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Exploring the contribution of distal P4 promoter elements to the oncoselectivity of Minute Virus of Mice

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Abstract

Minute Virus of Mice (MVM) shares inherent oncotropic properties with other members of the genus *Parvovirus*. Two elements responsible, at least in part, for this oncoselectivity have been mapped to an Ets1 binding site adjacent to the P4 TATA box of the initiating promoter, P4, and to a more distal cyclic AMP responsive element (CRE), located within the telomeric hairpin stem. Here the CRE overlaps one half-site for the binding of parvoviral initiation factor (PIF), which is essential for viral DNA replication. We used a degenerate oligonucleotide selection approach to show that CRE binding protein (CREB) selects the sequence ACGTCAC within this context, rather than its more generally accepted palindromic TGACGTCA recognition site. We have developed strategies for manipulating these sequences directly within the left-end palindrome of the MVM infectious clone and used them to clone mutants whose CRE either matches the symmetric consensus sequence or is scrambled, or in which the PIF binding site is incrementally weakened with respect to the CRE. The panel of mutants were tested for fitness relative to *wildtype* in normal murine fibroblasts A9 or transformed human fibroblasts 324 K, through multiple rounds of growth in co-infected cultures, using a differential real-time quantitative PCR assay. We confirmed that inactivating the CRE substantially abrogates oncoselectivity, but found that improving its fit to the palindromic consensus is somewhat debilitating in either cell type. We also confirmed that reducing the PIF half-site spacing by one basepair enhances oncoselectivity, but found that a further basepair deletion significantly reduces this effect.

Keywords: Parvovirus; Oncoselectivity; Cyclic AMP response element; Mutagenesis; Initiating promoter; Virus fitness

Introduction

The Parvoviridae are a family of small (20–25 nm), icosahedral, non-enveloped viruses, unique in that they package a linear single-stranded DNA genome. Several members of the genus *Parvovirus*, such as Minute Virus of Mice (MVM), LuIII and H-1, are known to be inherently oncoselective, demonstrating enhanced fitness and toxicity in many transformed human cell types, compared to their normal counterparts, under the same growth conditions *in vitro* (Rommelaere and Cornelis, 1991; Legrand et al., 1992; Deleu et al., 1999; Cornelis et al., 2005). *In vivo*, wildtype parvovirus infection can suppress the development of certain tumors, for example, H1 suppression of HeLa tumor growth in SCID mice (Rommelaere and Cornelis, 1991; Faisst et al., 1998; Cornelis et al., 2005). These viruses have additional qualities that make them promising for development into useful oncotherapeutic agents, including their low toxicity in humans and their small size, which would facilitate spread within a tumor (Rommelaere and Cornelis, 1991; Dupressoir et al., 1989; Cornelis et al., 2005).

The principal advantage of replication competent oncotropic viruses is their ability to effectively amplify their own 'dose' locally at the targeted therapeutic site through efficient viral replication in cancerous cells, potentially killing every cell within an infected tumor mass. In order for such viruses to be both efficacious and safe, they must simultaneously show little proclivity for killing normal host cells; that is, they must exhibit

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a high oncoselective index. Since this property is likely to be a major determinant of successful virotherapy, modifications to a virus being developed for oncolytic therapy are often designed to enhance this selectivity (e.g., Bischoff et al., 1996; Rodriguez et al., 1997; Hemminki et al., 2001; Everts and van der Poel, 2005). One major determinant of parvoviral oncoselectivity is the initiating viral P4 promoter. P4 drives expression of the non-structural protein, NS1 and NS2, which play multiple essential roles in the viral life cycle, and this promoter has been shown to be upregulated in *ras*-transformed rat fibroblasts (Spegelaere et al., 1991). Transient expression analysis of P4-driven reporter constructs has identified the promoter-distal cyclic AMP-Response Element (CRE) and the promoter-proximal Ets1 Binding Site (EBS), diagrammed in Fig. 1, as equivalent contributors to the oncoselectivity of P4 in such *ras*-transformed rat fibroblasts (Perros et al., 1995; Fuks et al., 1996).

Accordingly, the P4 promoter has become a favored target in attempts to develop safe, targeted and effective replication-competent oncolytic parvoviral therapeutic agents. For example, Malerba and colleagues (2003) modified the proximal region of P4 to include binding sites for Tcf, a transcription factor upregulated by *wnt* signaling, and showed that this selectively upregulated P4 activity and viral growth in cells with active *wnt* signaling, a feature common in colon cancer. Unfortunately, although this manipulation rendered both P4 promoter and P4-mutant virus Tcf-responsive, the majority of colon cancer cells tested remained poorly permissive to viral growth (Malerba et al., 2006).

