B) and which expressed LacZ, as shown by X-gal staining (Fig. 2C). The result resembled that obtained using the positive control: MS2-Ire (iron responsive element) hybrid-RNA and B42-Irp (iron regulatory protein) fusion protein (pRH3'-IRE+pYESTrp2-Irp). In contrast, co-transformation with the empty B42-expression vector and the MS2-v5 hybrid-RNA construct failed to give rise to cells that could grow in the absence of histidine and that expressed LacZ (pRH5'-v5+pYESTrp2; Fig. 2, B and C). Similarly, co-transformation of the B42-hnRNP A1 expression construct together with the control hybrid-RNA construct, in which the v5 sequence had been replaced by the Bluescript-polylinker sequence, failed to generate HIS prototrophy or LacZ positive cells (pRH5'-blue+pYESTrp2-A1; Fig. 2, B and C). The results indicate that activation of the yeast reporter genes was dependent on the presence of both the B42-hnRNP A1 fusion and the CD44 v5 exon sequences in the hybrid-RNA, strongly suggesting that hnRNP A1 interacted with v5 exon sequences in vivo.

**HnRNP A1 Represses Inclusion of Exon v5 in Vivo**—To examine whether the interaction of hnRNP A1 with CD44 exon

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**Fig. 1.** HnRNP A1 is part of protein complexes bound to CD44 v5 exon sequences in vitro. A, schematic representation of CD44 exon v5 and its regulatory subdomains. Subdomain L and M act as splice silencer elements (-) and prevent exon usage in cells or in conditions in which the exon is not used. Subdomain R contains splice enhancer elements (+), necessary for exon recognition, and exon silencer elements (-) [29]. B, silver staining of RNA-affinity precipitates from nuclear extracts of S194 mouse plasmacytoma cells separated by SDS-polyacrylamide gel electrophoresis. For precipitations, biotinylated 42-mer RNA oligonucleotides corresponding to the regulatory elements were employed. CD44 exon v5 and its regulatory subdomains indicated (L, M, and R). L40uraMS2, a construct, in which the v5 sequence had been replaced by the Blue-v5). A construct in which the v5 sequence had been replaced for a pBluescript-polylinker sequence, served as a negative control (pRH5'-blue).
FIG. 2. In vitro binding of hnRNP A1 to CD44 exon v5 in a yeast three-hybrid system. A, scheme of the experimental set-up. To test for the interaction of hnRNP A1 and v5 exon sequences, a fusion protein between hnRNP A1 and a transcriptional transactivation domain (TAD) was coexpressed in the yeast strain L40uraMS2 together with a bifunctional hybrid-RNA. The hybrid-RNA consisted of the CD44 v5 exon sequence and two tandemly repeated 21-nucleotide RNA recognition sites for the coat protein of bacteriophage MS2 (MS2-v5 hybrid-RNA). The L40uraMS2 strain contains the HIS3 gene and the lacZ reporter gene (black box) under the control of lexA-operator sites (LexA op). In addition, it expresses a hybrid protein in which the bacterial LexA DNA-binding domain (LexA DBD) is fused to the coat protein of bacteriophage MS2 (MS2 coat protein). Binding of the bifunctional hybrid-RNA to each of two hybrid proteins will activate transcription (indicated by arrow) of the reporter genes by recruiting the transcriptional activation domain to the LexA-operator sites in the promoter. B, L40uraMS2 yeast cells cotransformed with the expression constructs indicated plated on histidine-deficient plates. C, detection of lacZ expression in L40uraMS2 yeast cells cotransformed with the indicated expression constructs. Yeast cells were grown on histidine-deficient plates followed by transfer to nitrocellulose filters and X-gal staining. pRH5-v5, expression vector for MS2-v5 hybrid-RNA; pRH5-blue, expression vector for control hybrid-RNA in which the v5 exon sequence (except for the four 5'-most and the three 3'-most nucleotides) had been replaced for a pBluescript-polylinker sequence; pYESTrp2-A1, expression construct that gives rise to an hnRNP A1 protein fused to the B42 transcriptional transactivation domain; pYESTrp2, empty expression construct, expresses transactivation domain only. As a positive control to check the system in our hands, we used constructs expressing a B42-IRP (iron regulatory protein) fusion (pYESTrp2-IRP) and a MS2-IRE (iron-responsive element) hybrid-RNA (pRH3-IRE), respectively.

