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REPORT

Identification of cells deficient in signaling-induced alternative splicing by use of somatic cell genetics

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ABSTRACT

In recent years, a growing number of mammalian genes have been shown to undergo alternative splicing in response to extracellular stimuli. However, the factors and pathways involved in such signal-induced alternative splicing are almost entirely unknown. Here we describe a novel method for identifying candidate *trans*-acting factors that are involved in regulating mammalian alternative splicing, using the activation-induced alternative splicing of the human *CD45* gene in T cells as a model system. We generated a cell line that stably expresses a *CD45* minigene-based GFP reporter construct, such that the levels of green-fluorescent protein (GFP) expressed in the cell reflect the splicing state of the endogenous *CD45* gene. Following mutagenesis of this cell line, and multiple rounds of selection for cells that displayed aberrant levels of GFP expression, we isolated several cell lines that are at least partially defective in their ability to support regulated alternative splicing of endogenous *CD45* pre-mRNA in response to cell stimulation. Thus we have successfully isolated mutants in a mammalian alternative splicing pathway through use of a somatic cell-based genetic screen. This study clearly demonstrates the feasibility of using genetic screens to further our understanding of the regulation of mammalian splicing, particularly as it occurs in response to environmental cues.

Keywords: *CD45*; genetic screen; GFP; regulated splicing

INTRODUCTION

Recent estimates suggest that up to 60% of all human genes undergo some form of alternative splicing (Modrek & Lee, 2002). However, the mechanisms and factors by which this critical step in gene expression is regulated are only understood for a very few of these genes (for recent reviews, see Smith & Valcarcel, 2000; Caceres & Kornblihtt, 2002). Alternative splicing, which is defined as the differential use of splice sites within a given gene, results in the synthesis of multiple, distinct mRNAs. Because each distinct mRNA can encode for a unique protein, alternative splicing is a primary mechanism by which multiple protein functions may be generated from a single gene. Of further significance is the fact that the choice of which mRNA is produced is often tightly regulated, thus enabling distinct protein isoforms to be expressed in a tissue-specific manner or in response to environmental cues. In many cases, the variant proteins expressed from a given gene function in competition or opposition to one another. Thus small changes in the ratio of protein isoforms can have a dramatic effect on cellular function.

The regulation of alternative splicing in response to extracellular stimuli has been observed in a growing number of genes and in a variety of cell types. Examples of signaling-induced alternative splicing include glucose-induced intron removal from insulin pre-mRNA (Wang et al., 1997), insulin-induced changes in exon usage of the Protein Kinase C βII gene in skeletal muscle (Chalfant et al., 1998; Patel et al., 2001), and changes in the neuronal splicing of the *agrin* (Smith et al., 1997) and *Slo* (Xie & McCobb, 1998) genes induced by nerve growth factor (NGF) and stress hormones, respectively. In T cells, several genes have been shown to undergo activation-induced alternative splicing in response to antigen challenge, including *CD44*, *Fas*, and *CD45* (Akbar et al., 1988; Birkeland et al., 1989; Lui et al., 1995; Konig et al., 1998). For *CD44* and *CD45*, it is clear that activation of the small GTPase Ras is an obligate step in the pathway leading to alternative splicing (Konig et al., 1998; Lynch & Weiss, 2000). Ras

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activation has also been demonstrated to be critical in the NGF-induced alternative splicing of *agrin* (Smith et al., 1997), whereas calcium/calmodulin-dependent kinase IV (CaMK IV) induces splicing regulation of the *Slo* gene (Xie & Black, 2001). However, beyond these initial observations, almost nothing is known of the mechanisms by which signal transduction pathways induce specific changes in alternative splicing. Even in examples of tissue-specific regulation of alternative splicing, there is only a very limited picture of the factors and mechanisms that regulate specific splicing patterns in various tissues (Grabowski & Black, 2001).

Much of the limitation in understanding the mechanisms by which splice site choice is regulated in mammals has been due to a lack of methods by which to identify potential regulatory factors. In both yeast and *Drosophila*, the use of genetic screens has led to significant advances in the identification and characterization of proteins that determine splice site usage (Baker, 1989; Guthrie, 1991). However, in mammalian systems, the isolation of splicing regulatory factors has primarily been accomplished by means of biochemical fractionation and/or affinity purification using specific RNAs (Blencowe et al., 1995; Fu, 1995; Grabowski, 1998; Grabowski & Black, 2001). Although several splicing factors have been purified and characterized by such in vitro methods, these techniques are not well suited to the elucidation of pathways upstream of the regulated RNA that link cellular stimulation to changes in splice site selection. The availability of genetic methods to identify splicing regulatory proteins would be a significant addition to the repertoire of techniques with which to study alternative splicing in mammals, and would be particularly advantageous in the study of signaling-induced alternative splicing.

