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A POLYOMAVIRUS ENHANCER MUTANT CONFERS UBIQUITOUS HIGH TRANSCRIPTIONAL EFFICIENCY TO THE SV40 LATE PROMOTER

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SUMMARY: To identify expression plasmids with high efficiency of transcription and with a broad tissue and cell range, we have constructed a recombinant vector combining the late SV40 promoter and the polyomavirus regulatory region derived from a mutant (PyNB11/1) which displays a very wide host range. We show that these recombinant enhancer-promoters are efficient drivers for heterologous gene transcription and expression *in vitro* in all mouse and human cells tested. The most active combination we identified contained the mutant enhancer (PyNB11/1) in the late orientation. This construct was able to promote a high efficiency of expression without significant fluctuation between cells of different tissutal origin or different differentiative stage. A possible interpretation of these results is discussed.

Polyomavirus (Py) has a redundant regulatory region (R.R.) which binds a number of host cell factors (1-4). This property gives to the virus the ability to replicate in a variety of murine host cells, and its R.R. is an efficient enhancer in driving heterologous gene transcription in chimeric plasmids (5). Polyoma mutants with a modified host range were selected in a variety of cells of different tissutal derivation and at different differentiation stages (for a review see ref.6). One of such mutants (Py NB11/1), originally selected in murine neuroblastoma cells (7), was characterized by its ability to efficiently replicate in cell lines where the Py w.t. and other mutants were impaired in growth (8). The mutant virus regulatory region (R.R.) is characterized by a 91 bp duplication that creates in the junction site a sequence recognized by the NF1 host cell

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activator factor (9), which does not recognize the w.t. R.R. (10). Due to the special growth properties of Py NB11/1 we have used its R.R. to construct expression plasmids.

In the present work we report the construction of plasmids using Py NB11/1 R.R.s in early (E) and late (L) orientation and the SV40 late promoter to drive heterologous gene transcription. The results obtained show that the mutant Py R.R. is more efficient in the L orientation. This construct is much more efficient than the parental SV40 based plasmid (pSVL, Pharmacia) and the LTR of Rous sarcoma virus (11), that are generally considered efficient transcriptional enhancers. This advantage of our constructs has been observed in all the mouse and human cells tested. The higher activity observed is characterized by the ability to transcribe the reporter gene in all cells with comparable efficiency, whereas the other expression plasmids tested show significant variation depending on the tissue origin or on the differentiation stage of the host cells.

MATERIALS AND METHODS

Virus and plasmids.

The A2 polyomavirus mutant PyNB11/1 was previously described (7,10). The plasmids pSVL (Pharmacia) and pRSV-LTR (11) in which was cloned the bacterial gene for the synthesis of chloramphenicol-acetil-transferase (CAT), were used to compare the efficiency of the recombinant constructs. To normalize the CAT measurements to the efficiency of transfection we used the activity of the luciferase gene cotransfected with pRSV-Luc. (12).

Cell lines.

The following cell lines were used: mouse Swiss and NIH 3T3 fibroblasts, C2 myoblasts (13), embryonal carcinoma F9, human Hela, liver carcinoma HepG2 (14) and Jurkat (15) and COS-1, SV40 transformed simian cells (16). All cell lines were grown in cell culture dishes, in DMEM (Gibco) supplemented with 10% Fetal Calf serum, except Jurkat cells that was grown in bacterial dishes in RPMI medium (Gibco) supplemented with 10% Fetal Calf Serum.

Cell transfections and the chloramphenicol acetyl transferase assay.

Cells were transfected using the calcium phosphate precipitation method (17) with approximately 10^6 cells per 100 mm diameter dish.

Constructs were tested for the expression of the CAT gene in comparison with pSVL-CAT and pRSV-LTR-CAT. Transient CAT assays were performed using 2μg of plasmid DNA, 1 μg of pRSV-Luc DNA and 7μg of mouse genomic DNA as carrier per plate. Fortyeight hours after transfection the cells were disrupted by freezing and thawing; extracts were assayed for CAT activity according to the method of Gorman et al. (11). The luciferase assay was done according to de Wet et al.(12)

The CAT expression efficiency was measured by the percentage of conversion of chloramphenicol in the different acetilated forms counting radioactivity displaced in the spots after chromatography. These values have been normalized for the efficiency of transfection by the measurement of luciferase activity of the cotransfected plasmid pRSV-Luc.

