Effects of an internal transcription unit and its orientation on retrovirus titre and expression

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Abstract

Using the retroviral vector pMsp, constructs were produced with different coding sequences under the control of the Herpes simplex virus type 1 (HSV) thymidine kinase (*tk*) promoter, with the internal coding sequence in the same or reverse orientations with respect to long terminal repeat (LTR)-driven transcription and with or without an internal *tk* polyadenylation (polyA) signal. Following introduction of these constructs into ecotropic or amphotropic packaging cell lines by transfection or infection, it was found that, most consistently, those constructs in which the internal coding sequence/polyA signal component was inserted in the same orientation as LTR-driven transcription produced lower titres of virus than those in which this component was inserted in the reverse orientation. Also, in a construct containing a coding sequence in the same orientation as LTR-driven transcription, but lacking an internal polyA signal, virus titre was much greater than in the corresponding construct possessing the internal polyA signal. Additionally, although functional assays have previously demonstrated expression of the inserted sequences, transcription from the internal *tk* promoter was inefficient in all these constructs.

Abbreviations: HSV: Herpes simplex virus type I; IGF: insulin-like growth factor; LTR: long terminal repeat; *myc*: gene encoding Myc DNA-binding protein; polyA: polyadenylation; *tk*, gene encoding thymidine kinase

Introduction

Vector systems which utilise the transducing properties of avian and mammalian retroviruses have been widely used for the efficient introduction of cloned genes, including oncogenes and growth factor genes, into cell lines and primary cells. In many vectors both the inserted gene and a selectable marker gene are transcribed from the viral LTR promoter and individual mRNAs are generated by splicing of the viral transcript. Expression vectors in which the inserted gene is under the control of a separate promoter and can thus be regulated independently of viral transcription have been developed. In such vectors, however, prob-

lems may arise as a consequence of expression of the internal transcription unit. Previous reports have suggested that an internal promoter and polyA signal may (Shimotohono and Temin, 1981; Emerman and Temin, 1986) or may not (Cepko *et al.*, 1984) interfere with transcription and termination of transcripts from the viral LTR, thereby affecting virus titre. It has also been demonstrated that antisense RNA, which is expected to be transcribed from an internal promoter in 3'–5' orientation relative to the vector, may interfere with transcription of the sense (+) strand of the virus (Green *et al.*, 1986). Epigenetic suppression of either promoter by the other has been demonstrated in avian retrovirus systems (Emerman and Temin, 1984; 1986). Also, the presence of the viral LTR enhancer is likely to affect the level of transcription from an internal promoter but,

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Figure 1. DNA constructs used for transfection.

contrary to expectation, deletion of enhancer and other control regions from the viral LTR have been shown to increase transcription from an internal promoter (Yee *et al.*, 1987).

The retrovirus vector pMsp, which is derived from the myeloproliferative sarcoma virus (MPSV – Ostertag *et al.*, 1986), contains the *neo* gene, transcribed from the LTR promoter, as a selectable marker. This report assesses the effects of the orientation of inserted coding sequences and internal polyA signals on proviral transcription and virus titres.

Materials and methods

DNA constructs

The structures of the plasmids used in this work are shown in Fig. 1. Most of these have previously been described in full [p104-IGF-I, p105-IGF-I, p206-IGF-II and p207-IGF-II (Watts and MacDonald, 1990); p309 and 310 (Darnbrough and MacDonald, 1991)]. p308 is of the same structure as p309 except that the *myc* fragment is in the same orientation as LTR-driven transcription. The structure of the pMsp vector (Ostertag *et al.*, 1986) is also shown; coding sequences were cloned into the *Bam*HI and/or *Hind*III sites of the polylinker in the orientations indicated.

Plasmids were grown in *E. coli* HB101 and DNA for transfections was prepared by Triton lysis and puri-

fied by two cycles of caesium chloride density gradient centrifugation.

