Hypoxic-response elements in the oncolytic parvovirus *Minute virus of mice* do not allow for increased vector production at low oxygen concentration

Charlotte Servais,1 Perrine Caillet-Fauquet,2 Marie-Louise Draps,2 Thierry Velu,1 Yvan de Launoit2,3 and Annick Brandenburger1

Correspondence
Annick Brandenburger
abranden@ulb.ac.be

1Laboratoire de Cytologie et de Cancérologie Expérimentale, IBMM-IRIBHM, Université Libre de Bruxelles, 12 rue des Professeurs Jeener et Brachet, B-6041 Gosselies, Belgium
2Laboratoire de Virologie Moléculaire, Faculté de Médecine, Campus Erasme, Université Libre de Bruxelles, Route de Lennik 808, B-1070 Bruxelles, Belgium
3UMR 8117 CNRS, Université de Lille 1, Institut Pasteur Lille, Institut de Biologie de Lille, Lille, France

Vectors derived from the autonomous parvovirus *Minute virus of mice*, MVM(p), are promising tools for the gene therapy of cancer. The validation of their in vivo anti-tumour effect is, however, hampered by the difficulty to produce hightitre stocks. In an attempt to increase vector titres, host cells were subjected to low oxygen tension (hypoxia). It has been shown that a number of viruses are produced at higher titres under these conditions. This is the case, among others, for another member of the family Parvoviridae, the erythrovirus B19 virus. Hypoxia stabilizes a hypoxia-inducible transcription factor (HIF-1α) that interacts with a 'hypoxia-responsive element' (HRE), the consensus sequence of which (A/GCGTG) is present in the B19 and MVM promoters. Whilst the native P4 promoter was induced weakly in hypoxia, vector production was reduced dramatically, and adding HRE elements to the P4 promoter of the vector did not alleviate this reduction.

Hypoxia has many effects on cell metabolism. Therefore, even if the P4 promoter is activated, the cellular factors that are required for the completion of the parvoviral life cycle may not be expressed.

The parvovirus *Minute virus of mice*, MVM(p), replicates preferentially in oncogenic-transformed cells, where it completes a lytic life cycle (Rommelaere & Cornelis, 1991). This property is exploited in gene-therapy vectors derived from autonomous paroviruses (Russell et al., 1992; Cornelis et al., 2004). The MVM(p) genome is composed of two transcription units, encoding two sets of viral proteins, NS (non-structural) and VP (capsid), under the control of the P4 and P38 promoters, respectively (Cotmore et al., 1983). The non-structural protein NS1 plays an important role in viral DNA replication (Nüesch et al., 1998), is toxic in transformed cells (Caillet-Fauquet et al., 1990) and is required for transactivation of the P38 promoter, which is otherwise only expressed at a very low basal level (Rhode, 1985).

The mechanisms underlying the preferential expression of MVM(p) in transformed cells are only poorly understood, but it is at least in part linked to the upregulation of the P4 promoter in these cells compared with normal cells. We therefore wanted to determine whether P4 activity could be further enhanced and whether this could improve vector production. We have explored the possibility of producing MVM vectors under low oxygen tension (hypoxia). Gene expression and production of the human erythrovirus B19 virus are increased under these conditions (Caillet-Fauquet et al., 2004; Pillet et al., 2004). Regulation of promoter activity in hypoxia occurs through the interaction of the transcriptional regulator hypoxia-inducible factor (HIF-1) with a 'hypoxia-responsive element' (HRE) (Semenza, 2001). The MVM(p) promoter contains four putative HREs (consensus sequence A/GCGTG), two in each of the sense and antisense orientations.

The presence of the HRE in the promoter region is necessary, but not sufficient, for promoter regulation. Other cellular elements are probably involved in this regulation and the effect of hypoxia must be determined for each virus–host-cell combination (Ebbesen & Zachar, 1998). Replication of some viruses, such as human immunodeficiency virus (Polonis et al., 1991), Sendai virus (Ebbesen et al., 1991) and Kaposi’s sarcoma-associated herpesvirus (Davis et al., 2001), is increased in hypoxia. Production of other viruses, like *Simian virus 40* (SV40) (Riedinger et al., 1999)
and adenovirus (Pipiya et al., 2005; Shen & Hermiston, 2005), on the other hand, is reduced. The addition of HRE sequences to hypoxia-insensitive promoters leads to an increase of gene expression in hypoxia (Shibata et al., 2000; Ruan et al., 2001; Jeong et al., 2005; Wang et al., 2005).

HIF-1 is a heterodimer between HIF-1α and HIF-1β, the former being stabilized in hypoxia. The presence of Co ions in the culture medium also stabilizes HIF-1α, thus mimicking the effect of hypoxia (Wang & Semenza, 1995).

Vectors derived from MVM(p) retain the NS transcription unit; the transgene is substituted for capsid-encoding sequences under the control of the viral P38 promoter (Russell et al., 1992; Clément et al., 2002). We have investigated the effect of low oxygen tension and CoCl₂ on the activity of the P4 promoter and vector production.

