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SHORT REPORTS

# Study of the 2719 mutant of the c-H-*ras* oncogene in a bi-intronic alternative splicing system

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**C-H-*ras* proto-oncogene forms part of the signal transduction pathway of numerous external stimuli. This proto-oncogene is regulated by alternative splicing within its intron D due to the presence of the alternative intron D exon (IDX). The alternative splicing produces mRNA which encodes for the putative p19 protein, that lacks transforming potential. Herein, we demonstrated that SR proteins regulate the intron D splicing. Moreover, we studied the 2719 mutation of H-*ras* which has higher transforming potential than Ile12 and Val12 H-*ras* mutants and is also known to affect the 5′ splice site of the IDX. However, here we show that the 2719 mutant can still be spliced when the upstream 5′ splice-site is blocked. During these later studies, additionally, we generated a short 11 nucleotides 5′ terminal exon that was fully defined and spliced in a bi-intronic pre-mRNA. The definition of this mini-exon was also addressed in this work.**
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Alternative splicing of the c-H-*ras* gene is an important regulator of p21 mRNA. The presence of the alternative exon (IDX) between exon (E) 3 and E4A allows for the production of a second mRNA (see Figure 1) that encodes for the putative p19 protein, which lacks transforming potential (Cohen *et al*., 1989) and could act as a negative regulator of the p21 protein (Huang and Cohen, 1997). IDX was discovered when the Adenosine2719Guanosine mutant was isolated from a carcinoma cell line as having a higher transforming potential than codon 12 mutants (Cohen *et al*., 1989; Cohen and Levinson, 1988). As the Adenosine2719Guanosine mutant lowered the strength (Cohen *et al*., 1989) of the IDX 5′ splice site (5′SS), the study of this mutant could be of help in providing answers as to how the IDX 5′ SS is regulated.

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Therefore, we used human nuclear extracts to splice pre-mRNAs containing only this critical E3-D intron-E4A region to gain an indication of the level of alternative splicing – see Figure 1a, lane 2, and Codony *et al*. (2001). These experiments yielded more p21 than p19 products (see Figure 1a, lane 2); thus indicating that *in vitro* the p21 pathway is favoured in these mini-genes. Stepwise changes in SR proteins have been reported during mammary carcinogenesis (Stickeler *et al*., 1999); we then studied whether SR proteins could modulate this alternative splicing. We complemented splicing reactions with a pool of purified and native SR proteins (Figure 1a) showing that their balance and amount could favour the p19 mRNA pathway (lanes 4 and 5). High amounts of added SR proteins could completely inhibit the p21 pathway while still allowing the p19 pathway to proceed (compare lanes 3–6 of Figure 1a). This result would agree with the one reported by (Mayeda *et al*., 1993), showing that excess of the ASF/SF2 leads to internal exon inclusion, although we have not observed that this splicing pathway is regulated by ASF/SF2 (S Guil, unpublished results). The D2 intron 5′ SS was further characterized by assaying the Adenosine2719Guanosine H-*ras* mutant. The Adenosine2719Guanosine mutation is localized on the fourth base of the D2 intron (position 2719 of the c-H-*ras* gene), where a wild-type (wt) adenosine was changed to a guanosine. As expected, and according to the exon definition model of splicing, the D2 intron splicing did not occur *in vitro* when the mutant Adenosine2719Guanosine E3-D intron-E4A pre-mRNA was assayed (see Figure 1b, lane 3, and compare with lane 2, note the two p19 splicing products). Then, we mutated the E3 to a non-related 11-nt mini-exon (see Figure 2 legend). Surprisingly, this mutated pre-mRNA is still active in splicing reactions (see Figure 2a lanes 2 and 3), and we conclude that even when E3 is mutated to a very small exon (E11nt) the splicing of intron A still proceeds. Moreover, the substitution of the E3 by the E11nt resulted in intron B (D2) being spliced more efficiently in E11nt pre-mRNA than in the previous E3-D intron-E4A pre-mRNA (compare p19 products in Figure 1a, b, lanes 2, with product B (PB) in Figure 2a, lanes 2 and 3), indicating that the modifications favoured the alternative splicing of the D2 intron. To know how dependent the B intron splicing is on the two alternative 5′ SS, we mutated the