Transfection and infection of packaging cell lines

The ecotropic packaging cell line ψ -2 was obtained from Professor D. Onions. ψ -sp1-4, which carries a modified packaging provirus derived from ψ -2 (Watts and MacDonald, 1990), was obtained from Dr. R. Jarrett. The amphotropic cell line PA317 (Miller and Buttimore, 1986) was obtained from ATCC and the ψ -cre cell line (Danos and Mulligan, 1988) from Dr R. Mulligan.

Cells were transfected by the calcium phosphate method essentially according to Gorman (1985), using 15 μ g DNA and 2.5×10^5 cells. In order to isolate stable virus-producing cell lines, transfected cells were reseeded after 24h into the appropriate concentration of G418 (600–1000 μ g ml⁻¹). After 2–3 weeks, colonies of G418-resistant cells were picked and expanded in 200–400 μ g ml⁻¹ G418.

Alternatively, medium containing transiently secreted virus from PA317 was harvested at 48–60 h following transfection, 0.45 μ m filtered and diluted if necessary in DMEM/10% foetal calf serum/8 μ g/ml polybrene. ψ -cre packaging cells for infection were seeded at 10^4 cells cm⁻² 24 h prior to infection. Cells were infected with 2 ml of this virus stock for 2–3 h at 37 °C, and after 24 h were reseeded at a range of dilutions and G418 selected. Colonies were counted and picked as above.

Determination of virus titres

Virus titres were determined on 0.45 μ m-filtered supernatant medium obtained from cells grown from 50% to 100% confluence over a 24 h period. For determination of titres by G418-resistant colony formation, NIH 3T3 cells were infected as described for packaging cells with serial dilutions of virus, and G418-resistant colonies were counted after 2–3 weeks. Results are expressed as colonies ml⁻¹ of supernatant medium (cfu ml⁻¹). Viral RNA concentrations were determined by dot-blotting and hybridisation of ³²P-labelled probes (2×10^9 dpm μ g⁻¹) for pMsp or the appropriate inserted coding sequence to RNA extracted from pelleted virus essentially as described by Brown and Scott (1987). These data are expressed as pg viral RNA ml⁻¹ medium. 10^5 virions containing 2 RNA genomes of 7.5 kb are calculated to contain approximately 1pg of viral RNA.

Table 1. Titres and viral RNA concentrations of cell lines containing IGF-I and IGF-II viruses. Values were obtained from at least two determinations

Construct	Clone	Viral RNA (pg ml ⁻¹)	Titre on 3T3 cells (cfu ml ⁻¹)
p104	ψ -2C4-2	<0.2	n.d. ^a
p104	ψ -2C4-4	<0.2	n.d. ^a
p104	ψ -spC4-2	<0.2	n.t. ^b
p104	ψ -spC4-3	<0.2	n.t. ^b
p104	ψ -spC4-4	<0.2	n.t. ^b
p105	ψ -2C5-4	6	7 × 10 ³
p105	ψ -spB5-2	0.5	n.t. ^b
p105	ψ -spC5-1	50	1 × 10 ⁵
p105	ψ -spC5-4	n.t. ^c	5 × 10 ²
p105	ψ -spC5-5	20	1 × 10 ⁵
p206	ψ -2C6-1	0.5	n.t. ^b
p206	ψ -2C6-2	2	n.t. ^d
p206	ψ -2C6-4	2	n.t. ^d
p206	ψ -spC6-3	20	5 × 10 ⁴
p206	ψ -spC6-4	<0.2	n.t. ^b
p206	ψ -spC6-5	<0.2	n.t. ^b
p207	ψ -2B7-1	20	1 × 10 ⁵
p207	ψ -2B7-2	6	1 × 10 ⁵
p207	ψ -2C7-1	5	1 × 10 ³
p207	ψ -2C7-3	2	50 ^e

^a – Not detectable.

^b – Not tested: a viral RNA concentration of 0.5 pg ml⁻¹ or less in our experience routinely indicates a titre that is not detectable (eg. see ψ -2C4-2 above). Hence, titrations were not routinely performed on clones with RNA concentrations in this range.

^c – Not tested: sample lost during processing. From the titre on 3T3 cells observed, a value of 2–5 pg ml⁻¹ might be expected. However, as one of the main aims of this work was to isolate high titre clones, this clone was not investigated further.

