Alternative splicing provides a versatile mechanism of gene regulation, which is often subverted in disease. We have used customized oligonucleotide microarrays to interrogate simultaneously the levels of expression of splicing factors and the patterns of alternative splicing of genes involved in tumor progression. Analysis of RNAs isolated from cell lines derived from Hodgkin lymphoma tumors indicate that the relative abundance of alternatively spliced isoforms correlates with transformation and tumor grade. Changes in expression of regulators were also detected, and a subset sample was confirmed at the protein level. Ectopic expression of neuron-specific splicing regulatory proteins of the Nova family was observed in some cell lines and tumor samples, correlating with expression of a neuron-specific mRNA isoform of JNK2 kinase. This microarray design can help assess the role of alternative splicing in a variety of biological and medical problems and potentially serve as a diagnostic tool.

Microarray technology is purported as a key tool for genomewide analysis of transcriptomes in post-genomic biology (1). Early successes of this technology range from detailed genomewide analysis of gene expression during Drosophila development to the molecular classification of lymphomas (2–4) Classical microarray designs, however, largely ignore sources of transcript heterogeneity such as alternative pre-mRNA splicing, which affects more than 70% of human genes and leads to the generation of multiple mRNAs and protein products from a single primary transcript (5, 6). Alternative splicing is altered in disease, notably in cancer (7).

Pioneering steps have been made to extract information about alternative transcripts from conventional microarrays (8) and to develop microarray platforms specifically designed to detect alternative splicing (reviewed in Ref. 9). These include high resolution coverage of genomic sequence by tiling arrays (10), analysis of global effects associated with defects, in particular, RNA processing factors in yeast using intron-specific and splice-junction probes (11), multiplexed analysis of cancer cell lines on fiber optic arrays (12), and screening for alternative splicing events in human genes and different tissues using splice-junction oligonucleotides (6).

Work in a variety of genes and organisms has led to the hypothesis that the relative expression of general splicing factors in different cell types establishes patterns of alternative splicing in multiple genes (13–15). Tissue-specific regulators have also been found, e.g. the nervous system-specific proteins of the Nova family, which induce neuron-specific patterns of alternative splicing on target genes (16, 17).

Hodgkin lymphomas are malignancies of unknown pathogenesis. Despite their heterogeneous phenotype, cDNA microarrays have revealed the expression of a characteristic set of markers regardless of their B- or T-cell origin (18, 19). There is a need, however, for additional prognostic markers. Results obtained with our microarray design suggest that general patterns of alternative splicing, as well as expression of particular splicing regulators, can be indicative of cell transformation and tumor grade.

EXPERIMENTAL PROCEDURES

Biological Materials—Cell lines (Deutsche Sammlung von Mikroorganismen und Zellkulturen) were cultured in RPMI 1640 medium supplemented with 20% (L-540, HDML-2) or 10% (all other cell lines) heat-inactivated fetal calf serum. Lymph node tumor samples from lymphoma patients were purchased from BioChain (catalog number P1235161A-1, P1235161B-1).

Splicing Factors Data Base—A data base of splicing factors was built from the European Molecular Biology Laboratory (EMBL) Sequence Data base containing constitutive components of the spliceosome (snRNP) proteins and small nuclear RNAs, members of the SNR and hnRN families of splicing regulators, splicing factor kinases, and other RNA-binding proteins (see Fig. 1A). The genes encoding these proteins and RNAs were grouped by families or functions, together with their sequence information and links to orthologous genes in different species (eta.embl-heidelberg.de:8000/cgi/angela_restricted/cgimodel.py?fun=displayGroupFactorPage).

Microarray Analyses—Oligonucleotide microarrays were produced using the Geniom OnER system, Febit AG, Mannheim, Germany (20). Probes for bacterial genes conferring resistance to ampicillin, kanamycin, and chloramphenicol and the Drosophila gene Sex-lethal were used as negative controls. Preparation of biotinylated cRNA was essentially performed in the same manner as described previously (21). For generation of cDNA, total RNA was incubated with 100 pmol of T7-T(24)-primer at 70 °C for 10 min in an 11-μl reaction. cDNA synthesis was carried out in a 20-μl reaction containing 10 mm dithiothreitol, 500 μM dNTPs, 20 units of RNase OUT (Invitrogen), and 300 units of SuperScript II (Invitrogen) for 1 h at 50 °C. Second strand cDNA synthesis was carried out in a 150-μl reaction using the Geniom OneR system, Febit AG, Mannheim, Germany (20).