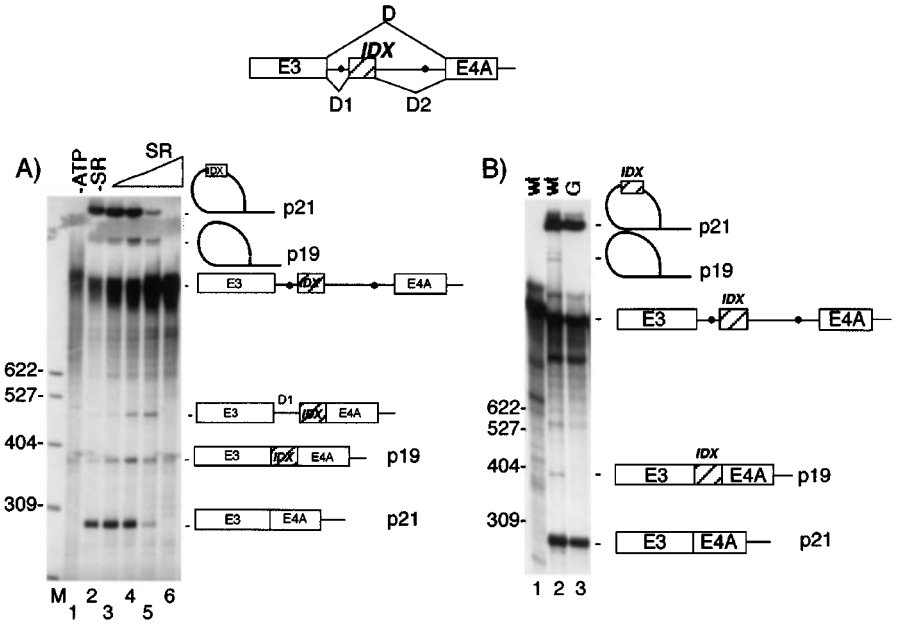


**Figure 1** Effect of the SR proteins on the alternative splicing of intron D. (**a**) Splicing reactions were complemented with a pool of native and purified SR proteins. In all figures (−) ATP indicates splicing reaction with no added ATP, other lanes show splicing reactions with ATP. Molecular weight markers are shown either on the lane M or on the left of the figures. Lane 2 did not contain SR proteins. SR proteins added in lanes: 3, 90 ng; 4, 180 ng; 5, 360 ng and 6, 720 ng. (**b**) Lane 1 and 2 contain the wild-type (wt) sequence (with N4 = A of the D2 intron 5′ SS, position 2719 of the c-H-*ras* gene), and lane 3 contains Adenosine2719Guanosine mutant (with N4 = G on the D2 intron 5′ SS). Lariat intermediates and products where mapped by RT–PCR as detailed elsewhere (Vogel *et al*., 1997). The splicing reactions assays, E3-D intron-E4A probe, RNA sequencing and RNA gels were described elsewhere (Codony *et al*., 2001). Similarly, Adenosine2719Guanosine mutant was obtained from ile12N(G) plasmid (Cohen *et al*., 1989). Total SR proteins were purified according to the technique described in Zahler *et al*. (1992)

5′ SS of the intron A. The N2 = T of the intron A 5′ SS was changed to N2 = G (X in Figure 2). Figure 2a shows that this mutation drastically reduced the splicing of the A products (notice the presence of a very low amount of the lariat A in lanes 5 and 6 in Figure 2a). When the Adenosine2719Guanosine also contained the second mutation N2 = G at intron A 5′ SS, it did not render products A (lanes 11 and 12), however now PB is obtained – notice the lariat B and PB in lanes 11 and 12 in Figure 2a and observe that these products are not so evident in lanes 8 and 9 in Figure 2a. When the intron A 5′ SS is not mutated, E4A splices more efficiently with the distal exon (either the wt E3 or the mutated E11nt), rather than with the proximal IDX in Adenosine2719Guanosine mutant. Whereas, when the intron A 5′ SS is mutated, D2 intron splicing could take place in Adenosine2719Guanosine mutant.

