Orientation-dependent gene expression with Epstein-Barr virus-derived vectors

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Abstract Episomal vectors, described for efficient and regulated expression of heterologous proteins in mammalian cells, have the advantage that they persist in multiple copies in the cell without integrating into the chromosome. To efficiently express heterologous proteins we used such a vector based on elements of the Epstein–Barr virus (EBV), namely the sequences coding for Epstein–Barr nuclear antigen 1 and the viral origin of replication. Because constitutive expression is often deleterious to the cell, we combined the interferon-inducible Mx promoter with this EBV-derived vector. This resulted in an efficient and strictly regulated expression of the reporter gene chloramphenicol acetyltransferase (CAT) and of the neurotransmitter receptor h5-HT_{1B}, reaching levels of 16 ng CAT/mg cytoplasmic protein and 1300 fmol receptor/mg membrane protein, respectively. For both proteins, the expression levels were influenced by the orientation of the expression cassette. The higher expression in the favored orientation did not result from a higher copy number of these episomes. Northern analysis revealed a transcriptional read-through from the thymidine kinase promoter on the episomal vector, which interfered with the transcription of the heterologous gene in the less favored orientation.

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Key words: Episomal vector; Epstein–Barr virus; Inducible expression; Mx promoter; Transcriptional interference

1. Introduction

Heterologous expression of recombinant proteins has found widespread use for the overproduction of therapeutically important proteins. A whole plethora of expression systems in various cell types has been developed. Mammalian cell systems are of particular interest, since they provide more authentic posttranslational modifications of the recombinant protein compared to prokaryotes and lower eukaryotes. Efficient vector systems for heterologous gene expression in mammalian cells are episomal vectors, e.g. vectors based on sequences of Epstein–Barr virus (EBV) [1]. EBV is a gammaherpesvirus with a double-stranded DNA of 172 kb that is episomally maintained in several cell lines. The latent origin of replication of EBV (oriP) and Epstein–Barr nuclear antigen 1 (EBNA1) are the only viral elements necessary and sufficient for stable episomal maintenance of the viral DNA in the host cell; consequently, these basic elements are included in the proposed mammalian expression vector. OriP comprises two non-contiguous regions, viz. the family of repeats (FR) and the dyad symmetry (DS) containing 20 tandem imperfect copies and four related copies, respectively, of a 30-bp repeat sequence. These 30 bp represent consensus sequences for EBNA1 binding [2–5]. EBNA1, the only virally encoded protein required for episomal maintenance, has no enzymatic functions correlated with replication such as an ATPase or helicase function. Therefore replication is performed by the host cell replication apparatus, which occurs in synchrony with the host chromosomes, i.e. once per cell cycle [6–9]. Replication is mainly unidirectional; it starts near the DS and is initially bidirectional but stops near the FR, which contains a replication fork barrier [10–12]. Upon cell division, the oriP-containing plasmids are bound, via EBNA1, to metaphase chromosomes, which interaction secures an equal distribution to the daughter cells [13,14]. Another role for oriP in the presence of EBNA1 is a transactivation function which depends on the cell line used and on the promoter driving the gene of interest, independently of its orientation [15]. How EBNA1 mediates this transactivation function can only be hypothesized; HIV Tat-associated 32-kDa protein, which strongly interacts with EBNA1, was recorded to be implicated in transactivation and might contribute to EBNA1-mediated transactivation [16]. The use of this episomal vector for heterologous gene expression has some important and unique characteristics compared to more conventional DNA-integrating vectors. First, high-level expression of the gene of interest can be obtained, because the EBV vector can reach copy numbers of more than 100/cell. Second, transfection efficiency in vitro is usually high, because no integration into the host genome is required. With respect to gene therapy, EBV sequences are often combined with retroviral, adenoviral or herpesviral sequences to further increase the gene delivery efficiency in vivo [17–19]. Finally, neither the gene of interest nor the host cell DNA will suffer from events peculiar to chromosome integration, such as silencing and gene disruption effects.