Received for publication, October 21, 2004
Published, JBC Papers in Press, November 16, 2004, DOI 10.1074/jbc.M411976200

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This paper is available online at http://www.jbc.org

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The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

§ Recipient of a PRAXIS XXI fellowship from the Portuguese Foundation for Science and Technology.

This work was supported in part by grants from Bundesministerium für Bildung und Forschung, European Union FP5, and Human Frontier Science Program Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: snRNP, small nuclear ribonucleoprotein particle; hnRNP, heterogeneous nuclear ribonucleoprotein; JNK, c-Jun NH2-terminal kinase; RT, reverse transcription.
reaction containing 40 units of DNA polymerase I, 10 units of DNA
ligase, 2 units of RNase H, 200 μM dNTP mix for 2 h at 16 °C. After T4
DNA polymerase treatment, phenol/chloroform extraction, and ethanol
precipitation, T7 transcription was carried out for 6 h at 37 °C in a 1 μl
reaction using the AMS Biotech Megascript T7 kit in the presence of
1.88 mM Biotin-11-CTP, 1.88 mM Biotin-16-UTP. The DNA template
was then digested for 15 min at 37 °C with 1 unit of DNase I, and the
labeled cRNA was purified using the RNeasy mini kit (Qiagen). cRNA
fragmentation was achieved by incubating 15 μg of cRNA in 40 mM Tris,
pH 8.1, 30 mM magnesium acetate, 100 mM potassium acetate in a total
volume of 0.6 μl for 35 min at 94 °C. The average fragment length was
100 nucleotides.

Microarrays were hybridized with 15 μg of fragmented cRNA in a
final volume of 20 μl. Detection, processing of raw data including
background correction, array-to-array normalization, determination of
gene expression levels, and calculation of fold change values were
performed as described (20, 21) using data from 13 perfect match/single
mismatch oligonucleotide pairs (for mRNAs and exons) or 11 pairs (for
splice junctions). Tiling arrays (full coverage of a sequence by oligonu-
cleotides offset by one nucleotide) were generated for selected sequence
features. At least six different RNA isoforms were used per cell line to
calculate median values of fold changes (data provided as Supplementary
Materials), which were categorized as up-regulation, down-regula-
tion, or no change based on a 2-fold threshold and the request that at
least 50% of the replicates showed consistent behavior, and less than
30% of the remaining data showed disparate results. Subsequent analy-
ses (including Spearman correlation clustering, gene trees, condition
trees, and analysis of variance analysis) used the Excel and Gene
Spring 6.2 (Silicon Genetics) software programs.

Semiquantitative RT-PCR Assays—For Nova assays, 5 μg of total
to plasmomic RNA, after treatment with RQ1 DNase (Promega), were
reverse-transcribed with SuperScript™ II reverse transcriptase (In-
vitrogen) using the oligonucleotides Ex1as 5'-GTGCTGTGGGAAC-
TATAAATTTAC-3' for Nova-1 or Nova-2-3(4) and 5'-TTGGGAC-
GATCAGCTTGG-3' for Nova-2. PCR was carried out using primers
Exlas and RI28 (5'-GCCGCCGACTCCCGGAAAAG-3') to amplify a
481-bp fragment of Nova-1 mRNA or primers Nova-2-5(1) (5'-CAA-
GAGGCCCTCTGAAACG-3') and Nova-2-3(4) to amplify a 388-bp frag-
ment of Nova-2 mRNA. The conditions used for amplification were
95 °C 5′ and 27 cycles of 95 °C at 30 s, 57 °C at 30 s, 72 °C, 1 min for Nova-1
and identical conditions except for an annealing temperature of 60 °C
for Nova-2. For JNK2 assays, reverse transcription was carried out using
2 μg of cytoplasmic RNA (treated with RQ1 DNase, Promega) with primers
JNK2 3′-1, 5′-ACTGCTGCATCTGAAAGGC-3′, or
JNK2 3′-2, 5′-TTACTGCTGCATCTGTC-3′ and murine mammary
mammary tumors virus reverse transcriptase (Promega). PCR amplification
was carried out using JNK2 3′-1 or JNK2 3′-2 primers in combination
with primers for the alternative exons 6b, 5′-GAGACTGTG-
GAAAGGTT-3′, and 6a, 5′-GAAATGGTGCTCCTAAAG-3′, under the
following conditions: 95 °C 1 min, 56 °C 1 min, 72 °C, 1 min. RNA titration
assays showed that under these conditions, amplification was linear and able to detect 2-fold differences in mRNA
amounts (not shown). Values obtained with various combinations of
oligonucleotides in five different RT-PCR experiments from two inde-
pendent RNA isolates were used to generate the data of Fig. 5D.