^d – Not tested: a viral RNA concentration of 2.0 pg ml⁻¹ in our experience routinely indicates a titre of 10² or less. (See clones ψ -2C7-3 above, ψ myc309.20, ψ myc310.18 in Table 3.) Hence, as, again, isolation of high titre clones was one of the principal aims of this work, titrations were not routinely carried out on clones with RNA concentrations of this value.

^e – *t*-tests showed that there was a significant difference between the titres obtained from the p104 and p105 clones and between those obtained from the p206 and p207 clones at 90% confidence.

Infection of target cells

NIH 3T3 cells were infected as described above for packaging cells, using virus stocks from either stable cell lines or transient transfections. The infection of PQXB hybridoma cells (Darnbrough and MacDonald,

1991) was carried out as described (Darnbrough *et al.*, 1992).

All manipulations were carried out under appropriate containment conditions according to ACGM guidelines. Amphotropic cell lines carrying viral constructs were handled in a class II hood under category 2 containment. Virus-producing cell lines and target cells were checked for helper virus using the XC assay (Rowe *et al.*, 1970) for ecotropic virus, and S+L– assays (Miller *et al.*, 1985) for amphotropic virus.

Northern blots

RNA was extracted from infected target cells by the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987), Northern blots prepared from total cellular RNA as described previously (Darnbrough and MacDonald, 1991) and hybridised to probes for inserted coding sequences as above, or to a probe for exon 1 of murine *c-myc* (Stanton *et al.*, 1984).

Results and discussion

Effects of internal transcription and polyA signal on virus titre

cDNA coding sequences for IGF-I (Jansen *et al.*, 1983), IGF-II (Bell, *et al.*, 1984) and feline *v-myc* (Stewart *et al.*, 1986) under the control of the HSV *tk* promoter (a weak promoter), and with the *tk* polyA signal immediately downstream, were inserted into the polylinker of pMsp, in the same and reverse orientations with respect to LTR-driven transcription. Additionally, the cDNA coding sequence for feline *v-myc* under the control of the *tk* promoter, but lacking the *tk* polyA signal was inserted into pMsp in the same orientation as LTR-driven transcription. The constructs, shown in Fig. 1, were introduced into ecotropic and amphotropic packaging cells in order to prepare virus stocks for the infection of cell lines and primary cells. Stable G418-resistant cell lines carrying these constructs were generated in two ways: IGF constructs were introduced by transfection into the ecotropic packaging cell lines ψ -2 (Mann *et al.*, 1983) and ψ -sp1–4 (Watts and MacDonald, 1990), and *myc* constructs by transfection into the amphotropic packaging line PA317 (Miller and Buttimore, 1986) and subsequent infection of ecotropic cells ψ -cre (Danos and Mulligan, 1988) by amphotropic virus expressed transiently from the transfected PA317 cells. The resul-

Table 2. G418-resistant colonies generated by transfection of PA317 and by subsequent infection of ψ -cre with transiently secreted virus

Construct	PA317 transfectants (colonies 2×10^5 cells $^{-1}$)		ψ -cre infectants (colonies ml PA317 medium $^{-1}$)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
pMsp	n.r. ^a	40	>500	500
p308	n.r. ^a	4	1 ^b	n.d. ^c
p309	n.r. ^a	2	50	27
p310	n.r. ^a	1	25	10

^a – not recorded.

^b – failed to grow following transfer to flask.

^c – not detectable.

tant cell lines were cloned and virus production was measured by dot-blot hybridisation of viral RNA from supernatant medium and by scoring G418-resistant colonies on NIH 3T3 cells. All constructs generated G418-resistant clones following transfection into packaging cells as described, with efficiencies of 4–12 colonies per 10^5 cells.