There are two primary characteristics of regulated alternative splicing that complicate the use of genetic screens to identify regulatory factors. First, mammalian alternative splice site choices do not tend to be "all-or-none," but rather involve relatively subtle ($<$10-fold) changes in isoform ratios under different environmental conditions or in differing cell types (Caceres & Kornblihtt, 2002). Thus, a reporter for alternative splicing must be one in which it is easy to detect and select for small changes in isoform expression. Second, regulation of splicing in mammals tends to be under the combinatorial control of multiple regulatory proteins (Smith & Valcarcel, 2000). Therefore, perturbing the levels of just one component of a larger regulatory complex may not result in a detectable change in isoform expression.

Two approaches toward using genetic selections to analyze mammalian splicing regulation have been reported to date. In one study, Roberts et al. (1996) demonstrated that they could induce a fibroblast to splice an α-tropomyosin minigene in a muscle-specific manner by introducing genomic DNA from smooth muscle cells. Thus far, the causal gene in that study has not been identified. In a second set of studies, Chasin and coworkers have used a reporter that encodes the DHFR enzyme in a splicing-dependent manner, to analyze the effect of *cis*-alterations in RNA sequence on the subsequent inclusion of a given exon (Chen & Chasin, 1993; Fairbrother & Chasin, 2000). In contrast, a screen to identify cells that are deficient in their naturally existing splicing regulatory machinery has never been reported. Such a mutagenesis screen would be most analogous to those that have been successful in identifying specific splicing regulatory factors in *Drosophila* (Baker, 1989). In addition, the use of a mutagenesis screen has the unique potential, with regards to signaling-induced alternative splicing, of potentially dissecting all steps of a regulatory pathway.

As a model system for studying signaling-induced alternative splicing, we have focused on understanding the regulation *CD45* pre-mRNA splicing. The *CD45* gene encodes a transmembrane protein tyrosine phosphatase that is expressed at high levels in all nucleated hematopoietic cells. In T cells, CD45 functions to maintain the T-cell receptor (TCR) in a primed state, allowing for activation of the TCR upon interaction with an antigen presenting cell (reviewed in Hermiston et al., 2002). Five distinct isoforms of CD45 are expressed in humans (Streuli et al., 1987). These isoforms arise from the regulated alternative pre-mRNA splicing of 3 of the 33 exons that encode for CD45. In resting T cells, these three variable exons are largely included in the mature message, whereas activated T cells express predominantly the R0 isoform in which all three variable exons are excluded (Akbar et al., 1988; Birkeland et al., 1989). It has been shown that alternative inclusion of the three variable exons (exons 4, 5, and 6 that encode a portion of the extracellular domain of CD45) influences the ability of the resultant protein to dimerize. Specifically, the smallest isoform, but not the larger isoforms, have been observed to exist as homodimers (Majeti et al., 2000; Dornan et al., 2002; Xu & Weiss, 2002). Because dimerization of CD45 protein inhibits its phosphatase activity, it is predicted that the change in CD45 isoform expression upon T cell activation results in a decrease in CD45 function (Majeti et al., 1998; Xu & Weiss, 2002). Indeed the physiological significance of CD45 alternative splicing is underscored by the finding that aberrant expression of the large isoforms of CD45 in humans correlates with susceptibility to autoimmune disease and HIV infection (Jacobsen et al., 2000; Tchilian et al., 2001).

We have previously described a cultured cell line (JSL1) in which we can induce alternative splicing of endogenous *CD45* pre-mRNA by stimulation with the phorbol ester PMA (Lynch & Weiss, 2000). Alternative splicing of *CD45* in these cells is indistinguishable in all respects from that observed upon activation of normal human primary T cells. Using the JSL1 cell line we have demonstrated that maximal activation-induced al-

ternative splicing of *CD45* occurs 48–72 h after PMA stimulation, similar to the time course of the change in CD45 protein expression observed upon TCR stimulation in primary T cells. Moreover, we have shown that Ras activation and protein synthesis are both critical steps in the pathway that leads to regulation of *CD45* splicing (Lynch & Weiss, 2000). JSL1 cells are derived from the well-characterized Jurkat T cell-derived cell line, which has been used extensively as a system to understand the myriad of cellular changes that occur upon T cell stimulation. In particular, Jurkat cells have proved to be amenable to somatic cell genetics and have been used for numerous mutagenesis-based screens that have resulted in the identification of factors critical in T cell signaling (Goldsmith & Weiss, 1987; Finco et al., 1998).

In this report, we describe a GFP-based splicing reporter that provides a sensitive read-out for relatively small changes in *CD45* isoform expression. Importantly, this reporter undergoes alternative splicing upon stimulation of JSL1 cells with PMA, resulting in a measurable change in GFP expression between unstimulated and stimulated cells. Using this reporter, we have successfully isolated cell lines that have lost the ability to regulate *CD45* alternative splicing in response to extracellular stimuli. Thus, we demonstrate for the first time the successful genetic selection of mammalian cells that have acquired mutations in some component of a specific splicing regulatory pathway. As such, this study not only represents a significant step toward furthering our understanding of the regulation of *CD45* pre-mRNA splicing, but also provides a prototype for a novel approach to the identification of factors involved in regulating other signaling-induced alternative splicing events.

