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Development of quantitative and high-throughput assays of polyomavirus and papillomavirus DNA replication

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ABSTRACT

Polyoma- and papillomaviruses genome replication is initiated by the binding of large T antigen (LT) and of E1 and E2, respectively, at the viral origin (ori). Replication of an ori-containing plasmid occurs in cells transiently expressing these viral proteins and is typically quantified by Southern blotting or PCR. To facilitate the study of SV40 and HPV31 DNA replication, we developed cellular assays in which transient replication of the ori-plasmid is quantified using a firefly luciferase gene located in cis to the ori. Under optimized conditions, replication of the SV40 and HPV31 ori-plasmids resulted in a 50- and 150-fold increase in firefly luciferase levels, respectively. These results were validated using replication-defective mutants of LT, E1 and E2 and with inhibitors of DNA replication and cell-cycle progression. These quantitative and high-throughput assays should greatly facilitate the study of SV40 and HPV31 DNA replication and the identification of small-molecule inhibitors of this process.

Introduction

Small DNA tumor viruses such as polyoma- and papillomaviruses rely widely on the host cell DNA replication machinery to replicate their double-stranded viral genome. Eukaryotic DNA replication is a complex process that is initiated by several factors including the origin recognition complex (ORC), Cdt1, Cdc6 and the mini-chromosome maintenance (MCM) complex, the alleged cellular replicative helicase (Johnson and O'Donnell, 2005; Masai et al., 2005; Nishitani and Lygerou, 2002). In contrast, small DNA tumor viruses like polyoma- and papillomaviruses encode a single initiator protein that performs multiple functions during viral genome replication. A well-studied example is the large T antigen (LT) of simian virus 40 (SV40). This multifunctional initiator protein can successively recognize the viral origin of replication, assemble into a double hexamer that melts and unwinds the DNA ahead of the replication fork, and interact with the host DNA replication factors such as polymerase α-primase, replication protein A (RPA) and topoisomerase I (reviewed in (Borowiec et al., 1990; Bullock, 1997)). The analogous protein from papillomavirus, E1, has similar activities but also requires the viral protein E2 to initiate viral DNA replication *in vivo* (reviewed in Hebner and Laimins, 2006). Papillomavirus E2 is both a replication and transcription factor that binds with high affinity to sites in the viral origin (Androphy et al., 1987). As a replication factor, E2 interacts directly with E1 to recruit it to the origin and favor its assembly into a double hexamer (Blitz and Laimins, 1991; Lusky et al., 1994; Mohr et al., 1990).

LT and E1 are structurally related members of the helicase superfamily III (SF3) (Clertant and Seif, 1984; Hickman and Dyda, 2005; Mansky et al., 1997). The C-terminal domains of LT and E1 have ATPase/helicase activity and are sufficient for oligomerization into hexamers (Li et al., 2003; Titolo et al., 2000; White et al., 2001). The central part of both proteins contains an origin-binding domain (OBD) which recognizes specific sequences in the origin (McVey et al., 1989b; Simmons et al., 1990a; Titolo et al., 2003a, 2003b; Wun-Kim et al., 1993). The OBDs of LT and E1 differ in their primary amino acid sequence but share a common fold. Interestingly, while the LT OBD can bind with high-affinity to its target binding site as a monomer, the E1 OBD needs to dimerize to achieve comparable affinity and specificity (Fradet-Turcotte et al., 2007; Titolo et al., 2003a, 2003b). Crystal structures of the bovine papillomavirus (BPV) and human papillomavirus (HPV) 18 E1 OBDs have revealed the nature of the

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dimerization interface and mutations that disrupt this interface have been shown to impair viral DNA replication (Auster and Joshua-Tor, 2004; Enemark et al., 2002; Schuck and Stenlund, 2005; Titolo et al., 2003a). Both LT and E1 also differ substantially in their N-terminal regions, although in either case these contain regulatory elements. The N-terminal domain of LT contains a unique J-domain required for replication *in vivo* (Sullivan and Pipas, 2002), a monopartite nuclear localization signal (NLS) (Kalderon et al., 1984a, 1984b) and several phosphorylation sites for different kinases that modulate either the nuclear import of LT (Rihs et al., 1991) or its assembly into a double hexamer at the origin (Cegielska et al., 1994; Moarefi et al., 1993; Mohr et al., 1987; Scheidtmann et al., 1984; Schneider and Fanning, 1988; Virshup et al., 1989, 1992; Weisshart et al., 1999). As for the N-terminal domain of E1, it contains a bi-partite NLS, a Crm1-dependant nuclear export signal (NES), and a binding site for cyclin A/E-cdk2, which regulates E1 nucleo-cytoplasmic shuttling by phosphorylation (Deng et al., 2004; Ma et al., 1999). In addition, this domain of E1 contains a binding site for the cellular protein p80, necessary for optimal viral DNA replication (Cote-Martin et al., 2008), and a caspase-3/7 cleavage site required for amplification of the viral genome in differentiated keratinocytes (Moody et al., 2007).