Table 1 shows the titres, measured by dot blots of viral RNA and by infection of 3T3 cells, obtained for IGF-I and II constructs with an internal polyA signal in both orientations. High titre cell lines ($>1 \times 10^5$ cfu ml $^{-1}$ or >20 pg RNA ml $^{-1}$) were obtained in both orientations with the IGF-II constructs (p206, p207), though titres were consistently higher with the reverse orientation construct (p207). The IGF-I constructs yielded high titre clones only in reverse orientation (p105). *t*-tests showed that there was a significant difference between the titres obtained from the p104 and p105 clones and between those obtained from the p206 and p207 clones at 90% confidence. The results of infection of ψ -cre cells with transiently secreted amphotropic *myc* virus (Table 2) were consistent with those obtained from the IGF constructs. In two experiments no clones could be isolated using p308 (*myc* coding sequence followed by internal polyA signal and in the same orientation as LTR-driven transcription). However, when ψ -cre cells were infected with transiently secreted virus from cells transfected with p309 (reverse orientation with polyA signal) or p310 (same orientation as LTR-driven transcription without a polyA signal), G418-resistant colonies were isolated at frequencies of 10–50 ψ -cre colonies ml $^{-1}$ of PA317 supernatant. The yields of both transfected and infected clones were reduced at least ten-fold in the *myc* constructs as compared to the pMsp vector alone. Among the G418-resistant ψ -cre clones isolated following infection with the *myc* constructs, both p309 (2

of 9 clones tested) and p310 (1 of 6) and yielded clones with titres higher than 10^5 cfu ml $^{-1}$ (all $1-2 \times 10^5$) although the majority of clones analysed showed low titres (Table 3). One stably transfected PA317 clone (one of three tested) also had a titre of 2×10^5 cfu ml $^{-1}$. The highest titres routinely observed in cell lines initially isolated for any of these constructs, including those with IGF-I or II, was 2×10^5 cfu ml $^{-1}$, irrespective of the construct, packaging cell line or route of isolation.

One of the stable virus-producing cell lines that had been obtained by transfection with p105, ψ -spC5–5, was further studied by re-cloning after fifty passages in culture. Titres of these new clones on 3T3 cells ranged from 2×10^4 to 5×10^6 cfu ml $^{-1}$ (Table 4). Thus, high titre virus production was maintained over a prolonged period in this original clone.

Cell lines producing amphotropic IGF-I virus were generated by infection of PA317 with ecotropic virus from the ψ -spC5–5 cell line. High titre PA317 clones were readily isolated, with nine of ten clones tested having titres of 5×10^4 – 2×10^5 on 3T3 cells (Table 5). It thus appears that infection of packaging cells with virus from an established virus-producing line is an efficient route for generating high titre cell lines. However, none of these clones showed titres in excess of those generated by transfection of ecotropic or amphotropic packaging cells.

The orientation of internal transcription and the presence of an internal polyA signal appear to affect LTR-driven transcription, and hence virus titre, in the case of all the constructs analysed. For both *myc* and IGF-I constructs containing an internal polyA signal, high titre cell lines were generated only from constructs with internal transcription in the reverse orientation to LTR-driven transcription. For the IGF-II constructs containing an internal polyA signal, high titre

Table 3. Titres of packaging cell lines transfected or infected with *myc* constructs. Values were obtained from at least two determinations

Clone	Viral RNA (pg ml ⁻¹)	Titre on 3T3 cells (cfu ml ⁻¹)
ψ myc309.2	2	n.t. ^a
ψ myc309.5	2	n.t. ^a
ψ myc309.6	<0.2	n.t. ^a
ψ myc309.13	<0.2	n.t. ^a
ψ myc309.14	50	2×10^5
ψ myc309.15	50	1×10^5
ψ myc309.16	<0.2	n.t. ^a
ψ myc309.19	<0.2	n.t. ^a
ψ myc309.20	2	1×10^2
ψ myc310.2	<0.2	n.t. ^a
ψ myc310.3	<0.2	< 10^2
ψ myc310.4	50	2×10^5
ψ myc310.7	2	n.t. ^a
ψ myc310.17	2	n.t. ^a
ψ myc310.18	2	< 10^2
ψ myc310.19	2	n.t. ^a
PA myc309.1	50	2×10^5
PA myc309.2	<0.2	< 10^2
PA myc310.1	<0.2	< 10^2