RESULTS

To develop a genetic screen for activation-induced alternative splicing of *CD45*, we needed to create a reporter construct that allowed *CD45* isoform expression to be readily assayed, preferably in living cells. One specific concern was that, similar to many examples of mammalian alternative splicing, the regulated change in *CD45* splicing involves at most a three- to fivefold change in the expression of any given isoform (Lynch & Weiss, 2000). For this reason, we needed a reporter construct in which we could detect, and select for, small changes in the read-out. Moreover, because we are interested in splicing changes that occur upon stimulation, we needed a read-out that is transient such that expression from the reporter construct following stimulation would not be obscured by gene product that might be present in the cell prior to treatment with PMA.

The three variable exons of *CD45* (exons 4, 5, and 6) are regulated independently but in response to the same stimulus (C. Rothrock, B. Cannon, & K.W. Lynch, in prep.). Previously we have shown that splicing of exon 4 is appropriately regulated by stimulation with PMA when it is in the context of a minigene in which it is flanked by constitutive exons 3 and 7 (Lynch & Weiss, 2000). We have also found that a minigene consisting only of exons 4, a small portion of exon 7, and their flanking introns, contains all the PMA-responsive splicing regulatory elements necessary for mediating alternative splicing of *CD45* exon 4 (C. Rothrock, B. Cannon, & K.W. Lynch, in prep.). Thus, consistent with the PMA-induced repression of exon 4 inclusion, the overall splicing efficiency of pre-mRNA derived from this minimal exon 4–7 is reduced upon treatment with PMA. We have used this minimal exon 4–7 *CD45* minigene as the basis of our reporter construct.

After testing a variety of fluorescent or enzymatic reporters, we chose to focus our efforts on using a destabilized version of GFP (d2EGFP) as a read-out. The d2EGFP has a PEST domain fused to the C terminus of the GFP protein, such that the half-life of the resultant protein in mammalian cells is approximately 2 h (Li et al., 1998). We fused the cDNA encoding d2EGFP downstream of *CD45* exon 7 to generate the splicing reporter construct 47GFP shown in Figure 1A. The 47GFP reporter is designed with a Kozak sequence and ATG at the beginning of exon 4. There are no suitable translation initiation sites within the GFP cDNA sequences included in the 47GFP reporter, and there are multiple stop codons within the intron of 47GFP. Consequently, GFP protein is expressed only when the pre-mRNA encoded by the 47GFP reporter is properly spliced. The prediction from this design is that GFP fluorescence should decrease upon PMA stimulation of cells expressing the reporter. In contrast, mutant cells that are defective in activation-induced alternative splicing of *CD45* should retain high GFP expression even in the presence of PMA.

We transfected JSL1 cells with a vector encoding the 47GFP minigene, and selected for individual cells that stably expressed the reporter. All subsequent experiments were done with cells derived from a single clone we will refer to as WT-47GFP, although multiple clones from the initial selection were indistinguishable from WT-47GFP with regards to GFP expression and regulation (data not shown). As shown in Figure 1B, pre-mRNA from the 47GFP reporter is spliced with high efficiency. As determined by a sensitive low-cycle RT-PCR assay (Lynch & Weiss, 2000), correctly spliced 47GFP mRNA is decreased three- to fourfold upon PMA induction, with a concomitant increase in the amount of unspliced pre-mRNA observed (Fig. 1B). This fold change in isoform expression is consistent with the PMA-induced alternative splicing of both the endogenous CD45 gene and other CD45 minigenes described previously (Lynch & Weiss, 2000). We have sequenced both products from the RT-PCR reaction to confirm their identity and the accuracy of the spliced