Western Blot—Total protein extracts were fractionated by electro-
phoresis on SDS-polyacrylamide gels and transferred to nitrocellulose
membranes using a semi-dry blotting chamber. Membranes were incu-
bated with 1× phosphate-buffered saline-0.1% Tween supplemented
with 5% nonfat milk and antibodies: rabbit polyclonal antihuman Nova-
1/3000; rabbit polyclonal antihuman PSF, 1/5000 (a gift from J. Patton,
Spring 6.2 (Silicon Genetics) software programs.

A Splicing factors /regulators (86) Splicing events (105)
- snRNP components (U1/U2) (12) Receptors (CD21, FGFR1, TNFR)
- snRNP components (U1/U2) (19) Signalling (PTPN7, RON, VEGF, TNFα)
- SR proteins (18) Cell cycle control (Mdm2, p16/14ARF)
- hnRNPs (19) Other splicing regulators (10)
- hnRNPs (19) Other RNA binding proteins (16)

B

FIG. 1. Experimental design. A, categories of splicing regulators
and splicing events analyzed in this study. The number/identity of
genes are indicated in brackets. TNF, tumor necrosis factor; TNFR,
tumor necrosis factor receptor; VEGF, vascular endothelial growth factor; ARF, alter-
native reading frame of p16. B, distribution of oligonucleotides to study
alternative splicing events. 13 oligonucleotide pairs (perfect match/mismatch,
only three are drawn) were selected for each alternative or
neighboring constitutive exon, and 11 (only one is drawn) were selected
for each of the splice junctions.

FIG. 2. Examples of changes in al-
ternative splicing in Hodgkin cell
lines. A, changes in expression of p16/
p14arf isoforms. The genomic structure of
the locus and splicing patterns are repre-
sented. Alternative transcription initia-
tion sites are indicated by brown arrows,
exons are indicated by boxes, and splicing
patterns are indicated by thin lines. Ar-
rows indicate increase (arrows pointing
up), decrease (arrows pointing down), or
no change (double-headed horizontal ar-
rows) in the detection of exons or splice
junctions in RNAs from Hodgkin cell lines
(indicated with a color code) as compared
with a reference non-tumor B cell line. B,
changes in expression of CD44 isoforms.
Exons v2–v10 are variable exons that can
be included or skipped in various combi-
nations, affecting the properties of the protein product. Genomic
structure, splicing patterns, and changes in splicing are indicated as in A.

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RESULTS

Experimental Design—Based on previous optimization efforts (22), 25-mer oligonucleotides were designed to analyze the expression of 86 mRNAs encoding proteins implicated in regulation of pre-mRNA splicing (Fig. 1A). Each mRNA was analyzed with 13 pairs of oligonucleotides, each pair containing one perfect match and a mismatch control with a single transversion at position 13. To detect ~100 splicing events, mostly involving alternative cassette exons, occurring in genes related to cell signaling, proliferation, adhesion, and apoptosis (Fig. 1A), 13 oligonucleotide pairs corresponding to constitutive or alternative exons, as well as 11 oligonucleotide pairs spanning alternative splice junctions, were selected (Fig. 1B). Microarrays were generated by light-activated in situ synthesis of the selected oligonucleotides (20).

Total cytoplasmic RNA was isolated from cell lines derived from Hodgkin lymphoma tumors at stages IIIb (HD-MY-Z), IV (HDL-2), or IVb (L-540) and from a non-tumor B cell line (LCL-H0) as a reference. After reverse transcription using an oligo(dT)-T7 RNA polymerase promoter primer and second strand synthesis, biotin-labeled cRNAs were generated by T7 transcription and hybridized to the microarrays. Hybridizations corresponding to at least six independent RNA isolates were carried out for each of the cell lines analyzed, resulting in ~70 independent measurements per cell line and sequence feature analyzed. -Fold changes between Hodgkin and reference cell lines for each sequence feature and their statistical analyses are provided as Supplementary Data. The ratios between alternative and constitutive exons and between the relevant splice junctions represent independent sources of information for analyzing the relative use of alternative splice sites.