^a – Not tested: a viral RNA concentration of 2.0 pg ml⁻¹ or less in our experience routinely indicates a titre of 10^2 or less. (See clones ψ -2C7-3 in Table 1, ψ myc309.20, ψ myc310.18 above.) Hence, as isolation of high titre clones was one of the principal aims of this work, titrations were not routinely carried out on clones with RNA concentrations of this value.

clones were obtained with internal transcription in both orientations, though the reverse orientation construct produced high titre clones more consistently. Also, a *myc* construct with internal transcription in the same orientation as LTR-driven transcription, but without an internal polyA signal, produced high titre clones, as previously reported (Darnbrough and MacDonald, 1991). Thus, although the titres of all the recombinant retroviruses used were reduced in comparison to the parental pMsp vector, the presence of the internal *tk* promoter itself did not affect titres in either orientation, since high titre clones were isolated from constructs containing coding sequences in the same orientation as LTR-driven transcription (with internal polyA – p206; lacking internal polyA – p310), and from those containing coding sequences in reverse orientation (with internal polyA – p105, p207 and p309). Both of these types of construct have been shown to result in expression of functional products (Watts and MacDon-

Table 4. Titres of packaging cell lines obtained by sub-cloning the original line ψ -spC5-5 at passage 50

Clone	Titre on 3T3 cells (cfu ml ⁻¹)
1	2×10^5
2	3×10^5
3	2×10^5
4	2×10^5
5	2×10^5
6	5×10^6
7	1×10^6
8	5×10^4
9	5×10^5
10	2×10^4

Table 5. Titres of PA317 packaging cells generated by infection with ecotropic virus from the cell line ψ -spC5-5

Clone	Titre on 3T3 cells (cfu ml ⁻¹)
1	1×10^5
2	2×10^5
3	1×10^5
4	1×10^5
5	2×10^5
6	n.d. ^a
7	2×10^5
8	2×10^5
9	5×10^4
10	2×10^5

^a – Not detectable.

ald, 1990; Darnbrough and MacDonald, 1991; Darnbrough *et al.*, 1992).

It has been reported that the presence of an internal polyA site in a retrovirus vector can drastically reduce virus titre, presumably because prematurely terminated transcripts that cannot replicate properly are generated (Shimotohono and Temin, 1981). The data presented here support this hypothesis, since virus titre is markedly reduced when an internal polyA signal is present and internal transcription is in the same direction as LTR-driven transcription, compared to the other situations studied. Whilst titres of viruses containing internal transcription units, with a polyA signal, orientated in the opposite direction to LTR-driven transcription are consistently higher, it is possible that the

existence of an 'antisense' RNA transcription unit in these viruses is causing a reduction in titre. However, this effect is clearly less marked than that seen in viruses containing transcription units in the former orientation. Also, these data suggest that the efficiency of transcription termination at the internal polyA signal may be dependent on the inserted coding sequence, termination being more efficient in p104 and p308 than in p206.

Packaging cell line construction

Titres of virus-producing packaging cell lines generated by transfection or infection with virus from transient transfection were similar. However, only 25% of G418-resistant clones isolated by either method showed high virus titres, although the infection route gave a higher yield of G418-resistant cell lines. This suggests that both transfection and transient expression may result in a high proportion of defective viral genomes, probably from aberrant transcription, replication or recombination events prior to integration. A further possible determinant of virus titre is positional effects at the integration site, and both transfection and infection may result in integration into unfavourable sites for virus transcription. In contrast, 90% of clones isolated following infection of amphotropic packaging cells with virus from a stable ecotropic virus-producing cell line showed high titres (suggesting that infection does not lead to integration at unfavourable sites). Thus, either direct transfection or infection with transiently expressed virus of the appropriate packaging cell line followed by screening of the resultant clones until a high titre clone was found, or infection of these packaging cells with virus from a previously isolated high titre cell line of a different tropism would be feasible means of generating a high titre clone of a particular, desired tropism.