Stable CAT transfectants were obtained by cotransfecting semiconfluent cells with the following amounts of DNA: 2 μg of various CAT plasmids , 0.2 μg of neomycin resistance expressing plasmid pIBW3 and 8 μg of mouse genomic DNA as carrier

(except for pNBL-NEO-CAT that was cotrasfected only with the DNA carrier). 36 hours after transfection cells were split 1:7 in selective medium containing 400 μg/ml of G418. The medium was changed every 2-3 days and, after 15 days, the surviving colonies were mixed together. The CAT activity of stable transfected clones was assayed using a constant amount of protein (40 μg). The protein concentration of the extracts was measured by the Biorad assay.

RESULTS

Construction of the plasmids.

We isolated a fragment of PyNB11/1 genome from the Bcl I (nt 5021) to Bgl I (nt 93) sites that includes all the R.R. and the origin of replication as well as the Late

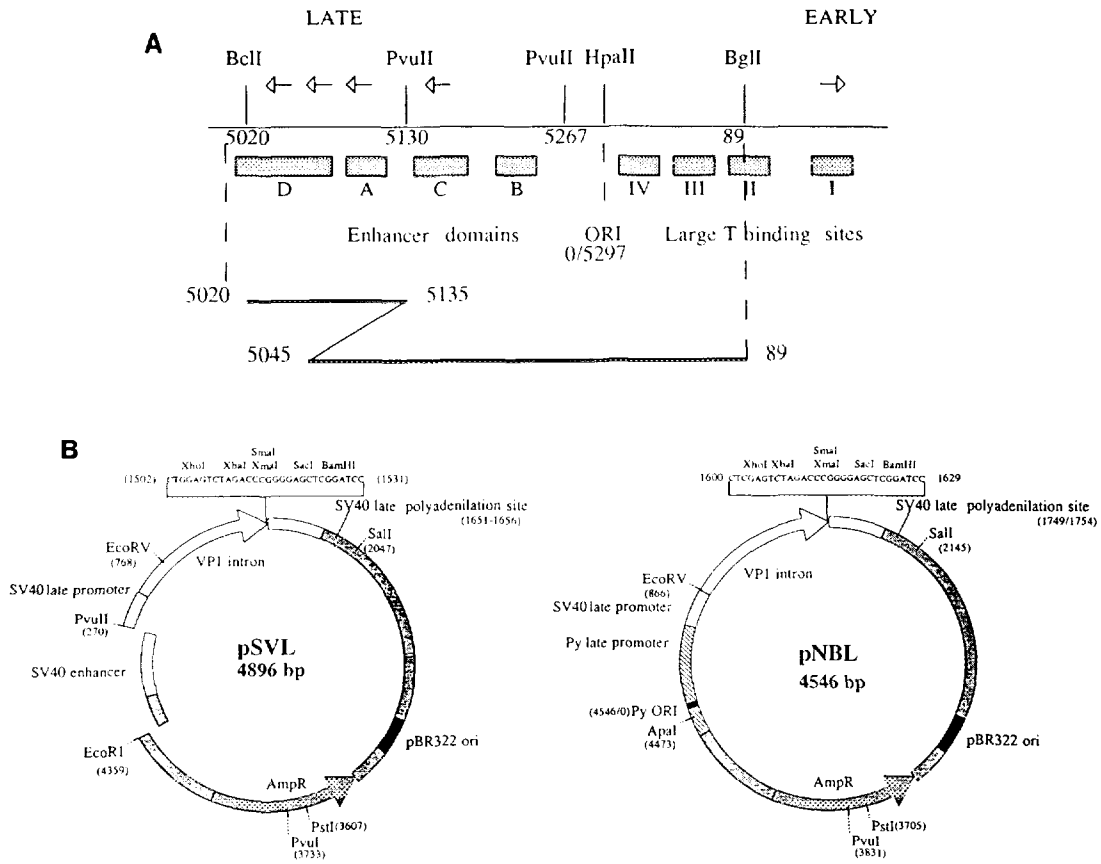

Figure 1. **Diagram of the construction of pNBL. A)** Schematic representation of the Py A2 enhancer with the localization of relevant sites. Arrows show transcription initiation sites. Lower bar shows the Py NB11/1 enhancer 91 bps duplication. **B)** Schematic representation of the substituted part of the SV40 enhancer in the pSVL plasmid (left) and the result of the insertion of the Py NB11/1 enhancer in the Late orientation. The insertion of Py NB11/1 enhancer in Early orientation results in the inversion of the inserted Py fragment. The relative position of the different sites in relation to Py Ori can be determined subtracting 311 bp.