v5 was functionally relevant, we transiently overexpressed hnRNP A1 and determined the behavior of a CD44 v5-minigene splice reporter. The minigene construct contains the murine CD44 v5 exon and adjacent intron sequences between preproinsulin exons 2 and 3 and their corresponding intron sequences. This construct had previously been shown to recapitulate cell type-specific and -inducible alternative splicing of the endogenous CD44-v5 exon (29). Because hnRNP A1 is an abundant protein in most cell types (39), we reasoned that it could be difficult to achieve overexpression. We therefore used the mouse erythroleukemia cell line CB3, which, due to loss of one hnRNP A1 allele and a proviral integration 3' to the coding sequence of the second allele, shows no detectable expression of hnRNP A1 protein (30, 40; and Fig. 3A). After cotransfection of the v5 minigene (for scheme, see Fig. 3B) with the empty expression vector, the majority of the minigene-derived mRNA included CD44 exon v5 (indicated by the larger PCR product of 361 bp; Fig. 3C, lane 1). Cotransfection with increasing amounts of an hnRNP A1 expression vector, however, led to exon skipping in a dose-dependent manner (Fig. 3C, lanes 2 and 3). An expression construct carrying the hnRNP A1 cDNA in antisense orientation did not repress v5 exon inclusion (Fig. 3C, lane 4), suggesting that expression of hnRNP A1 protein is required for splice silencing. Expression of the antisense cDNA reproducibly enhanced v5 exon inclusion slightly. This result could be explained by the residual expression of hnRNP A1 mRNA in CB3 cells (40). Surprisingly, inhibition of exon 5 v5 exon inclusion by hnRNP A1 was not confined to the hnRNP A1-deficient CB3 erythroleukemia cells but could also be shown in NIH-3T3 murine fibroblasts and in KLN205 mouse carcinoma cells. Although endogenous hnRNP A1 was easily detectable in both cell lines (see Fig. 3A), they showed predominantly inclusion of exon v5 in the minigene mRNA in the absence of additional hnRNP A1 (Fig. 3D, lanes 1 and 3). Transient overexpression of hnRNP A1 shifted the splice pattern to skipping of the v5 exon (Fig. 3D, lanes 2 and 4). To estimate the level of hnRNP A1 overexpression, we transfected NIH-3T3 cells with an epitope-tagged version of hnRNP A1. By Western blotting of cell extracts using an anti-hnRNP A1 antibody, we could determine the amount of epitope-tagged hnRNP A1 (which has a higher molecular weight due to the epitope tag) to be 35% of the endogenous hnRNP A1 level (data not shown). Taking into account the transfection efficiency of the cells under these conditions (13% cells transfected), it can be estimated that there is an approximately 3-fold overexpression of hnRNP A1 in the transfected cells. The repression of exon inclusion by overexpressed hnRNP A1 suggests that the endogenous levels of hnRNP A1 (and/or its functional state) in the two cell lines do not suffice for repression of v5 exon usage in the minigene pre-mRNA and that repression of CD44 v5 exon inclusion by overexpression of hnRNP A1 is not a peculiarity of the CB3 erythroleukemia cells.

hnRNP A1-dependent Exon Silencing Shows Exon Specificity—Using different combinations of variant exons, a multitude of variant CD44 isoforms can be generated under physiological conditions and in cancer (21). To assess whether hnRNP A1 is a general silencer of variant CD44 exons, or whether it regulates the usage of certain variant exons selectively, we used similar minigene constructs carrying either one of three other CD44 variant exons. When testing their splice patterns under the influence of hnRNP A1, inclusion of exon v6 was repressed similarly to exon v5 (Fig. 4, lanes 1 and 2, and lanes 5 and 6), whereas inclusion of exons v4 (Fig. 4, lanes 3 and 4) and v7 (Fig. 4, lanes 7 and 8) were not, indicating that hnRNP A1 does not cause general skipping of variant exon sequences. Rather, hnRNP A1-mediated splice silencing is exon-specific.