Estimation of virus titre

By comparing the viral RNA dot-blot data and the titres obtained on 3T3 cells (Tables 1 and 3), it can be concluded that a viral RNA concentration of $>20\text{pg RNA ml}^{-1}$ of medium indicates a titre in excess of $1 \times 10^5\text{cfu ml}^{-1}$. However, the relationship between virus RNA concentration and infective titre is both non-stoichiometric and non-linear. While dot blots indicate virion concentrations of up to $5 \times 10^6\text{ ml}^{-1}$ (50pg RNA ml^{-1}), titre did not exceed $2 \times 10^5\text{cfu ml}^{-1}$. This suggests that a high proportion of RNA-

Figure 2. Northern blots showing expression of proviruses following insertion into target cells. PQXB is a mouse hybridoma line (Darnbrough and MacDonald, 1991); BalbE δ t is a mouse macrophage line immortalised by SV40 (Kreuzburg-Duffy and MacDonald, 1991); J2/MR/2-1 is an immortalised mouse macrophage line carrying the *myc309.14* provirus (Darnbrough *et al.*, 1992). 10 μg of total RNA from each cell line was Northern blotted and hybridised to probes as follows: Cells: (a) NIH 3T3 infected with IGF-I.C5-5; (b) NIH 3T3 infected with IGF-II.C6-3; (c) uninfected PQXB 1/2; (d) PQXB/*myc309.14*; (e) PQXB/*myc310.4*; (f,h) BalbE δ t; (g,i) J2/MR/2-1. Probes: (a) IGF-I coding sequence; (b) *neo*; (c-g) *v-myc* coding sequence; (h,i) *c-myc* (exon 1). All probes were labelled to $2 \times 10^9\text{ dpm } \mu\text{g}^{-1}$ except for track b ($2 \times 10^8\text{ dpm } \mu\text{g}^{-1}$).

containing virions are either non-infectious or carry defective genomes which fail to integrate or express the *neo* marker. Thus, the dot blot method overestimates effective titres. However, in contrast with the reverse transcriptase assay often used to estimate virus concentration (which detects both virions containing or not containing genomic RNA), dot blotting measures only RNA-containing virions, and with the use of specific probes is able to quantitate virions containing specific cloned sequences. Both the dot blot method and the reverse transcriptase assay will overestimate viral titre and experimental titre should be determined by G418 resistance. Recently, a method for estimation of virus titre based on densitometric analysis of PCR products has been reported for simian immunodeficiency based retroviral vectors (Miyao *et al.*, 1995).

Expression of cloned genes

Fig. 2 shows the transcripts detected by Northern blotting of RNA from target cells infected with the IGF-I, IGF-II and *myc*-containing viruses. Full-length viral genome transcripts (7–8 kb.) are seen in 3T3 cells infected with recombinant viruses containing IGF-I or II genes (lanes a and b), the hybridoma PQXB1/2

infected with *myc* gene-containing recombinant viruses (lanes d and e) and the mouse splenic macrophage line J2/MR/2-1 (immortalised with p309 – lane g), but not in uninfected target cells (lane c) nor in the SV40-immortalised macrophage line, BalbE δ t (Kreuzburg-Duffy and MacDonald, 1991) (lane f). Transcription from the *tk* promoter in p105 is expected to yield a transcript of 0.85 kb. for IGF-I, but no transcript of this size could be seen, even after prolonged exposure (lane a). Thus, initiation of transcription from the *tk* promoter in this cell line is occurring only at low levels. In cells infected with virus from cells transfected with p206, two transcripts (lane b) of 7 and 6 kb. were observed; the smaller transcript is of the correct size to result from termination at the internal polyA signal. No transcripts of the correct size to have arisen from the *tk* promoter (1.2–2.1 kb.) were seen. Transcription from the *tk* promoter in the p309 construct is expected to yield a v-*myc* transcript of 2.2 kb. This is of similar size to the endogenous c-*myc* mRNA (shown by the exon 1 probe, lanes h and i) and cannot be resolved from it on Northern blots. The data therefore do not allow accurate quantitation of this transcript. However, there is no obvious difference in the overall levels of *myc* transcripts between infected and non-infected PQXB cells (lanes d and e), suggesting only weak transcription initiation from the *tk* promoter. In some cells containing the p309 construct, a processed viral transcript of 4.5–5.5 kb. was observed (lanes d and g). It is possible that this is a spliced derivative of a full length primary transcript from which the *neo* coding region has been spliced out. Such a transcript would contain RNA sequences complementary to the mRNA for v-*myc* and would therefore be detected by the DNA probe used.