The fact that a 5′ terminal mini-exon is fully active in the splicing reaction, led us to study the definition of this E11nt exon, since, to our knowledge, this was the first time that such a short cap-terminal 5′ exon had been seen to be spliced in competition with a cap-distal 5′ exon. It has already been reported that CBC, cap structure and U1snRNA play an important role in cap-proximal exon definition (for a review see Lewis and Izaurralde, 1997). We therefore tested if the cap-proximal exon of the E11nt pre-mRNA also requires these factors. First, the effects of the 5′ SS mutation on intron A (see lanes 5 and 6 and lanes 11 and 12 in Figure 2a) already demonstrated that the disruption of the U1snRNA base-pairing with the cap-proximal 5′ SS did not allow for correct E11nt definition. As expected, GpppG- and $m^7$GpppG-capped E11nt were well defined – see products A lanes 3–6 in Figure 2b – and as expected less of the PA was produced when the pre-mRNAs were $m_3$GpppG and ApppG capped (see absence of the same products in lanes 7–10 in Figure 2b). This indicates that cap-structure and CBC participates in this E11nt exon definition. It was interesting to observe that not only is splicing of the intron A affected in $m_3$GpppG- and ApppG-capped pre-mRNAs, but there is also a remarkable inhibition of the products from cap-distal splicing B (compare products B in lanes 3–6 with lanes 7–10 in Figure 2b, and compare lane 2 with lanes 4 and 9 in Figure 2c). Previously, it was demonstrated that SR proteins may rescue splicing activity in depleted extracts – as U1-, U2AF- or CBC-depleted extracts – (see Crispino *et al*., 1994; Lewis *et al*., 1996; MacMillan *et al*., 1997; Tarn and Steitz, 1994). We studied whether SR proteins may rescue splicing of the $m_3$GpppG- and ApppG-capped E11nt pre-mRNA. Figure 2c shows that splicing of the cap-distal intron B is clearly activated by the presence of increasing amounts of SR proteins (see PB in lanes 4–7 and lanes 9–12 in Figure 2c), while the cap-proximal intron A splicing is rescued to a lesser extent (see products A in lanes 4–7 and lanes 9–12 in Figure