Repression of CD44 v5 Exon Inclusion Requires the Exonic Sequence Elements to Which hnRNP A1 Binds in Vitro—HnRNP A1 could be shown to associate with RNA sequences of CD44 exon v5 in vitro and in vivo (see Figs. 1 and 2). Moreover,
hnRNP A1 can be cross-linked by UV irradiation to these sequences (data not shown). Thus, exon specificity in hnRNP A1-dependent splice silencing could be the result of sequence preference in RNA binding of hnRNP A1 itself or of an hnRNP A1-containing protein complex. To verify that exon silencing of the v5 exon by hnRNP A1 depended on the exon sequences to which it binds in vivo, we made use of minigene constructs containing mutant v5 exons. In the mutants, either the L or M exonic silencer subdomains or both had been replaced by blue poly linker sequences of the same length (29; see Fig. 5A). HnRNP A1 did not bind to the polylinker sequences either in vitro or in vivo (see Figs. 1 and 2). When we cotransfected the minigene constructs into NIH-3T3 cells together with the empty expression plasmid, the mutant v5 exon sequences were included in the minigene mRNA to a slightly higher level than empty expression construct (pcDNA3-A1) orientation, in the amounts indicated. In the case where 2 μg of pcDNA3-A1 was cotransfected, the total amount of pcDNA3-based DNA was brought to 8 μg with the empty pcDNA3 plasmid. Numbers on the left indicate sizes (in base pairs) of DNA marker bands (lane M). D, RT-PCR analysis of CD44 v5-minigene RNA in NIH-3T3 murine fibroblasts and KLN205 mouse carcinoma cells after cotransfection with 2 μg of pETv5-minigene plasmid and 8 μg of either the hnRNP A1 expression construct (pcDNA3-A1) or the empty expression vector (pcDNA3). Results of a typical experiment are shown, values for exon inclusion (% exon incl; expressed as percentage of v5-containing RT-PCR products relative to total RT-PCR products) are boxed.

hnRNP A1 is dependent on v5 exon sequences. A, schematic illustration of mutant CD44-v5 exons in the minigenes used for cotransfections in B. Open boxes, regulatory subdomains L, M, and R of CD44 exon v5; grey boxes, unrelated sequence (blue) derived from the polylinker of the Bluescript plasmid (Stratagene), used to replace v5 exon sequences; hatched boxes, dimerized 9-bp sequence corresponding to purine-rich enhancer element from exon 5 of the cardiac troponin T gene (31). B, RT-PCR analysis of NIH-3T3 cells transiently cotransfected with minigenes containing the mutant CD44-v5 exons shown in A and either an empty expression construct (-A1) or a construct expressing hnRNP A1 (+A1). The figure shows the results of a typical experiment. Values for exon inclusion (% exon incl; expressed as percentage of v5-containing RT-PCR products relative to total RT-PCR products) are boxed.
could not be examined by an R domain replacement mutant. We could ask, however, whether the silencer domains L and M were sufficient to mediate hnRNP A1 repression if subdomain R was replaced by a different splice enhancer, or whether sequences specific to the R subdomain contributed to repression. With this reasoning, we generated a minigene construct in which the R subdomain of exon v5 had been replaced by a duplicated 9-bp purine-rich exon enhancer sequence from the cardiac troponin T (TnT) alternative exon 5 (31). Although the TnT sequences led to a lower level of exon inclusion (76.2%) than that of the wild-type v5 exon (90.7%), hnRNP A1 could inhibit exon inclusion of the chimeric exon only by 30.2% (Fig. 5B, lanes 9 and 10), compared with 50.5% in the case of the wild-type exon (lanes 1 and 2). This result argues in favor of a contribution of R sequences to repression of v5 exon usage by hnRNP A1.

The results obtained by the v5 exon mutants thus indicate that hnRNP A1-dependent repression of v5 exon inclusion depends on all three regulatory subdomains of the exon and strongly suggest that the binding of hnRNP A1 to these sequences is crucial for exon skipping.