Another feature we focused on is the usage of inducible expression systems. Constitutive expression of proteins is often deleterious to the host cell and frequently results in cell death or loss of highly expressing clones. Therefore, we

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integrated a type-I interferon (IFN)-inducible Mx promoter [20] in the EBV-derived expression vector. Although this IFN-inducible Mx system is an endogenous system and thus probably suffers from pleiotropic effects caused by the inducing agent, it certainly has proven its utility for regulated expression of a variety of recombinant proteins [21–26]. This combination resulted in a tightly controlled expression of the reporter protein chloramphenicol acetyltransferase (CAT) and of the neurotransmitter receptor h5-HT_{1B}. In both cases, the expression levels depended on the orientation of the expression cassette relative to other sequences of the EBV-derived vector. A higher expression level was obtained when the expression cassette had the same orientation as the EBV sequences and was opposite to the eukaryotic selection cassette. Although it is well documented that the expression levels obtained with episomal vectors are correlated with vector copy number [27,28], Southern blot analysis revealed that this was not the case. In contrast, we provide evidence that interfering transcripts from the neighboring resistance gene co-determines the expression level of the heterologous gene. As EBV-derived expression vectors become more widely used, this aspect should be taken into account for further research or application purposes.

2. Materials and methods

2.1. Recombinant DNA constructions

The basal EBV-derived vector p220.2 [3] contains the viral sequences oriP and EBNA1, as well as the hygromycin B (hyg) selectable marker gene driven by a thymidine kinase (TK) promoter for selection in eukaryotes. The expression cassettes Mx/CAT/poly(A) or Mx/h5-HT_{1B}/poly(A), isolated by double restriction digestion from pMxCAT and pMxh5-HT_{1B}, respectively [26], were inserted in both orientations at the *Sal*I–*Xba*I site of p220.2 to obtain p220.2MxCAT orientation 1 (Or1) or p220.2Mx5-HT_{1B} Or1 and at the *Xba*I-*Bam*HI site to obtain p220.2MxCAT Or2 or p220.2Mx5-HT_{1B} Or2 (Fig. 1).

2.2. Cell culture and transfection

Vero (monkey) as well as MG63, HEK293 and HEK293EBNA1 (man) cells (Invitrogen, San Diego, CA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Transfections were carried out with standard calcium phosphate DNA coprecipitation procedures, using 30 μg DNA.

As the use of EBV vectors usually results in higher transfection efficiency, the transfected cells were seeded at lower density before selection with hyg (Vero, 300 U/ml; HEK293, 150 U/ml; MG63, 50 U/ml). After a selection period of 3 weeks, only single cell clones were isolated for further study. Stable clones, containing EBV-derived vectors, were kept under continuous selection [3].

2.3. Induction

Cell cultures were grown to subconfluency. For induction of the Mx promoter, the medium was replaced by DMEM with 10% serum containing 1000 U/ml human IFN-α (PeproTech, Hill, NJ, USA). After 24 h induction, cells were washed and lysates were prepared.

2.4. Reporter protein quantification

CAT expression was assayed with a quantitative home-developed CAT-ELISA [24] and the results were statistically analyzed using the Student's *t*-test.

2.5. Radioligand binding

Cell cultures were transferred to Petri dishes and grown to subconfluency. After 24 h induction, cells were washed with phosphate-buffered saline (PBS) and stored at −70°C for membrane preparation. h5-HT_{1B} receptor expression levels were determined by radioligand binding with [^{3}H]alniditan as described previously [29]. Briefly, h5-HT_{1B} receptor cell membrane preparations were incubated with 5 nM [^{3}H]alniditan for 30 min at 37°C. Non-specific binding of the radioligand was estimated in the presence of 10 μM sumatriptan.