promoters and the CAP site. (Fig.1-A). This fragment was inserted in pSVL at the Pvu II site deleting the EcoR I fragment (Fig 1-B). The orientation of the inserts was determined by the digestion with EcoR V that cuts only in the pSVL and Apa I that cuts only in nt. 10 of the Py fragment. The plasmids obtained were called either pNBE when the insert was in E orientation or pNBL when the insert was in L orientation with respect to the SV40 promoter. In the two recombinant plasmids as well as in the parental pSVL we used the Chloramphenicol Acetyl Transferase (CAT) gene as reporter gene. The CAT gene was cloned at the BamH I site of the polylinker. The unique Sal I site downstream from the SV40 polyadenilation sequence was used to insert into pNBL-CAT the bacterial neomycin resistance gene under RSV-LTR transcription control in the same orientation of transcription of the reporter gene.

Enhancer efficiency of the polyoma R.R. in dependence of its orientation.

In order to determine the comparative efficiency of the two constructs the CAT derivatives of recombinant Py enhancer-SV40 promoter and the parental pSVL expression plasmids were utilized to transfect NIH 3T3 and Hela cells. Results reported in Fig.2 clearly show that pNBE-CAT and pNBL-CAT are far more efficient than pSVL-CAT, and that the Py R.R. in late orientation is significantly better in promoting CAT expression than in early orientation. This difference has been observed in several other cell lines (data not shown).

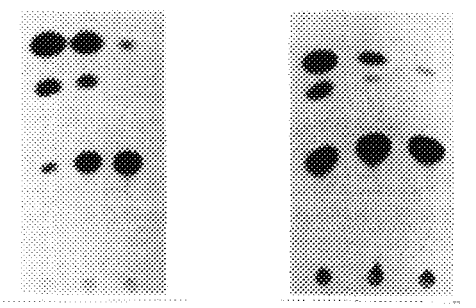

Figure 2. **Transcriptional efficiency of pNBL, pNBE and pSVL.** Mouse NIH-3T3 and human Hela cells were transiently transfected with the CAT derivatives of the three plasmids.

Table 1: Comparision of CAT expression efficiency of pNBL and pRSV with pSVL in different cell lines

CELL LINES	NBL	RSV	SVL
3T3NIH	120	15	1
3T3SWISS	63	4	1
C2	3	3	1
F9	23	3	1
COS	0,4	0.04	1
HELA	15	1	1
HEPG2	12	5	1
JURKAT	4	1	1

Efficiency of CAT expression of the plasmids in murine and human cells in transient transfection.

The plasmids pNBL-CAT, pRSV-LTR-CAT and pSLV-CAT were used to transfect murine, simian and human cells of different tissutal derivation. To determine the advantage given to the plasmid by the substitution of the SV40 enhancer with the Py enhancer the reported data were normalized to the activity of pSVL-CAT (Table 1). With the exception of COS cells, the presence of the Py enhancer produces a significant increased expression, ranging for 4 to 120 times the values obtained with SVL-CAT. The advantage of SVL in Cos-1 cells is likely to be due to the endogenous expression of SV40 Large T that allows the replication of pSVL-CAT. The RSV-LTR enhancer seems generally more efficient than pSVL (Table 1).

In order to better visualize the results of Table 1, a histogram is reported in Fig. 3 that shows that pNBL-CAT is by far the most active in all cells tested with the exception of COS cells. These results also show that pNBL is the only one of the plasmids tested whose ability to promote gene expression is relatively constant in cells of different species and tissue derivation.

Transcription efficiency in stable transfectants.

