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Short communication

Single-cell chemiluminescence imaging of parvovirus B19 life cycle

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ABSTRACT

Human parvovirus B19 (B19V) is a single-stranded DNA virus. The genome encodes a multifunctional non-structural protein (NS), two capsid proteins (VP1, VP2) and other small non-structural proteins (7.5 kDa, 9 kDa, 11 kDa). Within sensitive cells, B19V can achieve a productive replicative cycle or, on the contrary, establish persistence; differences in its expression profile have been yet investigated following *in vitro* infections by methodologies enabling information on the entire infected cell population. Conversely, the present study reports quantitative data on the production of B19V analytes (DNA, RNAs, and proteins) at single cell-level, underlining cell-to-cell differences through the viral specific macromolecular synthesis process. Microscope imaging assays (*in situ* hybridization and immunocytochemical assays), exploiting chemiluminescence as principle detection and targeting viral nucleic acids and antigens, have been performed on a permissive cell line following *in vitro* infection. Chemiluminescence, involving the emission of photons deriving from a chemical reaction, provided the localization and quantitative detection of analytes down to a few molecules within infected cells. In our experimental conditions, B19V transcriptional activity, leading to the production of NS and VP RNAs, has been detected early in the viral cycle (from 12 h post-infection, hpi) and before genome replication, starting at 24 hpi. The analysis of VP RNAs and related proteins suggested an inhibitory effect on capsid protein translation, as a post-transcriptional regulation events. Indeed, high levels of VP transcripts have been detected at early stages of infection while the proteins accumulated within cells only at 48–72 hpi.

Human parvovirus B19 (B19V), member of the *Erythrovirus* genus in the *Parvoviridae* family, is a widely spread human pathogenic virus (King et al., 2011; Gallinella, 2013). The genome, a single-stranded DNA molecule, 5596 bases in length, is composed of a unique internal region, containing the coding sequences for viral proteins, flanked by two repeated inverted terminal regions. Close to the left terminal region, the p6 promoter is able to direct the transcription of pre m-RNAs that undergo different splicing and cleavage-polyadenylation events with the production of at least thirteen classes of mature viral transcripts (Fig. 1).

The unspliced mRNAs, ending in the middle of the genome, encode a multifunctional non-structural protein (NS); other classes of RNAs, ending at the right of the genome, are single or double-spliced and encode two capsid proteins (VP1 and its amino-terminal truncated version VP2). Small RNAs coding for additional small non-structural proteins (7.5 kDa, 9 kDa and 11 kDa) complete the B19V transcriptome.

In the replicative cycle of B19V within permissive cells, the first step on viral macromolecular synthesis is the conversion of the incoming single-stranded genome in a double-stranded molecule; then, the viral genome becomes active starting transcriptional and replicative activities in a complex interaction with cellular machinery and cell cycle phases, as for other viruses in the *Parvoviridae* family (Gallinella, 2013).

B19V transcription and replication have been extensively investigated *in vitro* on primary cells and cell lines presenting different degrees of viral permissiveness (Luo et al., 2011; Wong et al., 2008; Munakata et al., 2006; Zakrzewska et al., 2005; Bonvicini et al., 2008a,b, 2006). Among cell lines, UT7/EpoS1 megakaryoblastoid cells are the most widely used because they can support B19V productive infection even if with a very low viral yield (Zhi et al., 2006; Guan et al., 2008; Bonvicini et al., 2008a, 2008b). The methodologies commonly used for viral nucleic acids detection are the southern/northern blot analysis (Shimomura et al., 1993; Gallinella et al., 2000), RNAse protection assays (Guan et al., 2011), and quantitative PCR-based assays (Bonvicini et al., 2008a,b; Wong and Brown, 2006). A chemiluminescence (CL) *in situ* hybridization (ISH) imaging assay has been previously developed for monitoring B19V replication kinetics following UT7/EpoS1 infection (Bonvicini et al., 2007). Differently from blotting and data obtained by PCR assays, that represent an averaged result over the entire cell population, the CL microscope imaging method allowed evaluating

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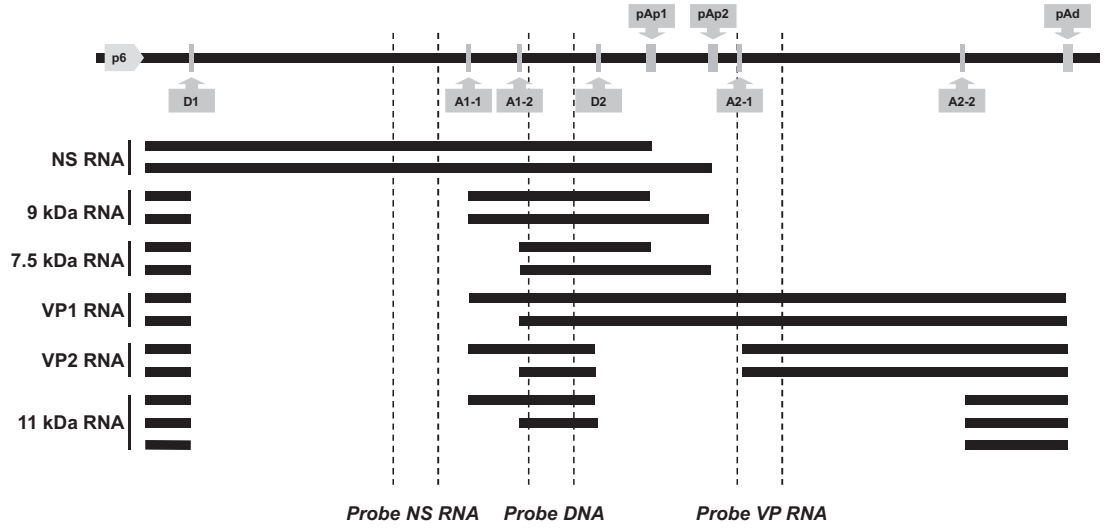