Expression of Alternative mRNA Isoforms—The ability of the microarrays to detect different mRNA isoforms was first evaluated by analyzing the expression of p16 and p14ARF mRNAs, which encode pivotal regulators of the tumor suppressors p53 and Rb (23). p16 and p14ARF mRNAs are generated from a single locus through alternative sites of transcription initiation and the use of alternative first exons. It was previously reported (24) that expression of p16 is increased in HDML-2 cells, expression of p14ARF is increased in HD-MY-Z cells, and expression of either gene is undetectable in L-540 cells. The microarray results were fully consistent with these previous observations (Fig. 2A), indicating that our microarray design is able to distinguish between the expression of alternatively spliced mRNA isoforms.

Analysis of ~100 splicing events documented 20–30% changes in splicing between the different Hodgkin cell lines and the non-tumor B cell line, which showed various levels of concordance among the different tumor lines. This is illustrated in Fig. 2B by the complex pattern of alternative splicing of the gene encoding the cell adhesion molecule CD44, which has been implicated in tumor metastasis (25). Unsupervised clustering analysis of data obtained from 16 RNA preparations corresponding to four independent RNA isolates/cell line showed appropriate grouping of the samples according to the cell line and closer clustering of cell lines derived from grade IV tumors versus grade III and non-tumor B cell lines (Fig. 3). Table I summarizes the statistical analysis of the data. Taken together, the results indicate that the analysis of splicing patterns allows us to distinguish Hodgkin cell lines from the non-tumor B cell line and to group together cell lines derived from the same tumor grade.

Expression of Splicing Factors and Regulators—Changes in the expression of spliceosomal components and other proteins related to splicing regulation were also detected (Table II). These include members of the hRNP and SR protein families, snRNP components such as SAP155, SAP62, and PSF, splicing co-activators such as SRm 160 and 300, and neuron-specific splicing regulators such as Nova-1 and -2. A subset sample of these changes was verified at the protein level (Figs. 4 and 5). 90% of the microarray data that were tested at the RNA and/or protein level were consistent with the results obtained by independent experiments and/or literature reports. Not unexpectedly, microarray and other expression data were not always comparable. For example, microarray data detected only a putative increase in expression of the U5-associated factor PSF (ptbas in the microarray) in HD-MY-Z cells, whereas increases were experimentally observed in the three Hodgkin cell lines (Fig. 4C).

Ectopic Expression of Nova Proteins—The neuron splicing regulator Nova-1 was initially discovered as a self-reactive antigen expressed in lung and breast tumors (26), and the presence of antibodies in serum correlates with the development of paraneoplastic opsonosomal myelocytosis ataxia, a motor control disorder (27). A related protein, Nova-2, is also recognized by the sera of paraneoplastic opsonosomal myelocytosis ataxia patients and is normally expressed in the central nervous system with a distribution distinct from that of Nova-1 (28).

The microarray data indicated expression of Nova mRNAs in the grade III HD-MY-Z cell line and expression of Nova-2 in grade IV HDML-2 cells (Table II). These patterns of expression were confirmed at the transcript and protein level (Fig. 5, A...
and B). Analysis of other Hodgkin cell lines and tumor samples confirmed expression of Nova-2 in another grade IV cell line and in malignant Hodgkin and non-Hodgkin lymphoma tumors (Fig. 5, B and C).

Recent results have identified pre-mRNAs containing Nova-2 binding sites, the alternative splicing of which was altered in Nova-2 knock-out mice (17). One of these, the JNK2 pre-mRNA, is alternatively spliced to generate mRNA isoforms that encode kinases that play key roles in cell signaling, proliferation, and apoptosis and that have different affinities for their transcription factor substrates (29).