In order to analyze the efficiency of transcription of the plasmids after stable transfection we cloned the neomycin resistant gene under RSV-LTR enhancer promoter at the SalI site (see figure 1) in the same orientation of transcription as that of CAT (pNBL-neo). This construct was compared in transient transfection with the previously analyzed plasmids. Results reported in Fig.4A show that pNBL-neo-CAT is approximately 1/3 less efficient than pNBL-CAT. This result can be interpreted as competition between pNBL and RSV-LTR promoters for cellular activator factors due to the high number of plasmids in the transient transfected cells. In stable transfection pNBL-neo-CAT is more efficient, even more so than pNBL-CAT (Fig.4B). The lower efficiency of pNBL-CAT in stable, than in transient, transfection may be due to the fact

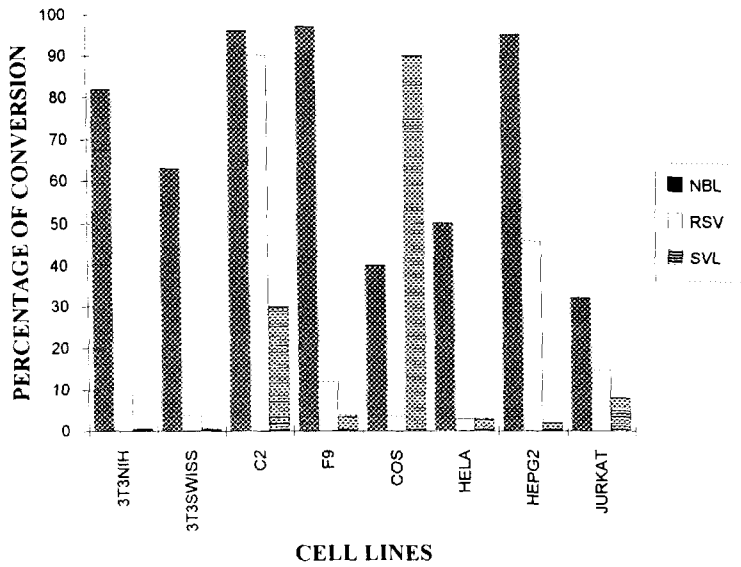

Figure 3. **Histogram of transcriptional efficiency in different cell lines of pNBL, pRSV-LTR and pSVL.** The different cell lines indicated were transiently transfected with the CAT derivatives of the three plasmids. Values are the ratio of acetilated to non acetilated chloramphenicol expressed in percent.

that these results were obtained in uncloned cultures of transfected cells selected with neomycin, and therefore this low difference can be ascribed to a fraction of cells that lost the CAT expressing plasmid during the selection for neomycin resistance.

DISCUSSION

The selection of efficient enhancer-promoter sequences for transcription of genes in eukaryotes is, at present, an important subject of research. This attention is motivated by the interest of selecting regulatory signals with characteristics of ubiquity or tissue specificity, differentiative stage specificity and high efficiency of expression, that can serve for different purposes such as: gene therapy, animal transgenization, specific protein production, etc.

Viral regulatory regions from retrovirus (LTR) and DNA tumor virus, as well as the promoter-enhancer regions of some eukaryotic genes, have been extensively used for the construction of expression plasmids. Many of them are now commonly used and commercialized. Polyoma and SV40 R.R.s have been largely used to construct expression plasmids. The Py R.R. has been considered a moderately efficient promoter-enhancer element (5), whereas SV40 R.R. has been more extensively used and several constructs are commonly available.

In the present work we report the construction of expression plasmids derived from a commercial SV40 driven construct in which the enhancer element was substituted