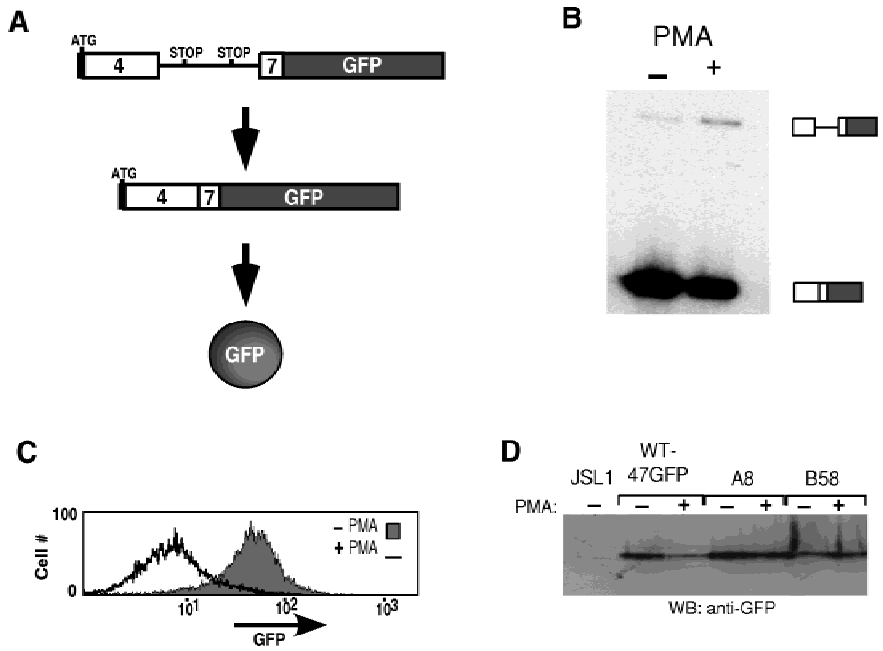

FIGURE 1. Isolation of cells that express GFP in a manner reflective of CD45 alternative splicing. **A:** Schematic of the 47GFP minigene that encodes fluorescent GFP only when appropriately spliced. Unspliced message contains multiple stop codons upstream of the GFP reading frame. **B:** Representative RT-PCR analysis of RNA expression from the 47GFP reporter in resting (−PMA) or activated (+PMA) WT-47GFP cells. Quantitation of multiple experiments (>4) reveals a three- to fivefold increase in the ratio of unspliced to spliced 47GFP RNA after 3 days stimulation with PMA. **C:** GFP fluorescence histogram of resting and activated WT-47GFP cells as analyzed by flow cytometry. Quantitation of multiple experiments (>4) reveals a six- to sevenfold decrease in the mean fluorescence intensity of WT-47GFP cells after 3 days stimulation with PMA. **D:** Western blot analysis of GFP expression in parental JSL1 cells, WT-47GFP cells, and two of the mutants isolated by this screen (see Fig. 3) in the absence and presence of 3 days treatment with PMA. For each sample, we ran whole-cell lysate from approximately 0.5 million cells on a 12.5% SDS-PAGE gel. The same lysates run in parallel, and probed with numerous other antibodies, indicate that the overall protein level in each lane is equivalent.

product. In addition, we do not detect any signal in this assay in the absence of reverse transcriptase, indicating that the upper band in Figure 1B is indeed unspliced pre-mRNA instead of contaminating genomic DNA (data not shown). Finally, a similar change in splicing is not observed in control minigenes, even in those that are spliced with similar efficiency as 47GFP RNA (C. Rothrock, B. Cannon, & K.W. Lynch, in prep.). Thus we conclude that the splicing of RNA from the 47GFP reporter is regulated in a manner consistent with that of the endogenous *CD45* gene.

Importantly, the PMA-induced decrease in splicing of the 47GFP RNA is clearly reflected in a decrease in GFP protein expression in these cells, as assayed by both flow cytometry (Fig. 1C) and western blot analysis (Fig. 1D). Comparison of the mean GFP fluorescence intensity of resting and stimulated cells by flow cytometry reveals an approximate sixfold decrease in GFP expression upon PMA stimulation, which is within twofold of the change in isoform expression of the reporter as quantitated by RT-PCR (Fig. 1B). Significantly, the change in GFP expression detected by flow cytometry parallels the time course required for *CD45* alternative splicing in that less than a twofold change is observed after 24 h, and maximal change is detected only after 60 h (data not shown). Furthermore, in control experiments to look at the effect of PMA on the expression of d2EGFP cDNA in JSL1 cells when transcribed from the same promoter as that used for 47GFP, we detect no more than a twofold change in GFP expression over the same 24–60 h time course. Finally, we note that the migration of GFP-reactive protein on the western blot shown in Figure 1D is consistent with the predicted translation of the spliced 47GFP fusion protein (approximately 45 kDa). Together, these data strongly suggest that the expression of GFP in WT-47GFP cells is largely representative of the RNA isoform expression and alternative splicing of CD45.

Previous studies have demonstrated that recessive mutations can readily be generated in the JSL1-parental Jurkat cell line by treatment with the chemical mutagen ethyl methanesulfonate (EMS) (Goldsmith & Weiss, 1987). Specifically, EMS mutagenesis-based screens have been used to elucidate the critical role of factors such as Lck and Lat in the initial steps of T cell signaling (Goldsmith & Weiss, 1987; Finco et al., 1998). The relative ease of generating recessive mutations in Jurkat cells has been attributed to the fact that these cells are only partial diploids due to the inherent genetic instability of cultured cell lines. Given the success of

chemical mutagenesis to induce T cell signaling mutants Jurkat cells, an EMS-based screen should likewise be well suited to generating cells that are deficient in their ability to undergo activation-induced splicing regulation of *CD45*.

