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A Parvovirus B19 synthetic genome: sequence features and functional competence

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ABSTRACT

Central to genetic studies for Parvovirus B19 (B19V) is the availability of genomic clones that may possess functional competence and ability to generate infectious virus. In our study, we established a new model genetic system for Parvovirus B19. A synthetic approach was followed, by design of a reference genome sequence, by generation of a corresponding artificial construct and its molecular cloning in a complete and functional form, and by setup of an efficient strategy to generate infectious virus, via transfection in UT7/EpoS1 cells and amplification in erythroid progenitor cells. The synthetic genome was able to generate virus with biological properties paralleling those of native virus, its infectious activity being dependent on the preservation of self-complementarity and sequence heterogeneity within the terminal regions. A virus of defined genome sequence, obtained from controlled cell culture conditions, can constitute a reference tool for investigation of the structural and functional characteristics of the virus.

1. Introduction

Human Parvovirus B19 (B19V) is a human pathogenic virus, member of the Erythroparvovirus genus in the Parvoviridae family (Cotmore et al., 2014). Infection is widespread and can be associated with an ample range of pathologies and clinical manifestations, whose characteristics and outcomes depend on the interplay between viral properties and the physiological and immune status of the infected individuals. B19V shows a selective tropism for erythroid progenitor cells in the bone marrow, exerting a cytotoxic effect and causing a block in erythropoiesis that can manifest as transient or persistent erythroid aplasia. Common manifestations of infection are erythema infectiosum in children or post-infection arthropathies mainly affecting adults, and the virus has been implicated in a growing spectrum of other different pathologies, among them myocarditis, encephalitis and connective tissue diseases. Infection in pregnancy may be transmitted to the fetus, leading to possible fetal death and/or hydrops fetalis (Gallinella, 2013; Qiu et al., 2017).

Structural features of B19V are common to viruses in the family (Gallinella, 2013; Qiu et al., 2017). One molecule of linear single-stranded DNA of either positive or negative polarity, about 5600 nt in length, is encapsidated in T=1 isometric virions, approximately 25 nm in diameter. The genome is composed of a unique internal region, 4830 nt long, containing all the coding sequences, flanked by two inverted, repeated terminal regions, 383 nt long, that serve as origins of replication. Within these, the distal 365 nt present a site of dyad sequence symmetry that allows for the single-stranded DNA molecule to adopt terminal stem and loop hairpin structures. Due to the occurrence of distinct sequence asymmetries, each terminal region can be present in either one of two alternative sequences, each the inverted complement of the other (usually referred to as flip/flop), and different combinations of these alternative sequences at both termini can give rise to four different genome isomers (Luo and Qiu, 2015).

In vitro replication of B19V is limited to a few cell types (Bonvicini et al., 2006; Gallinella et al., 2000). As an appropriate experimental system, an expanding population of differentiating erythroid progenitor cells (EPCs) can be obtained from peripheral blood mononuclear cells (PBMC) (Filippone et al., 2010; Wong et al., 2008). Such cellular system replicates in vitro the process that occurs in vivo in the bone marrow environment (Hattangadi et al., 2011; Merryweather-Clarke et al., 2011), and, notwithstanding its complexity, it is so far the best cellular model for supporting B19V replication and production of infectious virus. In this system, permissiveness to viral replication and infectious virus production is higher for cells at the proerythroblast stage, while restriction either to replication or release of infectious virus applies to cells at earlier or later stages, respectively (Bua et al.,

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2016). Apart from EPCs, only a few myeloblastoid cell lines, in particular the UT7/EpoS1 cells (Wong and Brown, 2006), can also support viral replication, but in these systems only a very limited production of infectious virus is obtained.

The availability of native virus from clinical isolates is still a major requirement for in vitro infection experiments, and genetic studies, as well as the possibility of obtaining recombinant viruses, have lagged far behind those of other viruses in the family. Central to the development of genetic studies for B19V is the availability of complete genomic clones that may possess functional competence. Clone pB19-M20 first showed the ability to replicate and generate infectious virus following transfection in UT7/EpoS1 cells (Zhi et al., 2004). Further systematic analysis on pB19-M20 indicated the relevance of the terminal regions and of the different coding sequences for the maintenance of the functional competence of the cloned viral genome and its ability to generate infectious virus (Zhi et al., 2006). Moreover, a comparative analysis between different available genomic clones of B19V obtained from different viral isolates, pB19-M20, pB19-FL and pHG1, demonstrated a critical role of the catalytic domain of the viral phospholipase (PLA2-like motif in the VP1u region) for the maintenance of the infectivity of virus produced following transfection of the cloned viral genomes (Filippone et al., 2008). Finally, genomic clones may be complemented by helper virus functions and show replicative activity in otherwise non-permissive environments (Guan et al., 2009).