2.6. Southern blot analysis

Low-molecular-weight DNA was prepared as described [30]. In order to minimize experimental inaccuracies, we accurately determined the cell number (in triplicate on two different dilutions) before lysing the different samples. Furthermore, at the start of the DNA extraction procedure, pMa58 plasmid DNA [31] was added to the cell lysis buffer at a final concentration of 500 ng/ml and used as an internal control for experimental reproducibility and DNA recovery. Briefly, 300 000 cells were spun down, washed twice with HBS and subsequently lysed in 600 μl Hirt extraction buffer (10 mM EDTA, 0,6% SDS; pH 7.6) for 20 min at 37°C. The cell lysate was scraped off the plates and transferred to an Eppendorf tube. 150 μl of 5 M NaCl was added and the tubes were gently inverted 10 times not to brake the chromosomal DNA. After an overnight incubation at 4°C, the solution was centrifuged for 30 min at 12 000 × *g* at 4°C. The supernatant was transferred to a new Eppendorf tube and purified by phenolization (3×) and ether extraction (2×). Finally, the DNA was precipitated, washed with 70% ethanol and dissolved in 50 μl of 0,1×TE. After digestion, the DNA was separated on a 1.2% agarose gel and subsequently transferred to a GenescreenPlus membrane (Du Pont, Wilmington, DE, USA). The membrane was hybridized with a ^{32}P-labeled random-primed probe and washed as prescribed by the manufacturer. Results were visualized with a PhosphorImager (Bio-Rad, Richmond, CA, USA) and data were analyzed using Quantity One software (Bio-Rad).

2.7. Northern blot analysis

Cells were grown to subconfluency and treated with 1000 U/ml IFN-α. After 6 h incubation, total RNA was isolated with an RNeasy kit (Qiagen, Chatsworth, CA, USA). Isolated RNA was separated on a 1% agarose gel in 20 mM phosphate buffer (pH 7.0) and transferred to a Nylon66 membrane (Amersham Pharmacia Biotech, Rainham, UK). Subsequent hybridization and revelations were performed as described above.

2.8. Western blot analysis

To detect EBNA1, nuclear lysates were prepared. After washing with ice-cold PBS, cells were lysed with buffer B1 (10 mM HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 2 mM Pefablock, 5% mercaptoethanol, 1 mM aprotinin and 1 μg leupeptin) for 10 min on ice. After centrifugation for 10 min at maximum speed, the pellet was lysed in buffer B2 (20 mM HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 400 mM NaCl, 1% NP40, 20% glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 2 mM Pefablock, 5% mercaptoethanol, 1 mM aprotinin and 1 μg leupeptin) for 10 min on ice. After centrifugation, the proteins in the supernatant were separated on a sodium dodecyl sulfate–10% polyacrylamide gel under denaturing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). EBNA1 was detected using a 1:8000 dilution of a polyclonal rabbit anti-EBNA1 antibody, followed by incubation with biotinylated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech) and finally with horseradish peroxidase-linked streptavidin (1:1000; Amersham Pharmacia Biotech). Detection was performed by chemiluminescence according to the manufacturer's recommendations (Du Pont).

2.9. RT-PCR

RNA was isolated as described above. 2 μl (5–10 μg) RNA was used for an RT-PCR reaction (Access RT-PCR kit; Promega Biotech, Madison, WI, USA) and subsequently screened with specific primer pairs. The primer sequences used were (1) GTC AAC AGC GTG CCG CAA GA, (2) CCA GGA AGC TCC TCT GTG TC, (3) CAG CCT GAC CTC CTC TCA GT and (4) AGA GTC AGC AGT AGC CTC AT. Reverse transcription was performed at 48°C for 45 min. For amplification, 35 cycles were applied (30 s at 94°C, 1 min at 60°C and 2 min at 68°C).