The presence of four HRE consensus sequences (A/GCGTG) in the P4 promoter of MVM (Fig. 1d) could allow for the modulation of its activity by hypoxia. An upregulation of P4 would lead to a higher expression of the non-structural protein NS1, leading to increased DNA amplification and expression of capsid proteins in wild-type virus or transgene expression in vectors, following transactivation of the late promoter P38. P4 activity was monitored in three cell lines, human NBE (new-born kidney) and 293T (derived from HEK293) and simian COS-7, that have all been used for the production of vectors derived from MVM(p) (Brandenburger & Velu, 2004). Cells were transfected by FuGENE 6 (Roche) with the reporter plasmid pP4-luc, which expresses the firefly luciferase under control of the early parvoviral P4 promoter. Four hours after transfection, medium was replaced and cells were incubated for 24 h either in a chamber containing 20% O₂ and 5% CO₂ (normoxic conditions) or in an Anoxomat Mart 8000 anaerobic chamber filled with a gas mixture containing 5% CO₂, 89% N₂ and 6% O₂ (hypoxic conditions). A control plasmid, pGL3-luc, was transfected in parallel. This plasmid expresses luciferase from the SV40 promoter, which has been shown to be insensitive to oxygen tension (Wang et al., 2005). Luciferase activity was measured in cell lysates with the luciferase assay system (Promega). P4 activity was upregulated under hypoxia in NBE (2-5-fold) and COS-7 (3-7-fold) cells, but not in 293T cells (Fig. 1a). The latter cells were very sensitive to reduced oxygen tension and showed an important lethality in hypoxia. Growth of NBE and COS-7 cells was not

![Fig. 1. Effect of oxygen tension and Co ions on P4 promoter activity. (a) Potential vector-producing cells were transfected with the reporter plasmids pP4-luc (MVM P4 promoter; filled bars) or pGL3-luc (SV40 promoter; shaded bars) and incubated for 24 h at normal oxygen tension (20%) or in hypoxia (6 or 1%). (b, c) NBE cells were incubated with different concentrations of CoCl₂ for (b) 24 h or (c) 48 h after transfection. Luciferase activity is expressed relative to expression in normoxia. It was determined for two dilutions of cell extract. Results are from: (a) four (NBE), two (COS-7) or one (293T) experiment(s); (b, c) four (0–200 μM), three (400 μM) or two (600–1000 μM) experiments. (d) HRE consensus sequences in the MVM(p) P4 promoter region are shown with their orientation (black arrows). The grey arrow indicates the transcription-initiation site for the NS messenger. Coordinates are according to Astell et al. (1986).](image-url)
affected significantly in hypoxia over a period of 4 days (results not shown). Further decreasing oxygen tension to 1% in NBE and COS-7 cells did not increase P4 promoter activity [Fig. 1a and results not shown (COS-7)]. P4 activity was upregulated similarly in NBE cells in the presence of Co ions (Fig. 1b, c). At high CoCl₂ concentrations (above 200 μM), P4 activity was further increased, especially after a 48 h treatment (Fig. 1c), but these concentrations were cytotoxic (results not shown). P4 could be upregulated via HIF-1 because HIF-1α was stabilized following growth of NBE cells both in the presence of CoCl₂ and in hypoxia at days 1 and 2 (results not shown). For the detection of HIF-1α, cell extracts were prepared 24 or 48 h after treatment in hypoxia or CoCl₂ in the presence of protease and phosphatase inhibitors (Mottet et al., 2003).

To evaluate the activity of the P4 promoter in the sequence environment of a vector, we checked the expression of the viral NS1 protein from the MVM-IL2 vector pORIGEN (Clement et al., 2002). No significant induction of NS1 could be observed by Western blotting after a 24 or 48 h treatment in hypoxia (results not shown). The effect of Co ions on overall NS1 expression was ambiguous, but it seems that, at high concentrations of CoCl₂, phosphorylation of NS1 is reduced (results not shown). Our results indicate that the P4 promoter is induced only weakly in hypoxia. The induction is detected by the very sensitive luciferase assay, but is more difficult to reveal by Western blotting of NS1. A weak induction of P4 activity could be sufficient to boost MVM vector production. For the erythrovirus B19 virus, a weak upregulation of the viral promoter (threefold) was concomitant with a sevenfold increase in particle production (Pillet et al., 2004). Moreover, although the B19 promoter only contains one HRE consensus sequence compared with four in MVM P4, viral amplification is largely facilitated in hypoxia (6% O₂) (Cailet-Fauquet et al., 2004).