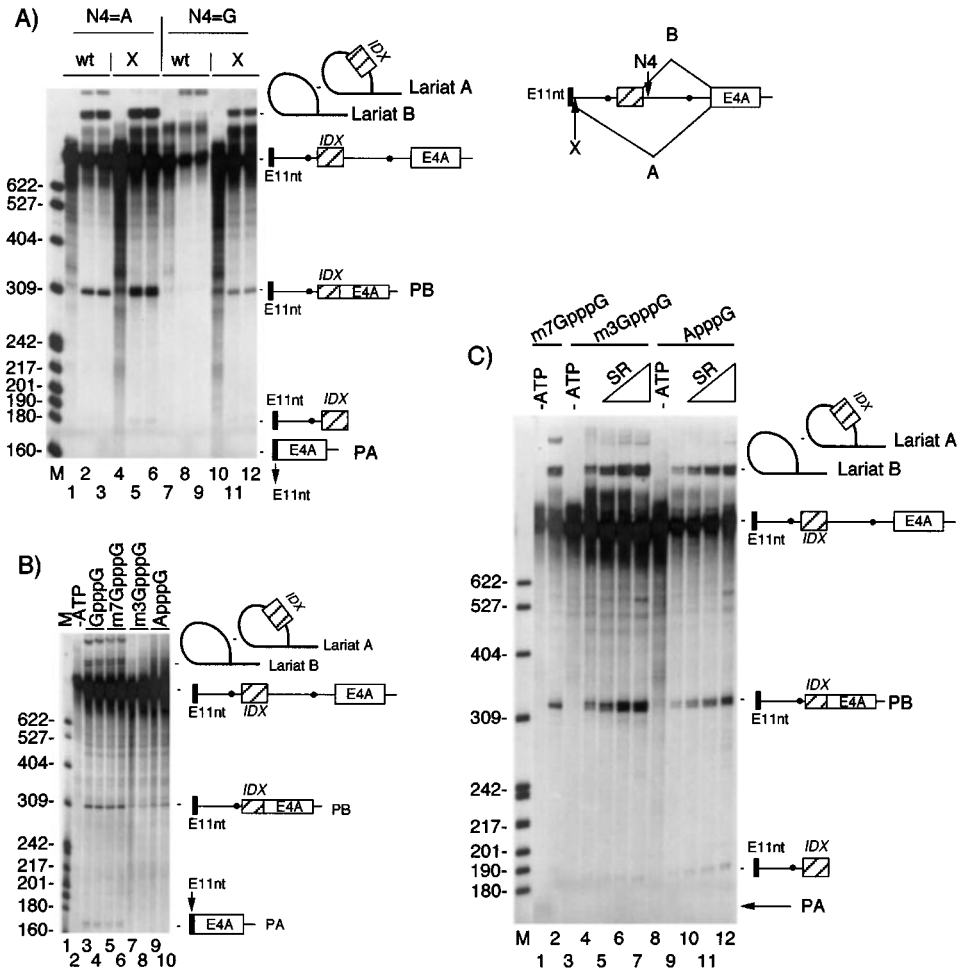


**Figure 2** Splicing of the mini-gene E11nt pre-mRNA containing wt and Adenosine2719Guanosine mutation on the D2 intron 5′ SS. For, E11nt plasmids, we first deleted E3 and cut the furthermost 5′ terminal 24-nt portion of the D1 intron, and ligated it into the Bluescript SK (−) polylinker region previously cut with *Acc*65I. Thus, the final transcribed pre-mRNA contained the 5′ terminal sequence *GGGCGAATTGG/GTAC***CTAGCC** (the D2 intron sequence is in bold and the added Bluescript SK (−) plasmid sequence is in italics). The newly created 5′ SS (similar to the consensus YRG/GTRAGT) is underlined. We called this assay 'A' for the (Δ5′)D intron splicing (cap-proximal) and 'B' for the D2 splicing (cap-distal); splicing products follow the same nomenclature: lariat A/B or PA/PB (see diagram in the upper part of Figure 2). In some gels the PA was barely detected and we therefore followed the splicing reaction by monitoring the lariat A product. (**a**) The plasmid containing the E11nt pre-mRNA was mutated at the E11nt 5′ SS by a PCR punctual protocol (Deng and Nickoloff, 1992; Zhu, 1996) with the two mutated oligodeoxynucleotides mutN2=G: GGC GAA TTG GGG ACC TAG CCA GGG; and complemmutN2=G: CCC TGG CTA GGT CCC CAA TTC GCC. The (N2=T), '(wt)', in lanes 1–3 and 7–9, was changed to (N2=G), 'X', in lanes 4–6 and 10–12. Together with this mutation on the E11nt 5′ SS, we also tested the Adenosine2719Guanosine H-*ras* mutant. N4=A is the wt pre-mRNA (in lanes 1–6) and N4=G is the mutant pre-mRNA (lanes 7–12). Each splicing reaction was performed twice (lanes 2 and 3, 5 and 6, 8 and 9, and 11 and 12) to better evaluate the level of product production. (**b**) E11nt pre-mRNA capped with the caps stated in the figure were spliced in two independent experiments (two lanes with each cap). (**c**) Shows the splicing rescue of the E11nt pre-mRNA (capped with $m_3$GpppG and ApppG) by addition of SR proteins. Amount of SRs in 25 $\mu$l: lanes 5 and 10, 30 ng; lanes 6 and 11, 60 ng; lanes 7 and 12, 120 ng

2c). The above observations showing that there is also an inhibition of the cap-distal splicing B with $m_3$GpppG- and ApppG-capped pre-mRNAs, lead us to investigate if CBC also affects splicing B. Splicing of E11nt pre-mRNA in CBC-depleted extracts (ΔCBC) showed a clear inhibition of the cap-proximal intron A – see products A in Figure 3a, and compare lane 3 with lane 4; but there is less inhibition of the cap-distal intron B and therefore products B are clearly obtained (Figure 3a). The addition of rCBC (CBP80 plus CBP20) recovers the cap-proximal product A (compare lane 6 with lanes 7 and 8 of Figure 3), but there is not a significant increase of the cap-distal product B. This result led us to conclude that only the presence of cap structural analogs alter the E11nt pre-mRNA in such a way that the sequence E11nt to 3′ end of IDX is recognized as an entire 5′ terminal exon unit; however, either by SRs rescue assays or by CBC depletion/complementation assays, E11nt pre-mRNA behaves as bi-intronic. This allows us to conclude that CBC depletion renders different results when compared to the presence of cap analogues.