HnRNP A1-dependent Exon Silencing Is Relieved by Oncogenic Cell Signaling—We previously found that constitutively activated Ras is capable of activating inclusion of CD44 exon v5 in RNA of a T-lymphoma cell line (29). This link between oncogene action and up-regulation of CD44 variant exon inclusion may be related to the generation of CD44 variant isoforms during tumor progression. Concerning the action of Ras on splicing, an obvious question was whether repression of v5 exon inclusion by hnRNP A1 would be sensitive to Ras signaling. We performed transient three-factor cotransfections using the CD44-v5 minigene, the hnRNP A1 expression construct, and either an expression vector for activated Ha-Ras or a corresponding control plasmid. Cotransfection into CB3 erythroblasts cells of the minigene with the hnRNP A1 expression construct and the control plasmid led, as expected, to skipping of the v5 exon sequence (Fig. 6A, compare lanes 1 and 3). In contrast, cotransfection with the Ras expression construct largely prevented the silencing effect of hnRNP A1 (see Fig. 6A, lane 4). Surprisingly, similar experiments performed with NIH-3T3 and KLN205 cells showed only a marginal Ras effect (data not shown). One possibility is that Ras cannot efficiently induce the same effectors pathways in NIH-3T3 and in KLN 205 cells as it does in CB3 cells, and it may not counteract the higher hnRNP A1 levels in these cells. Interestingly, however, constitutively active mitogen-activated protein/ERK kinase kinase 1 (MEKK1) (Fig. 6A, lanes 6 and 7) and an activated version of the small GTP-binding protein Cdc42 (Fig. 6A, lanes 8 and 9) reverted repression of v5 exon inclusion by hnRNP A1. A similar signaling-induced relief of hnRNP A1-dependent silencing was observed for the v6 exon (data not shown). MEKK1 and Cdc42 have in common their ability to activate the Jun-kinase (JNK) pathway (41, 42), and both molecules can be activated by Ras signaling (43). To exclude an effect of the signaling molecules on the expression level of hnRNP A1, we cotransfected an epitope-tagged version of hnRNP A1. Coexpression of Ras, MEKK1, or Cdc42 did not down-regulate the amount of hnRNP A1 protein (data not shown for Ras, and Fig. 6B).

We conclude that oncogene-driven signaling pathways antagonize the activity of an exonic splice-silencing complex in which hnRNP A1 plays a decisive role.

**DISCUSSION**

Variant CD44 isoforms are generated by alternative pre-mRNA splicing and are involved in a variety of physiological and pathological processes, among which tumor progression has received most attention. Despite this attention, the splice factors responsible for the regulation of alternative splicing have not been identified.

This paper describes a component, hnRNP A1, of an exon-specific splice-silencing complex, which is inactivated by signal transduction from Ras, MEKK1, or Cdc42. HnRNP A1 is shown here to bind to exonic regulatory sequences in CD44 exon v5 in vitro and in yeast cells in vitro. Transient overexpression of hnRNP A1 repressed recognition of the v5 exon in three different cell lines. The finding that exon v5 in both NIH-3T3 cells and KLN205 carcinoma cells was included despite abundant endogenous hnRNP A1, suggests that either the endogenous levels of hnRNP A1 do not suffice to efficiently repress exon inclusion from the v5-minigene pre-mRNA, or that the repressing activity of hnRNP A1 is down-regulated in these cells, e.g.
through post-translational modification. Transient overexpression of hnRNP A1 could exhaust modifying enzyme systems in these cells and thus lead to a pool of hnRNP A1 active in splice silencing. The 3-fold overexpression determined in NIH-3T3 cells is well within the range of physiological differences in hnRNP A1 protein levels observed between different tissues (44).

HnRNP A1 has previously been described as a factor that can antagonize selection of alternative 5'- and 3'-splice sites by certain SR proteins in vitro and in vivo (40, 45-48). Similarly to the hnRNP A1-dependent exon silencing of CD44 variant exons described in this paper, the antagonistic effect of transiently overexpressed hnRNP A1 on 5'- and 3'-splice site selection could be shown in cell lines expressing already high levels of endogenous hnRNP A1 protein (47, 48). Very recently, two other examples of hnRNP A1 binding in vitro to negative regulatory exon sequences have been described: to the human fibroblast growth factor receptor 2 K-SAM exon and to human immunodeficiency virus tat exon 2 (49, 50). In addition, intronic hnRNP A1-binding sites causing exon skipping have been found in pre-mRNA of hnRNP A1 (51). In the case of human immunodeficiency virus tat exon 2, hnRNP A1 was capable of inducing skipping of the exon in an in vitro splicing system (50). Recruitment of a MS2-hnRNP A1 fusion protein to a K-SAM exon carrying an MS2 recognition site resulted in exon skipping in transient cotransfection experiments (49). However, hnRNP A1 could not be shown to repress exon inclusion via the authentic hnRNP A1-binding exon element identified in vitro.