3. Results

3.1. Enhanced gene expression using episomal vectors

An expression cassette containing the CAT gene under con-

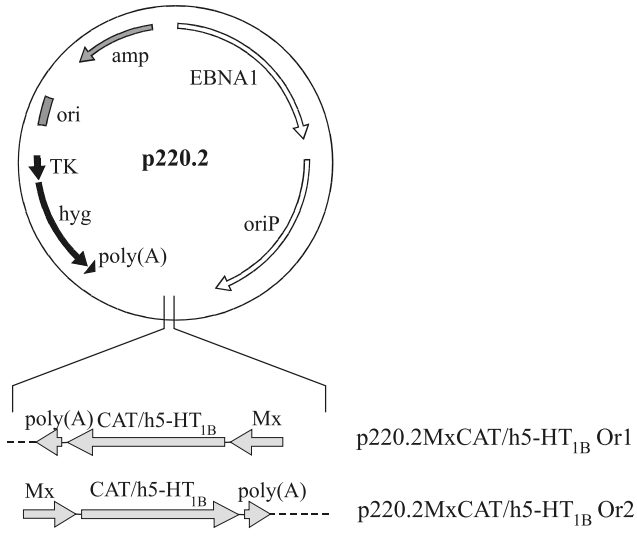

Fig. 1. The EBV-derived vector p220.2. White bars represent EBV sequences and black bars sequences for eukaryotic selection. The expression cassettes are inserted in both orientations (Or1 and Or2).

trol of the Mx promoter was cloned into the vector p220.2 in both orientations (Fig. 1). The episomal plasmids p220.2MxCAT Or1 and Or2 were then stably transfected into the cell lines MG63, HEK293 and Vero. To compare the difference in expression between an episomal and a chromosomally integrated expression cassette, the same cell lines were also transfected with the vector pMxCAT [26]. After isolation of stably transfected single cell clones, cells were left untreated or were induced with 1000 U/ml IFN-α for 24 h. For all cell lines tested, the CAT expression levels obtained with the episomal vectors are clearly higher as compared to the levels from the chromosome-integrating vector pMxCAT (Table 1). Non-induced levels, i.e. background expression, remained very low and were even not detectable in the majority of the clones (<0.1 ng CAT/mg protein).

3.2. Expression levels are influenced by the orientation of the expression cassette

A marked, statistically significant difference in expression level was consistently observed between the EBV-derived expression vectors p220.2MxCAT Or1 and p220.2MxCAT Or2 (Table 1). In the favorable Or1, the expression cassette is in the same orientation as the EBNA1 gene and oriP, and opposite to the orientation of the hyg expression cassette, whereas the expression cassette in Or2 is in the same orientation as the preceding hyg resistance gene (Fig. 1). Moreover, this difference in expression level is not cell line-dependent nor gene-specific, as it was observed in several cell lines and also when Vero was transfected with p220.2Mxh5-HT$_{1B}$ Or1 and Or2 (Fig. 1 and Table 1). As earlier studies demonstrated that the expression level of a heterologous gene on an episomal vector is directly proportional to the vector copy number [27,28], we investigated whether the more abundant expression in Or1 is due to a higher copy number of p220.2MxCAT Or1. Therefore, we selected four moderate and two high expressing Vero clones of both orientations for further analysis. We included the high expressing clones to really study the correlation between expression level and DNA copy number. Low-molecular weight DNA was isolated and quantified by Southern blot analysis. After quantification, these results in general did not show the presence of a higher copy number in Or1 as compared to Or2 (Fig. 2). On the contrary, an analysis of clones with a similar expression level of CAT protein (see Table 2), e.g. cl 1, cl 3 and cl 7 of Or1 vs. cl 3 and cl 4 of Or2, suggests that the respective copy numbers in Or2 are at least twice as high as in Or1. A comparison of clones with a similar copy number (e.g. cl 3 Or1 vs. cl 1 Or2, or cl 10 and cl 11 Or1 vs. cl 6 and cl 11 in Or2) shows a yield of CAT protein for Or1 that is twice as high as for Or2. We conclude that a higher protein expression in Or1 is obviously not related to a higher copy number of the expression vector. In a next step, total RNA of several clones of Or1 and Or2 was extracted before and after treatment with 1000 U/ml IFN-α for 6 h and used for Northern blot analysis, applying a CAT mRNA-specific probe (Fig. 2). After normalization with a GAPDH signal, the CAT protein levels correlated well with the CAT mRNA levels. A combination of both results and a calculation of the RNA/DNA ratio indicate that the latter value in Or1 is twice as high as in Or2 (Table 2). This clearly demonstrates that the higher expression in Or1 does not result from a higher gene copy number, but derives from a transcriptional advantage in Or1 or a disadvantage in Or2.