Fig. 1. Schematic illustration of B19V genome (NC_000883 in the NCBI Genome Database) and probes used in the present study. B19V genetic map (Ozawa et al., 1987; Yoto et al., 2006; Bonvicini et al., 2008a, 2008b) is shown with p6 promoter, splice donor sites (D1 and D2) and splice acceptor sites (A1-1, A1-2, A2-1, and A2-2), proximal and distal cleavage-polyadenylation sites (pAp1, pAp2 and pAd). Probe NS RNA (nt 1882–2033), probe DNA (nt 2210–2355) and probe VP RNA (nt 3180–3442) are diagrammed.

the fraction of infected cells and the amount of viral nucleic acids within the single cell. The peculiarity of CL imaging is that the light emission derives from a chemical reaction and is therefore highly specific for the used probe and unaffected by sample matrix constituents, which often occur when photoluminescence is used. The CL imaging allows obtaining not only the localization, but also a quantitative detection of target analytes, being the signal intensity proportional to the amount of enzyme label over a wide range of concentrations and down to a few molecules (Mirasoli et al., 2009; Roda et al., 2007; Bonvicini et al., 2013).

The present study reports quantitative data on the virus-specific macromolecular synthesis process in UT7/EpoS1 cells obtained by five different CL imaging assays, each one specific for B19V DNA, NS and VP1-2 transcripts, NS and VP1-2 proteins. The analytical methods rely on biospecific molecular recognition mechanisms (nucleic acids hybridization and antigen-antibody binding) and ultrasensitive CL detection of targets.

UT7/EpoS1 cells have been infected with B19V at a multiplicity of infection of 10^3 viral genome/cell following a previously optimized protocol (Bonvicini et al., 2008a, 2008b). At different time points post-infection (2, 12, 18, 24, 36, 42, 48 and 72 hpi), 50.000 cells have been collected and spotted on multi-well slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofer, Germany), fixed on methanol/acetone for 10 min at −20 °C and incubated for 2 h with PBS-BSA 2% (Phosphate Buffered Saline-Bovine Serum Albumin). As negative control, a B19V-mock infection has been performed and cells have been harvested at the same times post-infection.

Viral DNA and specific transcripts have been detected and quantified by CL-ISH assays as previously described with slight modifications (Bonvicini et al., 2007). In particular, cells before hybridization reactions have been incubated for 1 h at 37 °C with either 10 μg/mL RNase (DNA-free, Roche) or 10 U/mL DNase (DNase I recombinant, RNase-free, Roche) for B19V DNA or RNA analysis, respectively. Different digoxigenin-labeled B19V DNA probes of similar length and base composition, prepared by incorporating labeled dUTP (DIG DNA Labeling Mix, 10× concentration, Roche) in standardized PCR reactions, have been used in the hybridization procedures (10 ng of probe/25 μL of hybridization buffer); the dNTP labeling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-dUTP) used in the PCR reaction allows for the incorporation of DIG-dUTP every 20–25 nucleotides in the amplified product. B19V probes have been designed to hybridize specific sequences in B19 genome or transcripts (Fig. 1 and Table 1).

For DNA analysis, the specific probe and pretreated samples have been denatured by heating at 95 °C for 5 min, while for RNA, either NS or VP probes and pretreated samples have been denatured at 65 °C for 5 min. Subsequently, hybridization reactions have been performed at 37 °C for 12 h and hybrids have been revealed within cells with a horseradish peroxidase (HRP) anti-digoxigenin antibody, Fab fragments from sheep, (Roche) diluted 1:100 in PBS/BSA 1% for 1 h, followed by washing with PBS and CL SuperSignal West dura reagent (Thermo Fisher Scientific, Waltham, MA) addition. The specificity of hybridization procedures between probes and targets has been assessed performing DNA detection on samples treated with DNase, and RNA detection on samples RNase digested.

Viral proteins have been analyzed by means of two CL immunocytochemical (CL-ICC) assays. Briefly, after fixation and blocking procedures, cells were incubated with either the monoclonal mouse antibody against VP1 and VP2 proteins (MAB8293; Chemicon) diluted 1:200 in PBS/BSA 1% or the monoclonal human antibody against NS protein (a kind gift of S. Modrow, University of Regensburg, Regensburg, Germany) diluted 1:200 in PBS-FCS 10%

Table 1
Digoxigenin-labeled B19V probes.

Name	Position	Sequence (5′–3′)	Product size	GC content	*T* content	Annealing temperature (°C)
Probe NS RNA	1882–1901 2033–2014	GCGGGAACACTACAACAACT GTCCCAGCTTTGTGCATTAC	151 bps	44.1%	33	49
Probe DNA	2210–2229 2355–2336	CGCCTGGAACACTGAAACCC GAAACTGGTCTGCCAAAGGT	145 bps	54%	33	52
Probe VP RNA	3180–3199 3342–3323	TGGGTTTCAAGCACAAGTAG TGCACCAGTGCTGGCTTCTG	163 bps	46%	38	49

Position and sequence are referred to NC_000883 in the NCBI Genome Database.