Expression of the internal transcription unit is low relative to the rate of transcription from the viral LTR. This is consistent with other reports indicating that the *neo* coding sequence contains *cis*-acting elements which suppress transcription from nearby promoters (Artelt *et al.*, 1991). There may also be epigenetic effects (Emerman and Temin, 1984; 1986) which further suppress the expression of the internal promoter, although our data do not yield evidence of this. Despite the failure here to detect transcripts initiated from the *tk* promoter, it has been demonstrated previously that the inserted coding sequences are expressed at levels which are functionally significant – e.g. secretion of active IGF-I from 3T3 cells (Watts and MacDonald, 1990), growth enhancement of hybridoma cells by *myc* (Darnbrough and MacDonald, 1991) and immor-

talisation of primary cells following infection with the *myc* constructs (Darnbrough *et al.*, 1992). These earlier studies also demonstrated low levels of mRNA transcribed from the internal transcription units.

Biological containment

The results of XC and S+L– assays for helper virus indicated that 18% of ψ -2 and ψ -sp1-4 cell lines and 20% of 3T3 target cells infected with virus from helper free packaging lines carrying the IGF constructs secreted helper virus at high titres. None of the ψ -cre or PA317 packaging cells or infected target cells assayed carried helper virus.

It can thus be concluded that ψ -cre and PA317 offer a higher level of biological containment than the other two packaging lines, at least in the context of these coding sequences. This is to be expected from the structure of the packaging proviruses in these cells, but since it has been observed previously that the frequency of generation of helper virus is dependent on the particular combination of packaging and packaged proviruses (Miller *et al.*, 1986), helper virus formation should always be assessed on an empirical basis.

Conclusions

These experiments, and others reported elsewhere (Watts, 1990), used derivatives of the pMsp vector containing inserted transcription units with a weak promoter, expressing a product with a regulatory function, with or without an internal polyA signal, in two orientations with respect to LTR-driven transcription.

The pMsp vector has been shown to be a valuable system for the introduction of cloned coding sequences expressing a regulatory function into the cell types used here.

The constructs used, whilst allowing adequate levels of functional expression of the inserted coding sequence, showed only low levels of transcription from the internal promoter.

Virus titres were found to be reduced in those constructs containing an inserted transcription unit with an internal polyA signal when this unit was inserted in the same orientation as LTR-driven transcription. As the 3' LTR of the vector contains a polyA signal, it would clearly be both possible and advantageous to dispense with the internal polyA signal in constructs of this type. Indeed, some of the data presented here demonstrate that the absence of the internal polyA signal leads to

an increase in virus titre.

To achieve high level expression for isolation of a protein product from a pMsp vector derivative, a construct in which the cloned coding sequence is under the control of the LTR promoter or of a strong internal promoter would be more appropriate. More frequent transcription from the internal promoter in either orientation may result in more pronounced effects on virus titres, however.