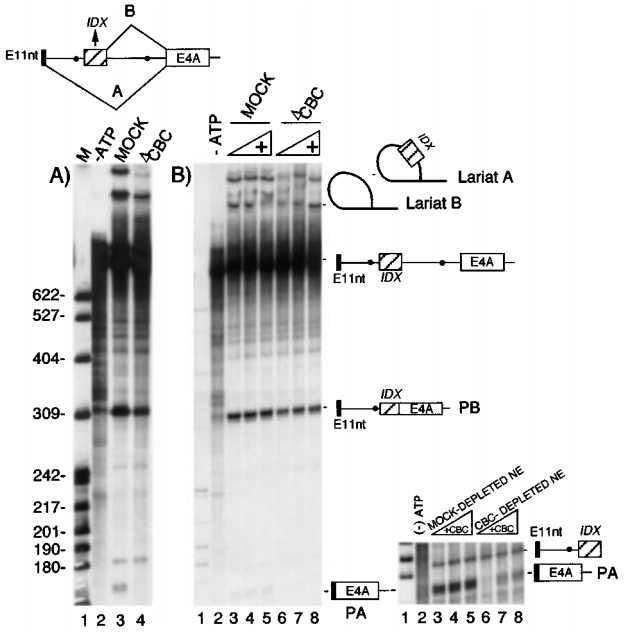


**Figure 3** CBC affects the splicing of the intron A that yields the PA product. (**a**) Lane 3 is a splicing reaction in a mock-depleted extract and lane 4 is a CBC-depleted extract (ΔCBC). (**b**) Reconstitution of the intron A splicing by complementation with rCBC (showed as +). Lanes 3–5 contained 5, 10 and 15 pg/$\mu$l of rCBC, respectively, in mock-depleted extract, and lanes 6–8 is the same but in CBC-depleted extract. The inset at the lower right of the figure shows details of an autoradiograph from the lower part of the left gel, after lengthy exposure to enhance the recuperation of the second splicing product PA, in lanes 7 and 8 (compare with lane 6), when rCBC is added to the splicing reaction. $MgCl_2$ concentration was 1 mM. Mock-depleted extract and rCBC were generously provided by Dr I Mattaj's group, and have been described in Izaurralde *et al*. (1994) and Lewis *et al*. (1996).Transcription of pre-mRNA capped with different nucleotides and rCBC complementation assays were performed as detailed in Izaurralde *et al*. (1994) and Lewis *et al*. (1996)

Our main conclusion in regard to H-*ras* alternative splicing is that SR proteins could push this towards the p19 mRNA pathway which lacks transforming potential. Interestingly, although the Adenosine2719 Guanosine mutant contains a 5′ SS mutation, it can still be spliced when the upstream 5′ SS is mutated. This led us to conclude that the IDX 5′ SS (even when mutated) undergoes a splicing reaction when the upstream 5′ SS is blocked. Hence, any specific blocking agent of the E3 5′ SS will not only block D intron splicing but also stimulate D2 intron splicing. It is interesting to observe that as few as 11 nts provide adequate room for the CBC and U1snRNA interaction and definition of the 11-nt 5′ SS. Another aspect is that although the distance between the cap and the distal IDX 5′SS is short (163 nts), but equal to the size of an average mammalian exon, CBC depletion did not drastically affect the splicing of the cap-distal intron B. Thus, depletion/complementation of rCBC did not inhibit/stimulate splicing of cap-distal intron B, as it does with the cap-proximal intron A. Therefore, the splicing of cap-distal intron B does not depend on CBC. This result agrees with a previous report (Lewis *et al*., 1996), in which the authors showed that the presence of a downstream 3′ SS – that is, of a downstream polypyrimidine track (PyT) – appears to make the cap-distal intron B independent of CBC. This observation indicates that CBC could not interact with the cap-distal U1snRNP, probably because this U1snRNP is not free due to its interactions with the 3′ SS complex across the IDX. Our results allowed us to distinguish between the CBC requirement and cap structure. It has been previously reported that cap structure only affects the cap-proximal intron in a bi-intronic pre-mRNA, for a review see (Lewis and Izaurralde, 1997). As expected, the cap structure in our bi-intronic pre-mRNA affects the cap-proximal intron A splicing, but surprisingly it also affects the distal intron B splicing (see lanes 7–10 in Figure 2b). This phenomenon is not clearly understood but we suggest that when CBC is present, cap and downstream polypyrimidine tracks are well recognized and the pre-mRNA behaves as if bi-intronic. If CBC is depleted, factors still binding to the 5′ SS of the cap-proximal exon may still recognise the $m^7$GpppG cap and the downstream PyT, and although they cannot correctly define the 5′ terminal exon to be spliced, the pre-mRNA still behaves as if bi-intronic. When the cap structure is changed to $m_3$GpppG or ApppG, either CBC or factors binding to the 5′ SS recognise neither the cap nor the downstream PyT. Therefore, the pre-mRNA behaves as monointronic, with E11nt to the 3′ end of IDX being recognized as one exon unit, and SR proteins only rescue cap-distal splicing of $m_3$GpppG and ApppG capped pre-mRNAs, in accordance with their action on any monointronic pre-mRNA. This specific SR rescue is a novel observation which has not been published in the literature until now.

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