3.3. Transcriptional interference

Two adjacent vector sequences can be considered to affect the transcription of the recombinant genes, namely the hyg expression cassette and the oriP sequence. To further investigate this phenomenon, a Northern blot analysis was performed with total RNA of various clones of Or1 and Or2.

Table 1
Overview of IFN-α-induced expression levels of the reporter gene CAT and the neurotransmitter receptor h5-HT$_{1B}$ in different cell types (average of *n* different clones). Comparison between chromosome-integrating and episomal vectors

	Reporter protein expression (ng/mg cytoplasmic protein)		
	pMxCAT	p220.2MxCAT Or1	p220.2MxCAT Or2
Vero	0 ($n=12$)[a]	4.7 ($n=11$)	2.2 ($n=13$)*
MG63	0 ($n=9$)[a]	4.0 ($n=6$)	1.8 ($n=5$)**
HEK293	0.7 ($n=8$)	2.3 ($n=6$)	1.8 ($n=5$)
HEK293EBNA1	0.2 ($n=7$)	2.1 ($n=18$)	ND
	Neurotransmitter receptor expression (fmol/mg membrane protein)		
	pMxh5-HT$_{1B}$	p220.2Mxh5-HT$_{1B}$ Or1	p220.2Mxh5-HT$_{1B}$ Or2
Vero	ND	950 ($n=9$)	300 ($n=5$)*

ND: not determined.
*$P<0.01$ (Or1-Or2).
***$P<0.001$ (Or1-Or2).
[a]Integration of pMxCAT DNA into the chromosome was confirmed by Southern blot analysis.

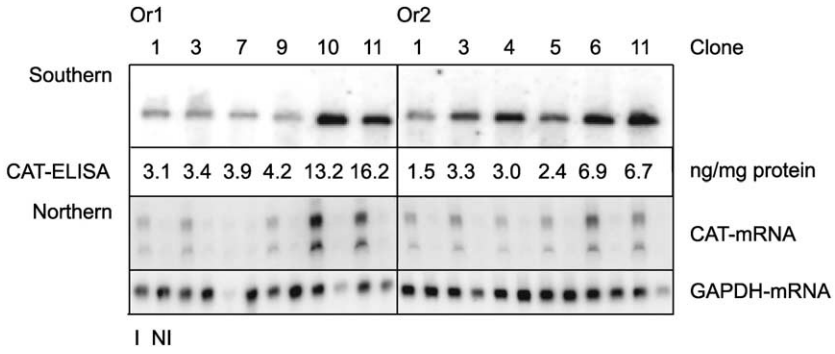

Fig. 2. Southern blot analysis, CAT–ELISA and Northern blot analysis of different clones of Vero p220.2MxCAT Or1 and Or2. Top: Episomal DNA isolated from different stable transfectants was digested with *Hin*dIII and subsequently separated on gel. After blotting, the membrane was hybridized with a labeled oriP fragment. Hybridization of the blot with a labeled pMa58 fragment allowed to correct for experimental inaccuracy. Bottom: After 6 h induction, total RNA was isolated and separated on gel. After blotting, the membrane was hybridized with a CAT mRNA-specific probe (Fig. 3B) and then with a GAPDH-specific probe as an internal control (I, induced; NI, non-induced).