To test vector production, NBE and COS-7 cells were co-transfected with a plasmid carrying the MVM-IL2 vector and a threefold molar excess of the helper pP38-VP, which expresses capsid proteins under the control of the natural P38 promoter. Cells were incubated for the indicated periods in hypoxia or in the presence of CoCl₂ (Fig. 2). Vector titres were determined by an ELISPOT assay and are expressed as transducing units (t.u.) (Cheong et al., 2003). A slight increase in vector production was observed (two- to threefold) at day 1 after transfection in hypoxia (6% O₂), but titres remained lower than those produced in standard conditions (day 3, 20% O₂). At days 2 and 3 after transfection, vector production decreased in hypoxia in both cell lines [Fig. 2a and results not shown (COS-7)]. It could be that cellular factors that are required for MVM amplification become limiting at longer incubation periods in hypoxia. We therefore tried to boost vector amplification by a short incubation in hypoxia (6% O₂), followed by incubation at normal oxygen tension. Induction of HIF-1α is indeed reversed rapidly upon return to normal oxygen tension (Huang et al., 1996). The return to 20% O₂ 24 h after transfection alleviated the decrease in vector production observed at days 2 and 3, but overall vector production was not increased compared with continuous incubation in normoxia (Fig. 2b).

![Fig. 2. MVM-IL2 vector production in NBE cells in hypoxia or in the presence of Co ions.](http://vir.sgmjournals.org)
At 400 μM CoCl₂, a significant decrease in vector production was observed, but this is probably related to the toxicity of CoCl₂ at this high concentration (Fig. 2c). Different concentrations of CoCl₂ within the non-toxic range (up to 200 μM) did not increase vector yield at day 3 after transfection (Fig. 2d).

Hypoxia could induce subtle changes in the phosphorylation pattern of NS1, thus affecting the viral life cycle. It is known that the different functions of NS1 are regulated by post-transcriptional modifications and in particular by changes in the phosphorylation pattern during the infection cycle of MVM (Nüesch et al., 1998). At the level of the NS1 protein, the effect of hypoxia on vector production could depend upon the balance of the two effects, increased expression of NS1 and reduced phosphorylation. This hypothesis does not exclude the possibility that other cellular factors involved in virus production are modulated by hypoxia at the level of promoter activity and/or post-transcriptional modification. The changes in NS1 phosphorylation could affect DNA amplification and/or transactivation of the P38 promoter and therefore capsid-protein synthesis. No difference in DNA amplification was observed at days 2 and 3 after transfection of vector DNA and growth in hypoxic (6 % O₂; 25, 50, 100 and 200 μM CoCl₂) compared with normal conditions (results not shown).

To check whether the decrease in vector production could be due to a limiting production of capsid proteins in hypoxia, we have measured the basal and transactivated activities of P38 in the absence and presence of NS1. P38 contains one consensus HRE sequence in the reverse orientation (Fig. 3b). A plasmid carrying the reporter cassette P38-luc was transfected alone or co-transfected with a plasmid that expresses the transactivator NS1 under the control of the hypoxia-insensitive SV40 promoter, pPSV40-NS. The basal activity of P38 increased by about 100-fold in hypoxia, whereas the transactivated level hardly changed (1-3-fold increase) (Fig. 3a). Although transactivation was about 60-fold weaker at 6 % O₂ tension compared with 20 %, the absolute amount of protein expressed was equivalent at both concentrations (Fig. 3a). An effect of hypoxia on P38 therefore cannot account for the decreased vector production in hypoxia. In keeping with this, substituting the pPSV40-VP helper plasmid, in which capsid proteins are expressed under the control of the SV40 promoter, for pP38-VP did not alleviate the decrease observed at days 2 and 3 (results not shown).

Finally, in an attempt to improve P4 upregulation, we added several HRE sequences upstream of the promoter. It has indeed been shown in several systems that the addition of HRE sequences could increase promoter activity in hypoxia (Shibata et al., 2000; Ruan et al., 2001; Jeong et al., 2005; Wang et al., 2005). Stretches of three HRE sequences one, two or three copies into the Pmel₁₃₅ restriction site, in front of the P4 promoter, in the MVM-IL2 vector. These modifications reduced vector production dramatically so that, in several experiments, it became undetectable (results not shown).

MVM(p), like other autonomous parvoviruses, is expressed preferentially in transformed cells and displays an oncotropic behaviour in vivo. The first autonomous parvovirus, H1, was indeed isolated from tumour material (Rommeleare & Cornelis, 1991). We therefore anticipated that its production could be increased under hypoxic conditions, which are prevalent in tumours (Menon & Fraker, 2005). Moreover, increased expression and amplification have been shown for the human erythrovirus B19 virus (Caillet-Fauquet et al., 2004; Pillet et al., 2004). As opposed to what had been suggested by Pillet & Le Guyader (2005), the production of vectors based on the parvovirus MVM(p) was reduced in hypoxia compared with 20 % O₂ tension. It is difficult to ascertain which step of the viral life cycle is blocked in hypoxia, as the mechanisms governing MVM amplification are not yet fully elucidated.

Acknowledgements

This work was supported by a grant from the 'Fonds National de la Recherche Scientifique' (no. 7.4544.02). C.S. was supported by the 'Fonds pour la Formation à la Recherche dans l’Industrie et dans l’Agriculture' (FRIA), P.C.-F. by Convention no. 99B210 between ‘Bruxelles Capitale’ and the Red Cross and A.B. by the ‘Fédération Luxembourgeoise contre le Cancer’. We thank Dr C. Michiels (FUNDP, Belgium) for helpful discussions.
References