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References

- Artelt P, Grannemann R, Stocking C, Friel J, Bartsch J & Hauser H (1991) The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells. *Gene* 99: 249–254.
- Bell GI, Merryweather JP, Sanchez-Pescador R, Stempien MM, Priestley L, Scott J & Rall LB (1984) Sequence of a cDNA clone encoding human preproinsulin-like growth factor II. *Nature* 310: 775–777.
- Brown AMC & Scott MRD (1987) Retroviral vectors, in Glover DM (ed.) *DNA Cloning – A Practical Approach*. Vol. 3 (pp. 189–212) IRL Press, Oxford.
- Cepko CL, Roberts BE & Mulligan RC (1984) Construction and application of a highly transmissible murine retrovirus shuttle vector. *Cell* 37: 1053–1062.
- Chomczynski P & Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159.
- Danos O & Mulligan RC (1988) Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA*. 85: 6460–6464.
- Darnbrough C & MacDonald C (1991) Enhanced growth characteristics of hybridoma cells infected with *myc*- and *ras*-containing retroviruses. *Exp. Cell Res.* 195: 263–268.
- Darnbrough C, Slater S, Vass MAV & MacDonald C (1992) immortalization of murine primary spleen cells by *v-myc*, *v-ras* and *v-raf*. *Exp. Cell Res.* 201: 273–283.
- Emerman M & Temin HM (1984) Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* 39: 459–467.
- Emerman M & Temin HM (1986) Quantitative analysis of gene suppression in integrated retrovirus vectors. *Mol. Cell. Biol.* 6: 792–800.
- Gorman C (1985) High efficiency gene transfer into mammalian cells, in Glover DM (ed.) *DNA Cloning – A Practical Approach*. Vol. 2 (pp. 143–190) IRL Press, Oxford.
- Green PJ, Pines O & Inouye M (1986) The role of antisense RNA in gene regulation. *Ann. Rev. Biochem.* 55: 569–597.
- Jansen M, van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabbay KH, Nussbaum AL, Sussenbach JS & Van den Brande JL (1983) Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature* 306: 609–611.
- Kreuzburg-Duffy U & MacDonald C (1991) Establishment of immortalized cell lines from mouse peritoneal macrophages following transformation with SV40 early region DNA deleted at the origin of replication. *Immunology* 72: 368–372.
- Mann R, Mulligan RC & Baltimore DB (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33: 153–159.
- Miller AD & Buttimore C (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* 6: 2895–2902.
- Miller AD, Law MF & Verma IM (1985) Generation of helper-free amphotropic retroviruses that transduce a dominant acting methotrexate-resistant dihydrofolate reductase gene. *Mol. Cell. Biol.* 5: 431–437.
- Miller AD, Trauber DR & Buttimore C (1986) Factors involved in production of helper virus free retrovirus vectors. *Somatic Cell Mol. Genet.* 12: 175–183.
- Miyao Y, Shimizu K, Tamura M, Yamada M, Tamura K, Nakahira K, Kuriyama S, Hayakawa T & Ikenaka K (1995) A simplified general method for determination of recombinant retrovirus titres. *Cell Struct. Func.* 20: 177–183.
- Ostertag W, Seliger B, Kollek R, Stocking C, Bergholz U & Smadja-Joffe F (1986) The myeloproliferative sarcoma virus retains transforming functions after introduction of a dominant selectable marker gene. *J. Gen. Virol.* 67: 1361–1371.
- Rowe WP, Pugh WE & Hartley JW (1970) Plaque assay techniques for murine leukaemia viruses. *Virology* 42: 1136–1139.
- Shimotohono K & Temin HM (1981) Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell* 26: 67–77.
- Stanton LW, Fahrlander PD, Tesser PM & Marcu KB (1984) Nucleotide sequence comparisons of normal and translocated murine *c-myc* genes. *Nature* 310: 423–425.
- Stewart MA, Forrest D, McFarlane R, Onions D, Wilkie N & Neil JC (1986) Conservation of the *c-myc* coding sequence in transduced feline *v-myc* genes. *Virology* 154: 121–134.
- Watts PL (1990) Retroviral vector-mediated expression of cloned growth factor genes in mammalian cells. Ph.D. thesis, University of Strathclyde.
- Watts PL & MacDonald C (1990) Production of biologically active insulin-like growth factor by cells transfected with a recombinant retroviral vector encoding IGF-I, in Spier R, Griffiths JB & Meignier B (eds.) *Production of biologicals from animal cells in culture*. (pp. 726–731) Butterworth-Heinemann, Oxford.
- Yee J-K, Moores JC, Jolly DJ, Wolff JA, Respass JG & Friedmann T (1987) Gene expression from transcriptionally disabled retroviral vectors. *Proc. Natl. Acad. Sci. USA*. 84: 5197–5201.

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