Careful analysis of the hybridization pattern obtained with a CAT mRNA-specific probe revealed the presence of different transcripts (Fig. 3A). For both orientations, a doublet was found corresponding to mRNA species of approximately 1550 and 1150 nucleotides (nt), respectively. The shorter transcript results from the use of an alternative splice donor site, present in the CAT sequences, with the splice acceptor site in the SV40 intron [32]. Besides these two transcripts, a third specific, but faint band was detected in Or2 only, corresponding to an mRNA of approximately 3200 nt. This mRNA seems to be constitutively produced and therefore not dependent on IFN-α-induced expression. We hypothesize that this transcript is initiated at the TK promoter, driving the hyg gene, and is terminated near the SV40 poly(A) sequences of the CAT expression cassette as a result of inefficient termination at the TK poly(A) sequences (Fig. 3C). To further test this hypothesis, we searched for the presence of hyg mRNA sequences that are not terminated at the TK poly(A) site. Specific primer sets were designed to identify incorrectly terminated mRNA by RT-PCR. The primer pairs 1/2 and 1/3 clearly show that not all hyg-mRNA transcription is correctly terminated at the TK poly(A) site and that considerable transcription read-through occurs (Fig. 3B). Furthermore, the use of primer pair 1/4 also gave rise to a faint band of approximately 3200 nt, which demonstrates transcriptional read-through as far as the 3′-untranslated region (UTR) of the expression cassette Mx/CAT (Fig. 3C).

3.4. Transcriptional activation

OriP has been described to exert a transcriptional activity upon binding of EBNA1 [15]. As our previous experiments did not exclude this possibility, we investigated the influence of EBNA1 on the activation of the Mx promoter. We used a HEK293EBNA1 cell line, overexpressing EBNA1; the overexpression was checked by Western analysis (data not shown). HEK293 and HEK293EBNA1 cells were stably transfected with the parental vector p220.2 or with the expression vector pMxCAT and p220.2MxCAT Or1. Southern analysis of episomal DNA showed that the overexpression of EBNA1 did not result in a higher copy number of the EBV-derived vector (Fig. 4). The fact that both cell lines showed a comparable copy number allowed to investigate the influence of EBNA1 overexpression on the transactivation of the Mx promoter. Stable cell clones were induced with IFN-α for 24 h or left untreated, cell lysates were prepared and CAT expression was quantified. CAT expression in non-induced HEK293 and HEK293EBNA1 cell clones was undetectable (detection limit <0.1 ng CAT/mg protein). CAT-ELISA of cell lysates of induced cells revealed that there was no significant difference between the expression levels in HEK293 and HEK293-

Table 2
RNA/DNA determination for various CAT-expressing clones

	CAT expression (ng/mg cytoplasmic protein)	RNA[a]	DNA[b]	RNA/DNA
Or1				
cl 1	3.1	2.2	5.9	0.4
cl 3	3.4	2.6	8.7	0.3
cl 7	3.9	ND	5.6	ND
cl 9	4.2	2.4	5.2	0.5
cl 10	13.2	9.4	14.4	0.7
cl 11	16.2	6.6	17.1	0.4
Or2				
cl 1	1.5	1.8	8.3	0.2
cl 3	3.3	2.3	10.9	0.2
cl 4	3.0	1.5	12.3	0.1
cl 5	2.4	1.7	12.5	0.1
cl 6	6.9	4.1	15.1	0.3
cl 11	6.7	3.3	16.1	0.2

ND: not determined.
[a]Normalized CAT/GAPDH Northern blot analysis data.
[b]Normalized CAT/pMa58 Southern blot analysis data.