Expression of LT and E1/E2 has been shown to be sufficient to support replication of a plasmid encompassing their cognate origin in transiently transfected cells. This type of assay has been used extensively to define specific DNA sequences required for origin–function *in vivo*. For SV40, the 64-bp core (nt 5193 to 34) was identified as the minimal origin however, replication was shown to be maximal when the adjacent 21-bp repeat region was included (Bergsma et al., 1982; Lee-Chen and Woodworth-Gutai, 1986). The middle portion of the core contains four binding sites for LT (5′-GAGGC-3′), arranged as two pairs of inverted repeats. This middle region known as site II is flanked on one side by an AT-rich region and on the other by the early palindrome (EP) (Borowiec et al., 1990; Bullock et al., 1997; Deb et al., 1987; DeLucia et al., 1983; Parsons et al., 1990; Tegtmeyer et al., 1983). Similarly, the HPV origin (nt 7721 to 100 for HPV31 (Frattini and Laimins, 1994)) was found to contain four binding sites for E1 (5′-ATTGTT-3′), also arranged as two pairs of inverted repeats, together with an AT-rich region (Chen and Stenlund, 2001; Holt and Wilson, 1995; Lee et al., 1997; Mendoza et al., 1995; Sun et al., 1996; Titolo et al., 2003a; Ustav et al., 1991). In addition and specific to HPV, the origin of replication contains three binding sites for the E2 protein (5′-ACCN$_6$GGT-3′) (Androphy et al., 1987).

Replication of an origin-containing plasmid (ori-plasmid) by LT, or E1/E2, in transiently transfected cells is typically detected by Southern blotting or PCR (Del Vecchio et al., 1992; Taylor and Morgan, 2003). In these assays, plasmid replication can be detected from either total or low-molecular weight DNA (i.e. Hirt-extracted DNA) that has been digested with *Dpn*I to restrict any input transfected ori-plasmid that failed to replicate and thus retained a methylation pattern characteristic of bacteria. The numerous steps involved in these transient DNA replication assays have limited their use for high-throughput studies such as those involving the characterization of large number of mutations or the screening of chemical or siRNA libraries. To overcome this limitation, we have developed facile and quantitative assays of SV40 and HPV31 DNA replication that rely on a dual-luciferase readout to measure the amount of replicated origin DNA directly from transfected cells.

Results and discussion

Development of a novel SV40 DNA replication assay based on a dual-luciferase readout

To facilitate the study of SV40 DNA replication, we set out to develop a cellular assay in which transient replication of an origin-containing plasmid (ori-plasmid) by large T antigen (LT) would result in increased expression of a firefly luciferase (Fluc) reporter gene encoded on the same ori-plasmid. A previous study has demonstrated that LT could boost the expression of several genes, including Fluc, present in cis of the SV40 origin (de Chasseval and de Villartay, 1992), thus making it likely that this strategy could be adapted to quantify SV40 DNA replication. First, we constructed a plasmid containing both

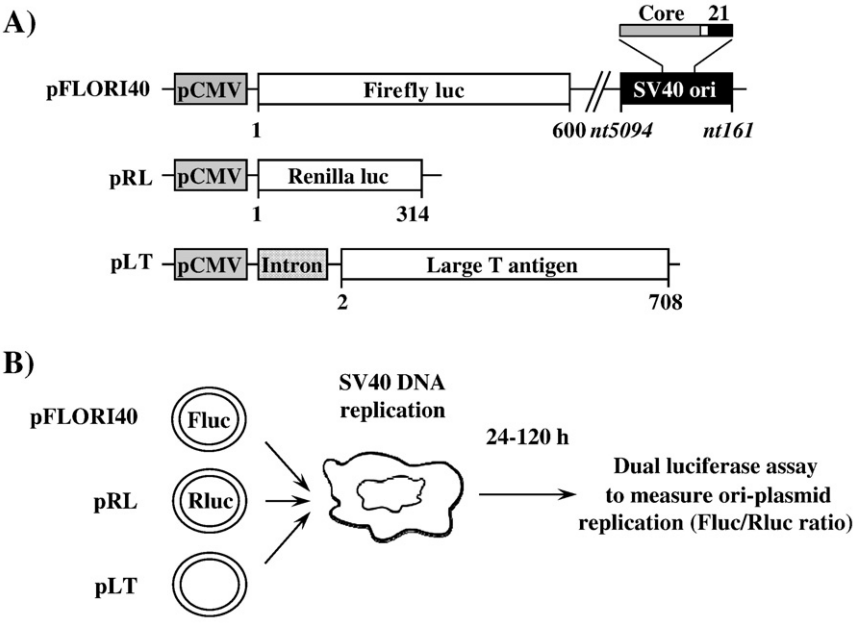

Fig. 1. Principle of the luciferase SV40 DNA replication assay. (A) Schematic representation of the three plasmids used in the assay. The name of each plasmid is written on the left. The location of the SV40 origin of replication is represented by a black box with the position of the core (grey) and 21 bp-repeat regions (black) enlarged above. The nucleotide (nt) sequence boundaries of the origin are indicated. The locations of the CMV promoter and intron are indicated by dark and light grey boxes, respectively. The coding regions of firefly and Renilla luciferase as well as those of LT are indicated by white boxes. Amino acid boundaries of each protein are indicated below each box. (B) Schematic representation of the assay. A plasmid expressing SV40 LT (pLT) is co-transfected in cells along with a second plasmid containing the SV40 origin of replication (pFLORI40) and a firefly luciferase reporter gene. A third plasmid expressing Renilla luciferase (pRL) is also transfected as an internal control to normalize for variations in transfection efficiency. Viral DNA replication is measured using a dual-luciferase assay, at different times post-transfection.