Using conditions described previously (Goldsmith & Weiss, 1987), treatment of 65 million WT-47GFP cells with 200 ng/mL of EMS for 24 h resulted in approximately 60% remaining cell viability. After 1 day of recovery, approximately 40 million cells were stimulated with 2 ng/mL PMA for 3 days and then analyzed by flow cytometry. We isolated cells by fluorescence activated cell sorting (FACS) gating for both GFP expression and for expression of the cell surface protein CD69. Expression of CD69 has been shown to be a reliable marker for Ras activation (D'Ambrosio et al., 1994). Because we are interested primarily in dissecting the steps of the splicing regulatory signaling pathway that occur downstream of Ras activation, we wanted to be able to differentiate between cells that were wild type with regards to Ras activation and those that had acquired mutations in signaling proteins such as Lck and Lat, that function upstream of Ras. Figure 2A shows the FACS plots for the resting and activated WT-47GFP cells following treatment with EMS. As for the unmutagenized cells, the bulk of the EMS-treated WT-47GFP cells have down-regulated GFP expression in response to PMA treatment and are all CD69-high. However, we did observe that approximately 3% of total cells appear to be CD69+ and still GFP-high. These few CD69+/GFP-high cells (~200,000 total) were collected, expanded, and allowed to return to a resting state (i.e., CD69-low). Even after this initial selection, we found that a notable fraction of the isolated, expanded cells remained GFP-high upon stimulation with PMA (Fig. 2B). Following two subsequent rounds of PMA stimulation and FACS sorting, the bulk of the cell population remained CD69+/GFP-high even after 3 days of PMA treatment (Fig. 2C). The cells that expressed the highest GFP levels in this final sort were plated in individual wells so that single clones could be isolated and further analyzed.

The vast majority of the individual clones isolated after the third FACS sort showed a significant decrease in PMA-induced GFP down-regulation compared to WT-47GFP cells (107 of 168 analyzed), and approximately 10% of these clones had less than a 1.5-fold change in GFP expression following PMA stimulation. Flow cytometry analysis of the GFP expression of a subset of the most GFP-unresponsive clones is shown in Figure 3A, and a western blot for GFP expression from a few of these clones is shown in Figure 1D. One possibility for the observed lack of PMA-induced change in GFP expression could be that the basal expression of GFP in these clones is outside of the window of sensitivity of our assays. However, although the basal level of GFP expression does vary between the individual

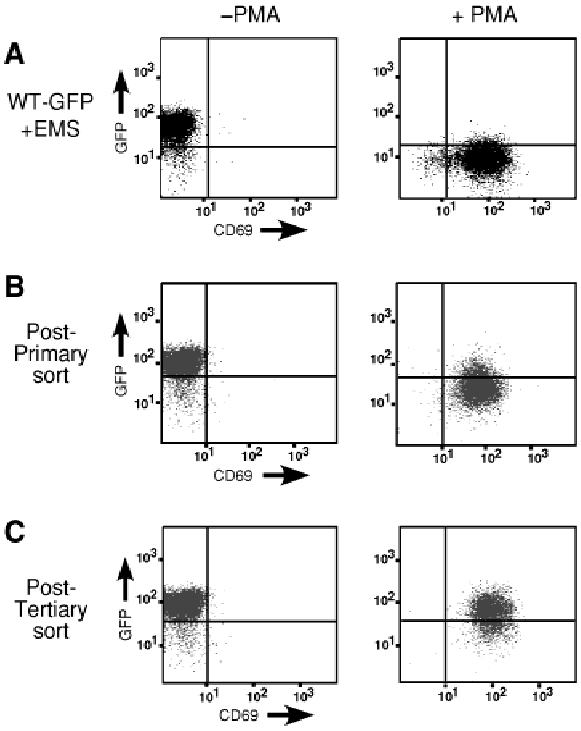

FIGURE 2. Iterative selection for cells that retain high GFP expression upon PMA stimulation. **A:** Dot plot of GFP and CD69-PE fluorescence as analyzed by flow cytometry. WT-47GFP cells, following EMS treatment, were left unstimulated (−PMA) or treated for three days with PMA (+PMA). The cells that expressed the highest 3% of GFP fluorescence in the +PMA sample were collected by FACS sorting. **B:** Dot plot of GFP and CD69-PE fluorescence of cells that were collected in the primary sort. After recovery, cells from primary sort were expanded and then left unstimulated (−PMA) or treated for three days with PMA (+PMA). Again cells that expressed the highest 3% of GFP fluorescence in the +PMA sample were collected by FACS sorting. **C:** Dot plot of GFP and CD69-PE fluorescence of cells that were collected in the tertiary sort. The cells that expressed the highest 5% of GFP fluorescence in the +PMA sample were collected by FACS sorting and distributed into individual wells of 96-well plates, so as to isolate and analyze individual clones.

clones, the distribution of GFP expression is around the mean observed for WT-47GFP (compare Figs. 3A and 1C). Thus the lack of GFP responsiveness appears unrelated to the basal expression of GFP.