A prediction of our results is that expression of Nova-2 in the HDML-2 cell line could alter JNK2 alternative splicing. The results of Fig. 5D verify this prediction as they show a significant enrichment of the Nova-2-induced JNK2 exon 6a in HDML-2 cells as compared with the reference cell line. Quantification of the results from five different experiments indicated a 2–3-fold increase in exon 6a utilization in Nova-2-

**Table I**

Statistical analysis of fold changes in splicing in Hodgkin cell lines

For each pair of Hodgkin lines, the fold differences in the use of exon/exon junctions between each cell line and the reference B cell line are compared. Similar behavior, fraction of instances in which changes or absence of changes, are similar between the compared cell lines. Similar discriminatory behavior, fraction of instances in which similar behavior is found for the two cell lines being compared, whereas different behavior is found for the third.

|                | HDML-2 (IV) vs. HD-MY-Z (IIIb) | HDML-2 (IV) vs. L-540 (IVb) | L-540 (IVb) vs. HD-MY-Z (IIIb) | All three cell lines |
|----------------|---------------------------------|-------------------------------|-------------------------------|---------------------|
| Similar behavior | 70%                             | 52%                           | 59%                           | 44%                 |
| Similar discriminatory behavior | 47%                             | 15%                           | 26%                           |                     |

**Table II**

Changes in observed in the expression of splicing factors in Hodgkin cell lines

Fold change refers to the fold difference in mRNA abundance between Hodgkin cell lines and the reference cell line. When no reference is made to specific lines, the changes affect all three Hodgkin cell lines.

| Factor | Family/function                  | Fold change |
|--------|---------------------------------|-------------|
| hnRNP E1 | hnRNP protein                    | 2–3 × down |
| 9G8    | SR protein                       | 2–3 × down  |
| U2AF65 | U2 snRNP auxiliary factor        | >5 × up     |
| SRm160,300 | splicing co-activators        | 2–3 × up   |
| SAP155 | U2 snRNP                         | >3 × up in HDML-2 & L-540 |
| SAP 62 | U2 snRNP                         | putative up in HD-MY-Z |
| PSF    | U5 snRNP-associated              | 2–3 × up in HD-MY-Z |
| NOVA-1 | neuron-specific splicing         | >3 × up in HDML-2 |
| NOVA-2 | neuron-specific splicing         |             |

**Fig. 4.** Changes in splicing factor levels in Hodgkin cell lines. Western blots were carried out using the indicated amounts of total extracts from the indicated cell lines and using antibodies specific for the splicing factors U2AF65 (A), SAP155 (B), and PSF (C). Reactivity with anti-β-tubulin antibodies was used to normalize loading.
expressing HDML-2 cells, a slight increase in Nova-1-expressing HD-MY-Z cells, and no effect in L-540 cells, which do not express either protein (Fig. 5E). These results further argue that expression of Nova proteins is sufficient to induce neuron-enriched patterns of splicing in non-neural cells.

DISCUSSION

Given the prevalence and relevance of alternative splicing in higher eukaryotes, it seems likely that many aspects of the phenotype of a cell, including those leading to tumor progression, are controlled by the relative expression of alternatively spliced isoforms of multiple genes. Our results indeed suggest that general patterns of alternative splicing, as well as expression of particular splicing regulators, can be indicative of cell transformation and of tumor grade. If this is the case, microarrays able to discriminate between alternatively spliced isoforms will obviously have important medical applications.

The fraction of changes observed between Hodgkin cell lines and the reference cell line is substantial, affecting 20–30% of the splicing events analyzed and showing a 2–3-fold better correlation for cell lines derived from tumors of the same grade (Table I). In the vast majority of cases, these changes are exon- or splice junction-specific, indicating that they do not correspond to a general increase or decrease in the output of a gene but that they represent bona fide changes in splicing rather than transcription. Intriguingly, some of the changes detected affect exons described previously as constitutive (e.g. in the CD21 gene), even when no changes were observed in other regions of the pre-mRNA described previously as alternatively spliced. This is consistent with the suggestion that significant, albeit not general, changes in the mechanisms of exon definition occur in tumor cells (7).

Given that 1) changes in the relative expression of general splicing factors can modulate alternative splicing of multiple genes (15) and 2) changes in the relative levels of general splicing factors are observed in different cell types or disease states (30, 31), it has been postulated that the relative expression of splicing factors acts as a “cellular code” that establishes patterns of alternative splicing in multiple genes (13, 14). Clustering analyses similar to those shown in Fig. 3, however, did not show the same degree of correlation between tumor grade and overall changes in the expression of splicing factors as they did with changes in splicing (data not shown). Similarly, splicing events were 5-fold more frequent than splicing factors in the list of array features that provided the best discrimination between cell lines (GeneSpring 6.2 analysis of variance software, data not shown). It is possible that global changes in splicing factor concentrations are not as predictive of tumor grade as the expression of isoforms of genes involved in cell cycle control, signaling, adhesion, etc., and therefore, that changes in a relatively small number of splicing regulators, or in their activities via post-translational modifications, are responsible for changes in a significant number of alternative