Interestingly, the silencing effect of hnRNP A1 could be detected using minigene constructs carrying CD44 exons v5 or v6, but not v4 or v7, thus indicating exon specificity of silencer action. The exon preference of the silencing effect seems to reflect special sequence requirements: inhibition of v5 exon inclusion by hnRNP A1 depends on the presence of exonic silencer sequences in subdomains L and M. The impaired inhibitory effect of hnRNP A1 on exon inclusion upon replacement of subdomain R by a heterologous splice enhancer from the TnT exon 5 could indicate additional sequence elements in subdomain R (harboring also the exon recognition element), contributing to repression of v5 exon inclusion by hnRNP A1. Alternatively, the TnT enhancer element could stimulate splicing through a molecular mechanism distinct from that of the enhancer in R and which could not be susceptible to hnRNP A1-mediated repression. The possibility of additional elements in subdomain R that add to the hnRNP A1 effect is compatible with linker-scan mutation analyses, which revealed silencing elements in the R subdomain of exon v5 (29). A consensus RNA-binding site (UAAGG(A/U)) had been obtained for hnRNP A1 by the SELEX procedure (52). However, there are no elements corresponding to this sequence in CD44 exon v5. One could envisage that hnRNP A1 binds, possibly in association with other partner proteins, through an exon-specific combination of distinct small sequence elements in a cooperative manner. These may be stretched out over the entire exon. Thereby, the silencing complex could displace positively acting factors binding to the exon recognition element within the 3'-part of the exon (subdomain R), or it might interfere with their potential to recruit spliceosomal components to the splice sites upstream and/or downstream of the exon. Sequence comparison of the CD44 variant exons v4 through v7 revealed numerous similarities. These similarities could indicate a common ancestor during exon evolution. In addition to these general similarities, we found stretches of nucleotides that are very similar between exons v5 and v6. However, it is too early to speculate on their significance in exon selection.

The repressing effect of hnRNP A1 on CD44 v5 exon inclusion was abolished in the presence of activated Ras and by dominant active MEKK1 or Cdc42, two molecules that are part of Ras-effector pathways (43). Like Ras, activated forms of Cdc42 have been shown to have transforming potential (53, 54). Furthermore, Cdc42 has been demonstrated to be necessary for Ras transformation in fibroblasts (53) and to induce invasive cell growth (55, 56). Thus, oncogenic cell signaling can interfere with hnRNP A1-dependent exon silencing. This interference could be due to the targeting of hnRNP A1 itself, or of associated or antagonizing splice factors. Phosphorylation of hnRNP A1 has been previously shown to increase in response to platelet-derived growth factor via a pathway requiring protein kinase C ζ (PKCζ) and has been suggested to control cytoplasmic localization of hnRNP A1 (57). This pathway is most likely not involved in relief of exon silencing by Cdc42 or MEKK1, because a constitutively active mutant of PKCζ could not release repression, and Cdc42 and MEKK1 did not induce an increase in hnRNP A1 phosphorylation. Consistent with the possibility that hnRNP A1 itself is a target in signaling-induced relief of exon silencing, we have observed changes in methylation and in phosphorylation of hnRNP A1 in response to MEKK1 or Cdc42 overexpression (data not shown). Whether these changes in modification are relevant for hnRNP A1-silencing function still needs to be explored.

We thus can summarize: hnRNP A1 can function as a decisive component of an exon-selective splice silencer complex. Oncogene-derived signals may converge at this complex, inactivating its function and thus leading to the generation of mRNAs for variant CD44 isoforms in the course of tumor progression. Furthermore, such regulatable and exon-discriminatory complexes could be the basis for controlling the elaborate splice patterns observed in highly complex genes, like CD44.

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