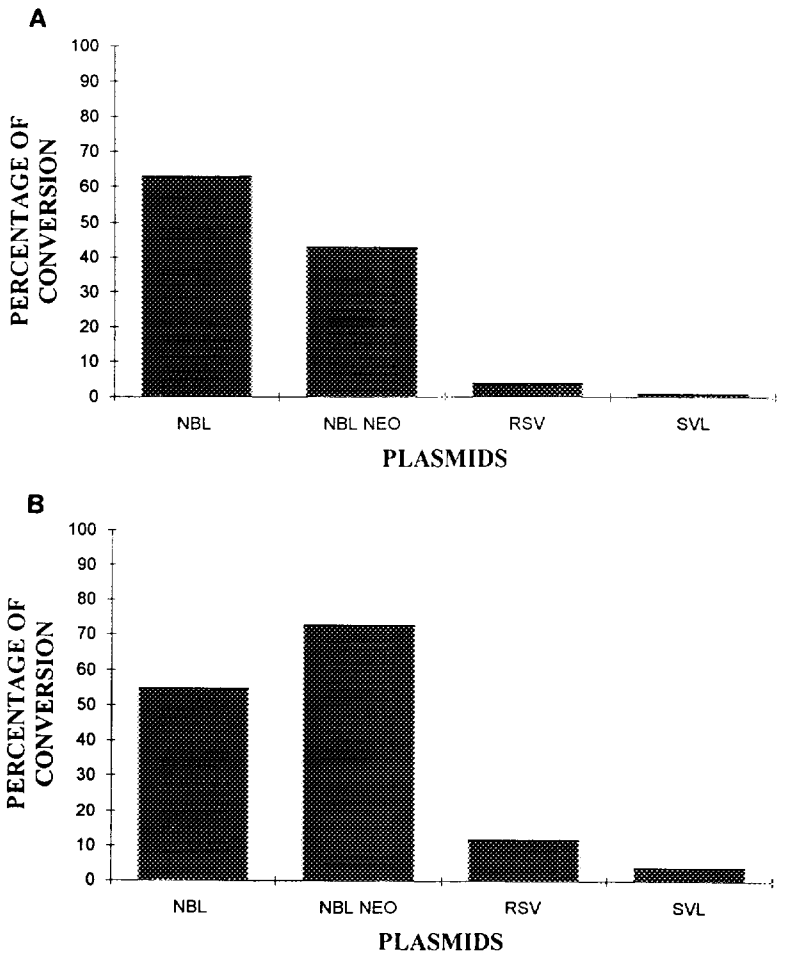

Figure 4. **Comparison of transcriptional efficiency of pNBL, pNBL-neo, pRSV- LTR and SVL in transient and stable transfection.** Swiss 3T3 cells were transiently (A) or stably (B) transfected with the CAT derivative of the plasmids. Stable transfected cells for each plasmid represent a pool of neomycin resistant clones.

with the R.R. of Py mutant (Py NB11/1) in both early and late orientation with respect to the retained SV40 promoter. This Py mutant has been selected for its ability to replicate in neuroblastoma cells (7) and was shown to replicate in cells where the Py wt was unable to grow (8). Since the replicative and transcriptional property of the Py R.R. appears to be strictly correlated (1) we have used this mutant R.R. to construct an efficient ubiquitous expression plasmid. The main advantages of this expression vector appear to reside in its ability to support a high level of gene expression in a variety of cell lines of different species and tissue origin. Thus it should be useful wherever ubiquitously high levels of gene expression are desired, particularly *in vivo* (see bellow). The reasons for high efficiency of expression observed with the pNBL vector are not totally clear at the moment. The Py NB11/1 enhancer promoter is by itself a mild transcriber

(unpublished results), but a direct comparison with the present constructs cannot be made since other differences between constructs may have their influence (e.g. SV40 promoter and VP1 intron). Unexpectedly, a construct in which the Py NB11/1 R.R. was replaced by the wild type R.R. was almost as efficient as pNBL in promoting gene expression in all cells tested (data not shown). This finding suggests that the main reason for the high efficiency of the pNBL vector is the environmental conditions in which the Py R.R. is found.

The finding that Py R.R. is more efficient in L than in E orientation can be ascribed to the fact that in L orientation both Py and SV40 late promoters are present, whereas in E orientation there is no Py promoter. Primer extension experiments show that the Py late promoters are also active (data not shown). Thus it is possible that the juxtaposition of the Py enhancer-promoter region next to the SV40 promoter is particularly important.

Recent work using pNBL in driving the expression of an antisense RNA in transgenic mice (Vignali et al. unpublished results) demonstrates that the transcription of the cloned sequences is extremely efficient in a variety of tissues; pNBL, therefore, appears a valid tool for *in vivo* gene expression.

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