For each of these clones in Figure 3A we confirmed that PMA treatment did induce normal levels of CD69 expression, indicating that these cells have not lost global susceptibility to PMA stimulation (data not shown). As a further confirmation of the integrity of early PMA-induced T cell signaling events, we analyzed phosphorylation of the kinase Erk 1/2 in some of the clones (Fig. 3B). In normal T cells, Erk 1/2 is phosphorylated as a result of Ras activation (Genot & Cantrell, 2000), and inhibition of Erk phosphorylation-dependent activation at least partially blocks PMA-induced *CD45* alternative splicing (K. Lynch & A. Weiss, unpubl. data). Consistent with the PMA-inducible expression of CD69 in the GFP-unresponsive mutants, we detect no difference in the PMA-induced phosphorylation of Erk 1/2

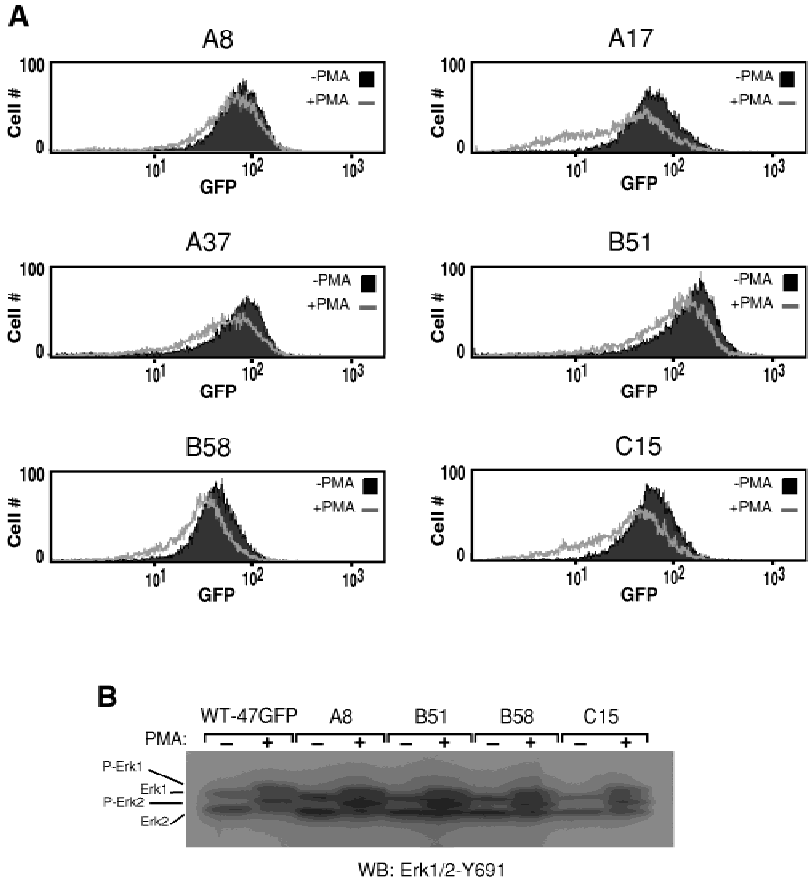

FIGURE 3. Analysis of GFP expression and Erk stimulation of individual clones isolated from mutagenesis screen. **A:** Fluorescence histogram of resting and activated cells as analyzed by flow cytometry. Each histogram is from an independently isolated clone following the tertiary sort shown in Figure 2C. The histograms shown here are from 3-day stimulations with PMA done in parallel with the stimulation of WT-47GFP cells shown in Figure 1C. **B:** Western blot analysis of Erk1/2 in WT-47GFP cells and four of the mutants shown in **A**. Whole-cell lysate of approximately 0.5 million cells harvested 15 min following PMA stimulation (or media alone, −PMA) were run on an 8% SDS-PAGE gel. The Erk-Y691 antibody we used detects both unphosphorylated and inducibly phosphorylated (i.e., Ras-activated) Erk 1 and Erk 2.

(Fig. 3B) between these mutants and WT-47GFP cells. Thus, the defects in the clones we have isolated in this screen are downstream of, or parallel to, Erk activation.

To confirm that the GFP-unresponsiveness of clones shown in Figure 3A is in fact due to a splicing defect, we first analyzed the RNA from the 47GFP reporter in these cells. Indeed, we do detect a decrease in PMA-induced alternative splicing in the clones in Figure 3A as compared to WT-47GFP, consistent with the fluorescence differences observed (data not shown). More importantly, however, we wanted to determine whether these cells were truly deficient in their ability to transduce a signal from PMA stimulation to alternative splicing of *CD45*. In particular, we wanted to rule out the possibility that the loss of GFP-responsiveness in the clones shown in Figure 3 was due to *cis*-mutations within the reporter construct itself. We have sequenced both spliced and unspliced reporter RNA expressed in several of our clones, and have detected no mutations. A more stringent test of whether the causal mutation in our GFP-unresponsive clones is a *cis*-acting mutation in the reporter, or alternatively, a *trans*-acting defect in the splicing machinery or signaling pathway, is to determine whether the endogenous CD45 gene expressed in each of the clones is alternatively spliced in response to PMA. To analyze the RNA isoform expression of the endogenous *CD45* gene we used an RT-PCR assay we have described in detail elsewhere (Lynch & Weiss, 2000). In short, this assay allows us to sensitively detect PMA-induced changes in the expression of the various isoforms of *CD45* (see Fig. 4A). Strikingly, at least two clones (A8 and B58) show almost no activation-induced alternative splicing of the endogenous *CD45* gene, and several others show a marked decrease in alternative splicing compared to WT-47GFP or parental JSL1 cells (Fig. 4A,B). Because the PMA-induced alternative splicing of both the reporter minigene and the endogenous *CD45* gene are defective in these clones, we conclude that we have effectively isolated cells that are deficient in the machinery required to mediate PMA-induced alternative splicing of *CD45*. We now refer to the clones