An alternative approach has been to modify the existing transcription factor binding sites within the viral P4 promoter. We previously reported the effects of mutations introduced into the distal region of MVM P4 that were designed to coordinately influence the binding of the cellular protein complex parvoviral initiation factor (PIF) and other cellular transcription factors that recognize the viral CRE (Burnett and Tattersall, 2003). PIF is a heterodimeric transcription factor that recognizes two CpG-containing tetranucleotide half-sites within the left-end hairpin stem (Christensen et al., 1997b), one of which overlaps the viral CRE (Fig. 1). When one of these half-sites was eliminated from the infectious clone without mutating the CRE, NS1 expression 48 h post-transfection was comparable to *wildtype* in both A9 and 324 K cells, whereas genomic replication was blocked at the dimer intermediate, and progeny virion production was completely abrogated, characterizing PIF binding as substantially more critical for replication than for P4 promoter activity (Burnett and Tattersall, 2003).

Significantly, the spacing between PIF half-sites is flexible (Christensen et al., 1999). Analysis using a degenerate oligonucleotide selection procedure allowed derivation of a consensus PIF recognition sequence, ACGPy N_1–N_9 PuCGPy, which describes the majority of the binding sites selected by PIF complexes (Burnett et al., 2001). Furthermore, in vitro off-rate determinations suggested that a spacing of 6 basepairs between the two half-site (or 8 between the two CpG dinucleotides) was optimal, with the affinity falling off by about 25% of maximum for each basepair added or subtracted from the optimum (Burnett et al., 2001). Thus, it appears that the spacing of 5 basepairs between half-sites found in the wildtype MVM left-end is significantly sub-optimal for PIF binding.

Clearly, PIF's potential ability to bind a wide range of differently spaced, and somewhat degenerate half-sites, presents some unique possibilities for the accommodation of other binding site specificities overlapping the PIF binding region. Thus, reducing the spacing between PIF half-sites, in the context of the viral left telomere, might alter the phenotype of the virus by lowering the avidity of PIF binding, allowing greater occupancy of the CRE by other cellular transcription factors, such as members of the CREB (cAMP-responsive element binding) protein and ATF (activated transcription factor) family. The CREB protein binding sequence has generally been accepted, in the literature, as the palindrome TGACGTCA (Montminy et al., 1986; Iguchi-Ariga and Schaffner, 1989), but this factor is known to be involved in the transcriptional upregulation of the retinoblastoma gene promoter, by binding to the asymmetric sequence ACGTCAC (Sakai et al., 1991; Gill et al., 1994). The MVM left-hand origin site also contains the sequence ACGTCAC and binds purified recombinant CREB and ATF1 directly and sequesters ATF/CREB family members when incubated in cell extracts (Perros et al., 1995; Christensen et al., 1997a; Burnett, 2002). This site is also known to be involved in the basal activity of the P4 promoter and to contribute to its *ras* transformation-associated stimulation (Perros et al., 1995).

Because the CRE appears to be a significant regulator of viral oncoselectivity, we originally generated a mutant, *no-cre*, in which the wildtype sequence and spacing of the PIF half-sites

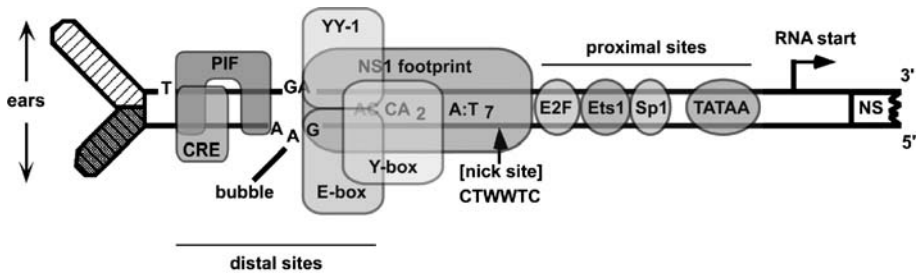

Fig. 1. The left-hand end of MVM in hairpin configuration. The left-hand telomeric hairpin lies immediately adjacent to the P4 promoter. The P4 proximal region features adjacent binding sites for cellular transcription factors E2F, Ets1 and Sp1. A binding site for viral NS1 sits between the proximal and distal regions, immediately proximal to the unpaired GAA-GA 'bubble' sequences. The P4-distal region features a CRE that overlaps one of two ACGT half-sites that coordinately recognize cellular PIF. An extrahelical thymidine lies immediately distal to the CRE.