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Footprinting the 'essential regulatory region' of the retinoblastoma gene promoter in intact human cells

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Abstract

The retinoblastoma tumour suppressor protein is a key cell cycle regulator. Protein–DNA interactions at the retinoblastoma (RB1) promoter, including the 'essential regulatory region', were investigated using novel DNA-targeted nitrogen mustards in intact human cells. The footprinting experiments were carried out in two different environments: in intact HeLa and K562 cells where the access of DNA-targeted probes to chromatin is affected by cellular protein–DNA interactions associated with gene regulation; and in purified DNA where their access is unencumbered by protein–DNA interactions. Using the ligation-mediated PCR (LMPCR) technique, the sites of damage were determined at base pair resolution on DNA sequencing gels. Our results demonstrate that, in intact cells, footprints were observed at the E2F, ATF and RBF1/Sp1 DNA binding motifs in the RB1 promoter. In addition, a novel footprint was observed at a previously unidentified cycle homology region (CHR) and at four uncharacterised protein–DNA binding sites.

In further experiments, nitrogen mustard-treated cells were FACS sorted into G_1, S and G_2/M phases of the cell cycle prior to LMPCR analysis. Expression of the RB1 gene is cell cycle-regulated and footprinting studies of the promoter in FACS-sorted cells indicated that transcription factor binding at the GC box, CHR binding motif and the 'essential regulatory region' are cell cycle dependent.

Keywords: Protein–DNA interactions; Tumour suppressor; Nitrogen mustard analogues; Footprinting; Cell cycle; FACS

1. Introduction

The retinoblastoma tumour suppressor protein (pRb) regulates cell growth at a key cell cycle restriction point that occurs prior to the G_1/S transition – in part though its interaction with E2F transcription factors. E2F are a family of transcriptional activators that consist of a heterodimer complex of one member of E2F-1 to E2F-6 and either DP1 or DP2. It is known

Abbreviations: C3-AA, acridine attached nitrogen mustard; C50-AMSA, amsacrine attached nitrogen mustard; RB, retinoblastoma; LMPCR, ligation-mediated PCR; CDE, cycle-dependent element; CHR, cycle homology region; HDAC, histone deacetylase

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that pRb has a preference for interaction with E2F-1, -2 or -3 heterodimers, whereas the retinoblastoma related proteins p107 and p130 can bind only to E2F-4 and E2F-4/-5, respectively (Kohn, 1999).

During G_0 and early G_1, the pRb is hypophosphorylated and in this state can sequester the transactivation domain of E2F forming a pRb/E2F complex that acts as a repressor of transcription. Other proteins associated with this complex include RbAp48 that may be responsible for the transcriptional repression of E2F-1 responsive genes (Nicolas et al., 2000) and histone deacetylase (HDAC) activity (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998).

pRb is hyperphosphorylated in mid/late G_1 by cyclin-dependent kinase activities that cause dissociation of the pRb/E2F inhibitor complex. E2F is then able to act as a transcriptional activator at genes whose promoters contain free E2F binding motifs. Once pRb-mediated repression is alleviated, the mammalian cell enters S-phase. Cyclin-dependent kinase activity is regulated by an array of inhibitory proteins, such as p21, that mediate normal cellular regulatory signals.

Analysis of the RB1 gene structure reveals a complex organisation of 27 exons scattered over 180 kb (Hong et al., 1989; Wiggs et al., 1988) that produce a 4.7 kb mRNA transcript that encodes a nuclear phosphoprotein consisting of 928 amino acid residues (Lee et al., 1987). The RB1 promoter is located upstream of the human RB1 gene within an unmethylated CpG-rich DNA sequence and contains no obvious CCAAT or TATA box, indicating that the transcription initiation complex is recruited to initiator-binding factors at other DNA binding motifs (De Fiore et al., 1999). A sequence within the promoter, often referred to as the 'essential regulatory region', has been shown to be important for both basal and regulated transcription. A schematic of the RB1 gene promoter showing the location of the 'essential regulatory region' and other sites of transcription factor binding is shown in Fig. 1A. Deletion analysis mapped the 'essential regulatory region' to bp 1844–1879 (labelled according to GenBank accession no. L11910) that contains the DNA binding motifs for E2F, ATF and Sp1 (Gill et al., 1994)—this region corresponds to −215 to −179 bp upstream of the ATG initiation codon. The Sp1 and ATF sites within the 'essential regulatory region' are thought to be essential activators of basal promoter activity (Sakai, Ohtani, McGee, Robbins, & Dryja, 1991). The sequences of the DNA binding motifs in the RB1 gene promoter and a summary of the transcription factors that could interact at these motifs, is shown in Table 1.

The role of transcription factors bound at the E2F binding motif at the promoter region of cell cycle-regulated genes is further complicated by their overlap with a bipartite repressor binding site, first identified at the promoter of CycA, referred to as a cycle-dependent element/cycle homology region (CDE/CHR) (Zwicker et al., 1995). The CDE motif lies within the GC rich core of the E2F binding site and consists of 5′-G/CGCGG/C-3′. The transcription factors that are thought to bind to the CDE site of CycA during G_0, are distinct from the E2F repressor complex (Liu, Lucibello Frances, Engeland, & Mueller, 1998). The CHR occurs within a few bp 3′ of the E2F/CDE site and consists of 5′-TTGG/A-3′ sequence that was identified in the promoters of CycA, cdc25C, cdc2, b-myc, survivin, Plk and the pocket protein p130 gene (Fajas et al., 2000). Further characterisation of the CDE/CHR binding proteins involved in regulation of these cell cycle-regulated genes is required prior to understanding their role, particularly in relation to the E2F repressor complex.

The mechanism by which pRb mediates RB1 promoter repression is complex and does not solely occur through interaction with E2F. It has been suggested that pRb autorepression is mediated by interactions associated with the ATF and Sp1 sites at the RB1 promoter (Gill et al., 1994). However, it has also been reported that pRb causes autoinduction (positive autoregulation) of the RB1 gene through interaction with the ATF binding site at the RB1 promoter and that pRb/ATF-2 interaction at the ATF binding site is thought to mediate this response (Park et al., 1994). Members of the Ets family of transcription factors, identical to the E4TF1-60 protein, bind to a site termed RBF1 that overlaps with the Sp1 and ATF sites of the 'essential regulatory region' and are thought to bind preferentially (Savoysky et al., 1994). p53 has been shown to bind to the promoter region at the 'p53 control element' and acts as a repressor of transcription through inhibition of the basal promoter activity (Shiio, Yamamoto, & Yamaguchi, 1992). Again the 'p53 control element' binding site occurs in the 'essential regulatory region' and overlaps with the RBF1 and ATF binding sites. Furthermore, in co-transfection assays p53 was able to stimulate or repress transcription of the RB1 gene at low or high levels, re-