Fig. 5. Functional expression of Nova proteins in some Hodgkin cell lines. A, expression of Nova-1 and Nova-2 mRNAs. RT-PCR reactions were carried out using RNAs isolated from the indicated cell lines and oligonucleotide primers corresponding to fragments of Nova-1 (481 bp) and Nova-2 (388 bp) open reading frames. M, gene ruler 50-bp DNA ladder (Fermentas). Asterisk, 500 bp. B, expression of Nova proteins. Western blots were carried out using total extracts from the indicated cell lines and an antibody that recognizes both Nova-1 (52 kDa) and two forms of Nova-2 antigens (50 and 70–75 kDa; Yang et al. (28)). M, molecular mass markers. C, expression of Nova-2 in lymphoma tumor samples. Nova-1 and Nova-2 knock-out mice tissues are used as markers of Nova antigens. D, induction of neuron-specific JNK2 alternative splicing by ectopic expression of Nova-2 in HDML-2 cells. The diagram represents the patterns of alternative splicing affecting JNK2 mutually exclusive exons 6a and 6b. Semiquantitative RT-PCR assays were carried out using primers in sequences downstream of exon 6 and primers specific for each of the alternative exons, amplifying products of 492 bp (6a) and 502 bp (6b). Asterisk, 500-bp fragment, gene ruler 50-bp DNA ladder (Fermentas). E, quantification of changes in relative expression of JNK2 isoforms containing exons 6a and 6b in Hodgkin cell lines as compared with the reference B cell line. Average (blocks) and standard deviation (error bars) values of five independent experiments are represented.
splicing events. Alternatively, because a large fraction of splicing factor genes are themselves alternatively spliced, mRNA-based analysis of their expression may not be as predictive as changes in the expression of individual isoforms. Microarrays designed to detect expression of alternatively spliced isoforms of splicing factors will help test this possibility.

Nevertheless, changes in splicing regulatory proteins were also detected. An interesting observation was that individual components of splicing complexes appeared to be regulated independently of other partners. This was the case for the 65-kDa subunit of the splicing factor U2AF (Fig. 4A) and for the U2 snRNP component SAP155 (Fig. 4B), the levels of which were increased without parallel increases in U2AF35 or some other components of U2. It is conceivable that a U2AF complex devoid of its 35 kDa subunit causes changes in 3′ splice site recognition (32) and that an excess of SAP155, which has repeats that are targets of cdk2 phosphorylation (33), can titrate the activity of this kinase and influence cell cycle progression.

Ectopic expression of Nova proteins was originally reported in lung and breast tumors, leading to the production of autoantibodies that are thought to be the cause for the subsequent development of motor disorders in these patients (26, 27). Our microarray analyses revealed expression of Nova-1 and -2 in some Hodgkin cell lines, observations that were independently confirmed and extended to other Hodgkin cell lines and tumor samples (Fig. 5). These findings could provide pathophysiological basis for explaining the clinical development of paraneoplastic opsoclonus-myoclonus in some Hodgkin patients (34).

Although Nova proteins have been implicated in neuron-specific splicing regulation (16, 17), changes in alternative splicing caused by ectopic expression of these proteins in tumors or tumor cell lines, with potential effects in the regulation of cell proliferation, had not been reported. Our observation that expression of Nova-2 correlated with increased expression of neuronal isoforms of JNK2, together with the key roles that these proteins play in cell signaling, proliferation, and apoptosis (29, 35), are compatible with the possibility that Nova-2 expression causes changes in alternative splicing that influence tumor progression.

In summary, although larger data sets and powerful computational tools will be required for full exploitation of this microarray design, the results reported in this study have provided new insights of potential medical relevance, including an explanation for clinical reports of motor disorders in Hodgkin patients and a correlation between global changes in alternative splicing and cell transformation and tumor grade.

Acknowledgments—We thank M. Piris and A. Sánchez for reagents and discussions. J. Patton and R. Reed for antibodies. We also thank members of Pehlt, EMBL, and Centre de Regulació Genòmica for advice and comments on the manuscript and give special thanks to F. Kafatos for continued support.

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J. Biol. Chem. 2005, 280:4779-4784.
doi: 10.1074/jbc.M411976200 originally published online November 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411976200

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