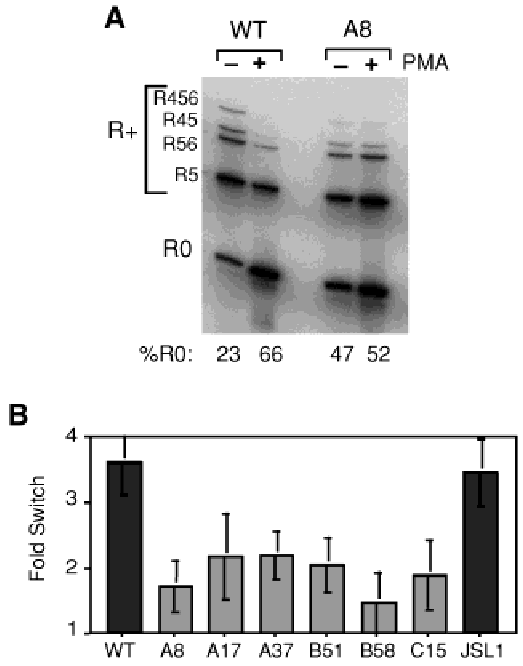

FIGURE 4. Analysis of activation-induced alternative splicing of the endogenous CD45 gene in mutant clones isolated from the GFP-expression screen. **A:** Representative RT-PCR analysis of RNA expression from the endogenous CD45 gene in resting and activated WT-47GFP cells and in one of the isolated mutants. As we have shown previously (Lynch & Weiss, 2000), five isoforms of CD45 RNA can be detected in JSL1 cells. These isoforms correspond to the exclusion of all three variable exons (R0), or the inclusion of various combinations of exons 4, 5, and 6 (R5, R56, R45, R456, or collectively called R+). The percentage of R0 isoform relative to total RNA for this experiment is given below the gel. **B:** Quantitation of the activation-induced alternative splicing of the endogenous CD45 gene in WT-GFP (WT), SRD mutants, and parental JSL1 cells. Fold Switch is defined as the increase in the ratio of R0/R+ isoforms of CD45 upon stimulation with PMA. The quantitation shown here is the average of at least six independent stimulations and RT-PCR assays using freshly isolated mutants as well as cells analyzed following subsequent freeze–thaw cycles.

shown in Figure 4 as Splicing Regulation Deficient, or SRD, mutants.

DISCUSSION

The use of genetic screens to identify mutations in mammalian splicing regulation components has frequently been considered an infeasible approach given the level of complexity of splicing regulation and the typically moderate changes in isoform expression. Here we report that, despite the relatively narrow window for selection, we have developed a reporter construct that provides a read-out representative of the efficiency of a regulated splicing event. Moreover, we demonstrate the successful use of this reporter to select for cells that are deficient in the signaling-induced regulation of *CD45* splicing. Although we have yet to classify our various SRD mutants into complementation groups, the differences between these clones in terms of GFP expression (see Fig. 3A) and endogenous *CD45* regulation (i.e., SRD clones A8/B58 compared to others) suggest that we have at least two distinct classes of mutations.

One particular concern with regards to the feasibility of generating mutants in regulated splicing is that most alternative splicing events in mammals are under combinatorial control (Smith & Valcarcel, 2000). Thus the disruption of a single regulatory factor is unlikely to result in a complete block in regulation. Our results are consistent with this prediction, in that none of SRD mutants we have identified are fully blocked with regards to PMA-induced alternative splicing of *CD45*. We have observed similar partial inhibition of *CD45* splicing regulation upon inhibition of signaling molecules as far upstream as Erk (K. Lynch, unpubl. data) and upon mutation of individual sequence elements within the PMA-Responsive Sequences of *CD45* pre-mRNA (C. Rothrock, B. Cannon, & K.W. Lynch, in prep.). Thus, the phenotypes of our SRD mutants identified in this report are consistent with that expected upon disruption of one of multiple elements critical for *CD45* splicing regulation. In addition, we note that the isoform profile for the endogenous *CD45* gene in many of our SRD mutants in the absence of PMA stimulation is intermediate between that seen in uninduced and stimulated wild-type cells (see Fig. 4A). This may reflect the likely possibility that at least some of the factors responsible for repression of exons 4, 5, and 6 in activated cells may also exert some influence on isoform choice in resting cells.