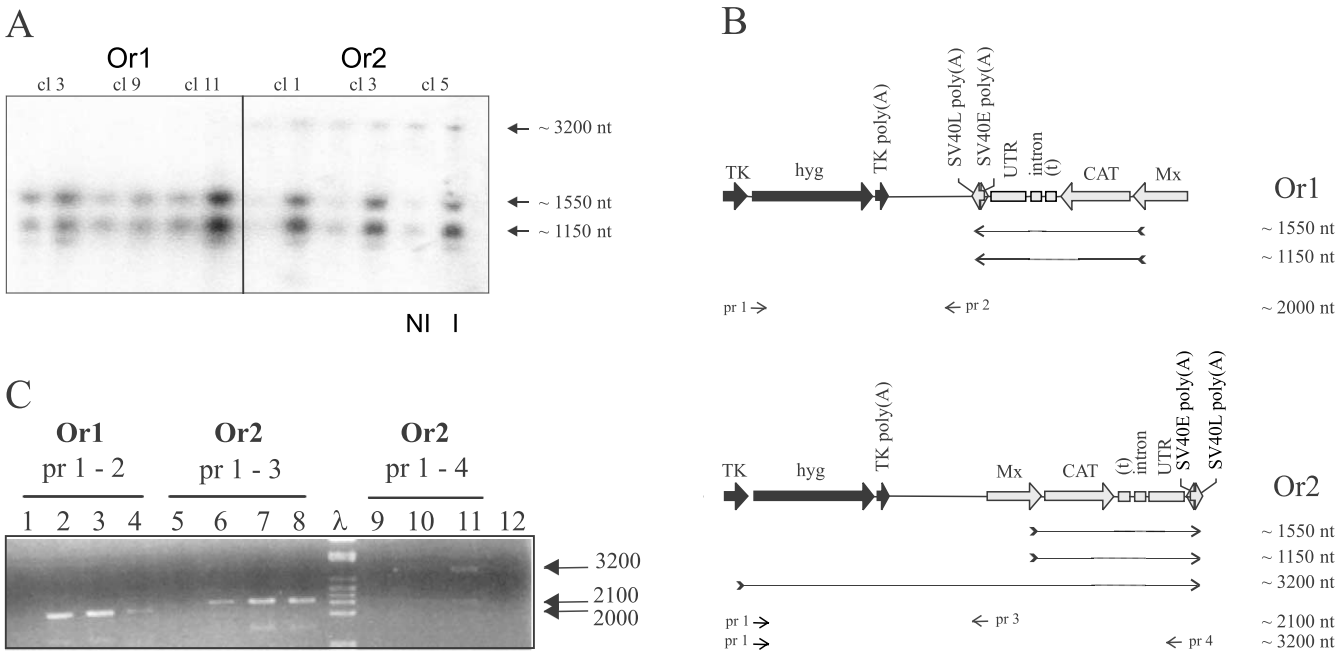

Fig. 3. A: Northern blot analysis of various stable Vero p220.2MxCAT Or1 and Or2 clones. Total RNA was isolated and separated on gel. After blotting, the membrane was hybridized with a CAT mRNA-specific probe (NI, non-induced; I, induced). B: Part of the expression vector showing the insert in both orientations with respect to the flanking selection marker module as well as the potentially different CAT mRNA products generated (t, part of the SV40 small-t antigen). C: RT-PCR analysis of stably transfected Vero p220.2MxCAT Or1 (lanes 1–4) and Or2 (lanes 5–8 and 9–11) cell clones. RNA was isolated and used in an RT-PCR reaction using the indicated primer pairs. As a control for DNA contamination, lanes 1, 5 and 9 contain RNA samples where RT was omitted from the RT-PCR reaction. Lane 12 contains Vero parental cells (negative control). λ represents λ DNA digested with *Pst*I (DNA size marker).

EBNA1 cell clones, viz. 2.3 ($n = 6$) and 2.1 ($n = 18$) ng CAT/mg cytoplasmic protein, respectively (Table 1).

4. Discussion

We recently demonstrated that EBV-derived plasmids, in contrast to other episomal expression vectors, are not prone to DNA rearrangements [26]. Therefore the applicability of this mammalian expression vector in combination with an inducible promoter was further examined. A higher expression of the reporter protein CAT and the neurotransmitter receptor h5-HT_{1B} was obtained with the EBV-derived vectors in the different cell lines tested as compared to chromosomally integrated versions of the expression cassette. This most likely results from the fact that they persist in multiple copies in the cell. Furthermore, they are also not susceptible to control mechanisms, peculiar to integration of foreign DNA into the host chromosome, such as silencing. Such mechanisms are probably responsible for the lack of CAT expression in several of the Vero and MG63 pMxCAT clones.