However, on the whole, none of these systems proved so far easily amenable to experimental manipulation or capable of generating infectious virus at significant levels, and additional effort to obtain a more coherent and efficient system to generate virus from cloned genomes may prove worthwhile. In particular, in this respect, a few issues should be addressed, namely: i) cloning the genome in a complete and functional form, reducing the system complexity and instability; ii) investigating the characteristics of the terminal regions relevant to the functional competence of the cloned genome; iii) avoiding any sequence bias, due to the stochastic effect of sampling from a particular isolate, with possible effects on the functional competence of the cloned genome; iv) optimizing the whole experimental procedure, in order to increase the yield of virus that may be recovered, and obtain a stock of virus effectively paralleling the infectivity of native virus.

In our study, we sought to address the above issues and establish a new model genetic system for Parvovirus B19. A synthetic approach was followed, by design of a consensus reference genome sequence as a working tool, by generation of a corresponding artificial construct and its molecular cloning in a complete and functional form, and by setup of an efficient strategy to generate infectious virus, via transfection in UT7/EpoS1 cells and amplification in successive rounds of infection in EPCs.

2. Materials and methods

2.1. Bioinformatic analysis

A set of B19V genomic sequences, genotype 1(a), deposited in the NCBI nucleotide database, were selected and retrieved for analysis (listed in the supplemental sequence dataset). Sequences were aligned and analyzed using the Clone Manager 9 Professional Edition Software (Scientific & Educational Software) and MEGA6 Software (Tamura et al., 2013).

2.2. Molecular cloning

Synthetic DNA inserts, cloned in pIDT vector plasmid, were obtained from IDT technologies. Restriction endonuclease (RE) and ligase enzymes were obtained from NEB and used according to manufacturer's directions. Subsequent manipulations and cloning steps were carried out by standard procedures. Plasmid clones were maintained by transforming SURE bacterial cells (Invitrogen) under ampicillin selection and subsequent growth in LB medium at 37 °C. Plasmid DNA purification was performed by Endofree Plasmid Kit (Qiagen). Inserts used for transfection assay were either excised from vector plasmid by RE cleavage, or amplified by PCR by using the Expand High Fidelity System (Roche) and primer HJ0 (pTGTCTTCTTTTAAATTTT). Plasmid derived inserts or PCR products were further separated by gel electrophoresis, purified by using Gel Extraction Kit (Qiagen) and quantified by UV absorbance determination.

2.3. Cells

UT7/EpoS1 cells were cultured in IMDM (Cambrex), supplemented with 10% FCS and 2 U/ml Epo (NeoRecormon, Roche), at 37 °C and 5% CO_2. Cells were kept in culture at densities between 2×10^5-1×10^6 cells/ml, and used for transfection experiments when at a density of 3×10^5 cells/ml. Erythroid progenitor cells (EPCs) were generated in vitro from peripheral blood mononuclear cells (PBMC) obtained from the leukocyte-enriched buffy coats of healthy blood donors, available for institutional research purposes according to the policy approved by the local Ethical Committee (S.Orsola-Malpighi University Hospital). PBMC were isolated using centrifugation in Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB) and cultured in a medium containing erythropoietic growth and differentiation factors, following previously established protocols with minor modifications (Bua et al., 2016). Isolated PBMC were cultured in IMDM supplemented with 20% serum substitute BIT 9500 (StemCell Technologies), and enriched with 900 ng/ml ferrous sulphate, 90 ng/ml ferric nitrate, 1 μM hydrocortisone (Sigma), 3 U/ml Epo (NeoRecormon, Roche), 5 ng/ml IL-3 and 20 ng/ml stem cell factor (Life Technologies). Cells were used for infection experiments when at day 8 of in vitro growth and differentiation.

2.4. Transfection

UT7/EpoS1 cells were transfected by using the Amaxa Nucleofection System (Lonza), with V Nucleofector Reagent and T20 program setting, at a ratio of 1 μg insert DNA for 10^6 cells. Following transfection, the cells were incubated at 37 °C in complete medium at an initial density of 10^6 cells/ml. Constant amounts of cell cultures were collected up to 12 days post-transfection (dpt), cells and cell-free supernatants (spn) were separated by centrifugation at 5000 rpm for 5 min, then used for analysis and/or successive infection experiments.

2.5. Infection

Cell-free supernatants obtained from transfected UT7/EpoS1 cells were used to infect EPCs cells at a ratio of 100 μL for 1×10^6 cells. As a control, B19V from a serum sample, identified in our laboratory in the course of routine diagnostic analysis and available for research purposes according to Italian privacy law, was used at a moi of 10^4 geq/cell (Bonvicini et al., 2013). The infection was carried out at 37 °C for 2 h, then cells were washed free of inoculum and expanded in complete medium at an initial density of 10^6 cells/ml. Constant amounts of cell cultures were collected at 2 and 48 h post-infection (hpi) and at 6 days post-infection (dpi), cells and cell-free supernatants (spn) were separated by centrifugation at 5000 rpm for 5 min, then used for analysis and/or successive infection experiments.

2.6. FISH ssay

Equal amounts of cell cultures, corresponding to 1.5×10^5 cells, collected at the appropriate time points following transfection or infection were processed by on slide FISH assay for the detection of viral nucleic acids, as described (Manaresi et al., 2015) with minor