Obviously the ultimate goal of any mutagenesis screen is to use the resultant mutant cell lines as a tool to identify the critical components of the regulation machinery by determining the causal defect in the mutants. Preliminary analysis suggests that the cellular levels of many known splicing factors, including PTB and U2AF65/35, are identical between the wild-type and SRD mutant cells. We have detected some slight difference in the ratio of SR proteins among the various mutants; however, we have yet to determine if these differences have any relevance to the splicing defect in *CD45*. A more unbiased approach to characterizing the SRD mutants would be to use a cDNA library to complement the splicing defect in these cells. To aid in complementation studies, the WT-47GFP cells used in our screen (and thus also our SRD mutants) were engineered to express a murine-derived retroviral receptor. We are currently optimizing a retroviral-expressed cDNA library derived from PMA-treated JSL1 cells, and plan to continue our characterization of the SRD mutants by selecting for cells that regain PMA-induced down-regulation of GFP following retroviral infection.

With regards to the generality of the approach described in this study, we predict that mutagenesis screens should be a feasible method to dissect most examples of alternative splicing. The greatest limitation to developing a screen for factors that govern alternative splicing is in the development of a reporter that

retains the splicing regulation of interest. The optimal design of such reporters is likely to be best determined experimentally for any given gene. Indeed, we have analyzed a number of reporters with GFP fused to differing configurations of CD45 exons, and in most of the constructs, regulation of splicing was disrupted by the presence of the GFP sequences. However, once a suitable reporter has been developed for a given splicing event, the use of genetic screens should be widely applicable to the understanding of regulated alternative splicing.

MATERIALS AND METHODS

Construction of 47GFP reporter

The sequences encompassing nt 18 of CD45 exon 4 to nt 16 of CD45 exon 7 from the minigene MG4 described previously (Lynch & Weiss, 2000) was isolated by PCR and flanked by a *Kpn*I site and Kozak sequence on the 5′ end and a *Not*I linker on the 3′ end. This was inserted into the *Kpn*I-*Not*I site of the vector pAct-Zeo, which is derived from the pcDNA3.1/Zeo vector (Invitrogen) in which the CMV promoter has been replaced with a hybrid promoter containing sequences from the chicken β-actin gene and the rabbit β-globin gene (Takata et al., 1994). The advantage of this vector is that transcription from the actin/globin promoter is minimally influenced by PMA stimulation. The GFP sequences in the 47GFP reporter were obtained by PCR from the pd2EGFP vector from Clontech (Palo Alto, California). We used primers that added a *Not*I site 5′ to the fourth nucleotide of the d2EGFP cDNA (so as to avoid the start codon), and flanked the stop codon immediately 3′ with an *Xba*I site. This GFP sequence was then insert into the *Not*I-*Xba*I site of the pAct-Zeo vector containing the CD45 sequences. The final vector, pAct-47GFP, was transfected into JSL1 cells by electroporation and stable clones were selected by resistance to Zeocin.

Cell culture and PMA stimulations

Cells were maintained as described previously (Lynch & Weiss, 2000). PMA stimulations were done by adding PMA (Sigma, St. Louis, Missouri) to a final concentration of 2 ng/mL (sorts) or 20 ng/mL (single clone analysis) to cells that were at early-log phase (~0.3 million cells/mL). The cells were then subsequently grown in the PMA+ media for 60–70 h prior to analysis.

Flow cytometry

Isolation of cell populations by fluorescence activated cell sorting was done as follows. After stimulation, 20–40 million cells were harvested by centrifugation and washed with PBS. Cells were then incubated on ice for 15 min with anti-CD69 antibody conjugated to the fluorescent label PE (BD Biosciences, San Jose, California) in a 1:100 dilution in PBS, collected by centrifugation, and washed again with PBS. Cells were then resuspended at a density of approximately 4 million/mL in PSB + 0.5% FCS and analyzed by FACS for GFP and PE fluorescence. For each sort, we collected the top 2–3% of cells that were brightest for both PE and GFP. Cells were collected into RPMI + 20% FCS, then allowed to recover for 1–2 weeks in a CO_2 incubator with periodic refreshment of media. For analysis of GFP fluorescence by flow cytometry, we repeated the protocol above, but with only 1 million cells per sample and no collection was done.

RT-PCR assay

The analysis of RNA isoform expression of the endogenous CD45 gene by RT-PCR was done as described previously (Lynch & Weiss, 2000). RT-PCR analysis of the RNA expressed from the 47GFP reporter was done similarly with the exception that 16, instead of 20, PCR cycles were used and the primers used annealed to a region just downstream of the promoter and to a region within the 5′ end of the d2EGFP coding sequence.

Western blotting

Western blotting for Erk and GFP was done as described previously (Yablonski et al., 1998). Erk antibody directed against Y691 was kindly provided by Melanie Cobb (Boulton & Cobb, 1991), and anti-GFP was obtained from Chemicon (Temecula, California). Both antibodies are rabbit polyclonal and were detected using an HRP-conjugated, goat anti-rabbit secondary antibody from Biorad (Hercules, California).

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