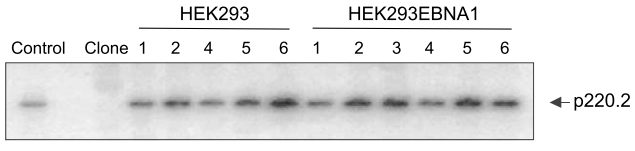

Fig. 4. Southern blot analysis of p220.2. Episomal DNA was isolated from stably transfected HEK293 and HEK293EBNA1 cell clones. Isolated DNA was linearized with *Hin*dIII, separated on a 1.2% agarose gel and transferred to a nitrocellulose membrane. The membrane was hybridized with a ^{32}P-labeled oriP fragment; the control consisted of p220.2 plasmid DNA digested with *Hin*dIII.

The expression cassette Mx/CAT/poly(A) was inserted in the parental vector p220.2 in two orientations, resulting in a markedly higher expression level with Or1 than with Or2. The difference in expression level is not cell line-dependent or gene-specific, as it is also observed when the Vero cell line was transfected with p220.2Mxh5-HT_{1B} Or1 and Or2. A similar orientation-dependent expression has been reported previously with a human IFN-γ transcriptional cassette inserted in both orientations in an EBV-derived vector [33]. The results clearly show that the expression cassette itself is not responsible for this orientation-dependent effect. Although several investigators reported that the expression level of a heterologous gene on an episomal vector is correlated with vector copy number [27,28], Southern blot analysis did not show that the higher protein concentration in Or1 was due to a higher copy number of the episomal vector but could be correlated with higher mRNA levels. A comparative Northern blot analysis of several stable clones of both orientations revealed the presence of a constitutive band of approximately 3200 nt in Or2 only. As this band was not influenced by treatment with IFN-α, we hypothesized that this mRNA was properly initiated at the TK promoter but not correctly terminated near the TK poly(A) sequence, which provides less efficient poly(A) signals. This was confirmed by RT-PCR using specific primer pairs discriminating between the various mRNA species. There is a close correlation between efficient termination of transcription and efficiency of the poly(A) signal [34]. Most eukaryotic mRNAs are cleaved posttranscriptionally at a specific site in the UTR, approximately 10–30 nt downstream of a consensus sequence, i.e. the highly conserved AAUAAA poly(A) signal. This sequence restricts transcriptional progression, resulting in cleavage of the mRNA near

this poly(A) site [34,35]. Both SV40E (early) and SV40L (late) as well as the TK poly(A) sequences contain this conserved hexanucleotide sequence. However, it was also shown that this AAUAAA sequence is insufficient for mRNA polyadenylation. Sequences such as GU- or U-rich elements more downstream of this cleavage site are also determining the strength of the polyadenylation signal and can actually regulate the choice between alternative upstream cleavage sites [35–38]. Polymerase release occurs closer to the poly(A) signal when the latter is stronger [34]. The SV40L poly(A) sequence, for instance, is more efficiently used than other polyadenylation signals [39,40]. The observed read-through of the hyg mRNA certainly proceeds into the Mx promoter for Or2 with a few transcripts going as far as the UTR. These incorrectly terminated mRNAs will interfere with transcription initiation at this promoter sequence and may thus account for the lower expression levels in Or2. Such interference effect is well documented for duplicated gene constructions and can occur over a distance of more than 1000 bases [41]. It has also been shown that binding of transcription factors to such an occluded promoter is reduced [42].

Besides the influence of the hyg expression cassette on neighboring transcription, an influence of oriP could not be ruled out. It has been demonstrated that the FR of oriP, upon binding of EBNA1, can act as a transcriptional enhancer. Although the extent of enhancement varies with promoter and cell type, it is exerted across 10 kb of viral DNA and is orientation-independent [10,15,43,44]. Therefore it is less likely that this contributes to the observed difference in expression levels between Or1 and Or2. The observation that the use of the MSV or CMV enhancer did not alleviate this orientation effect [33], further contradicts this assumption. Moreover, EBNA1 overexpression could not increase the transactivation function of oriP, as we obtained comparable CAT expression levels in HEK293 and HEK293EBNA1 cells. Our results show that read-through transcription from the TK promoter interferes with the efficiency of initiation at the Mx promoter in Or2, resulting in a decreased expression of the heterologous protein of interest. The observation should be taken into account when using this episomal vector system for expression of heterologous gene products.

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