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Replicative *cis*-Advantage of Polyomavirus Regulatory Region Mutants in Different Murine Cell Lines

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To determine the relative growth advantages of polyomavirus regulatory region mutants selected from Friend leukemic and neuroblastoma cells persistently infected with polyomavirus A2 wild type, different murine cell lines, some of which are capable of further differentiation in vitro, were used as hosts in mixed infection and transfection. The tests allowed the determination, through the measurement of *cis*-advantage in replication, of the most effective polyomavirus regulatory region constitutions for a given cell line and, in some cases, for specific stages of cell differentiation. Furthermore, different domains of the viral regulatory region were shown to have different effects—positive, neutral, or negative—depending on the host analyzed.

The regulatory region of the polyomavirus (Py) genome comprises multiple genetic elements regulating both viral replication (23) and early (5) or late (15) viral gene expression.

There are three sequence domains in this region that are essential for viral DNA replication: the ORI region domain (where DNA replication starts) (13) and two other domains, known as α and β (19), located in the late side of the ORI region domain and coinciding with the A and B domains of the Py transcriptional enhancer (11). It has been demonstrated that the minimal requirement for a plasmid to be replicated in Py T-antigen-producing cells is to contain in *cis* the Py ORI region domain and either the α or the β domain (19). Other studies have revealed that these domains can be functionally replaced by a heterologous enhancer region of either viral or cellular origin (8). This region of the Py genome was further characterized by deletion mapping, which led to the identification of four domains that have a cooperative effect on Py transcription and DNA replication (23): A (or α); B (or β); the C domain, which is located between A and B and coincides with the Ω domain we described (16); and the D domain, which has less-defined boundaries and extends for approximately 60 base pairs on the late side of A (nucleotides 5020 to 5080).

The α and β domains of the Py regulatory region are also involved in the determination of the viral host range. In fact, all the Py mutants which were selected for growth in nonpermissive murine cell lines, such as the embryonal carcinoma (EC) PCC4 (18) and F9 (14) cell lines, show cell-specific rearrangements (or point mutations) in these domains.

We recently characterized Py regulatory region mutants selected in differentiated cell lines and at different stages of differentiation in neuroblastoma (NB) cells (16). The kinetics of mutant selection in different cell lines showed that the occurrence of mutants is inversely related to the permissivity of the host cells and that in persistent infection mutants are selected for their *cis*-advantage in replication (6, 7).

We interpreted (1) the complexity of the Py regulatory region as an evolutionary advantage that enables the virus to show a modulated affinity in its interaction with cell regulatory proteins specific to different mouse tissues and to changing differentiative stages in a given tissue. To verify this interpretation, in the present work we compared the replicative advantages of different regulatory regions in cells derived from several tissues and at different stages of differentiation.

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MATERIALS AND METHODS

Mouse cell lines and growth conditions. 3T3 and 3T6 fibroblasts were used as standard hosts. The other cell lines tested were Friend leukemic (FL) cells (6), NB 41A3 cells (2), and EC F9 cells (17). The last two were used in different differentiative stages.

All cell lines were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) except the NB cells, which were grown in F-10 (GIBCO) medium. Both media were supplemented with 10% fetal bovine serum. The NB cells were grown in suspension (NB-s; less-differentiated stage) or in monolayer (NB-m; more-differentiated stage). Undifferentiated F9 cells (EC-nd) were induced into differentiating cells (EC-d) by the addition of retinoic acid (10^{-7} M) to the growth medium.

The killing effect of Py growth on the infected cells was estimated by trypan blue staining.

Viral strains and infection. The viral strains used were the PyA2 wild-type (wt) strain (21), the PyFL78N mutant (7), and the PyNB11/1 and PyNB10/6 (16) mutants. Their regulatory regions are shown in Fig. 1, and the differences in their restriction enzyme patterns are shown in Fig. 2. Viral titers were determined by using plaque assays and the hemagglutination test. All infections, single or double, were done with 100 PFU per cell.

Restriction enzyme analysis and blot hybridization. The viral DNA from infected cells was extracted by using the Hirt procedure (12) and digested by the appropriate restriction endonucleases (see Results). The restriction fragments were separated on 2% agarose gels and transferred to nitrocellulose filters according to the Southern procedure (22). Filters were hybridized to nick-translated ^{32}P-labeled DNA of the Py *BclI-BglI* small fragment cloned in pAT153 plasmid, as previously described (6). Autoradiography was done under conditions that maximized linear response (preflashed films exposed at −80°C with an intensifying screen). The relative intensities of the bands were measured by densitometric scanning of the autoradiographic films.

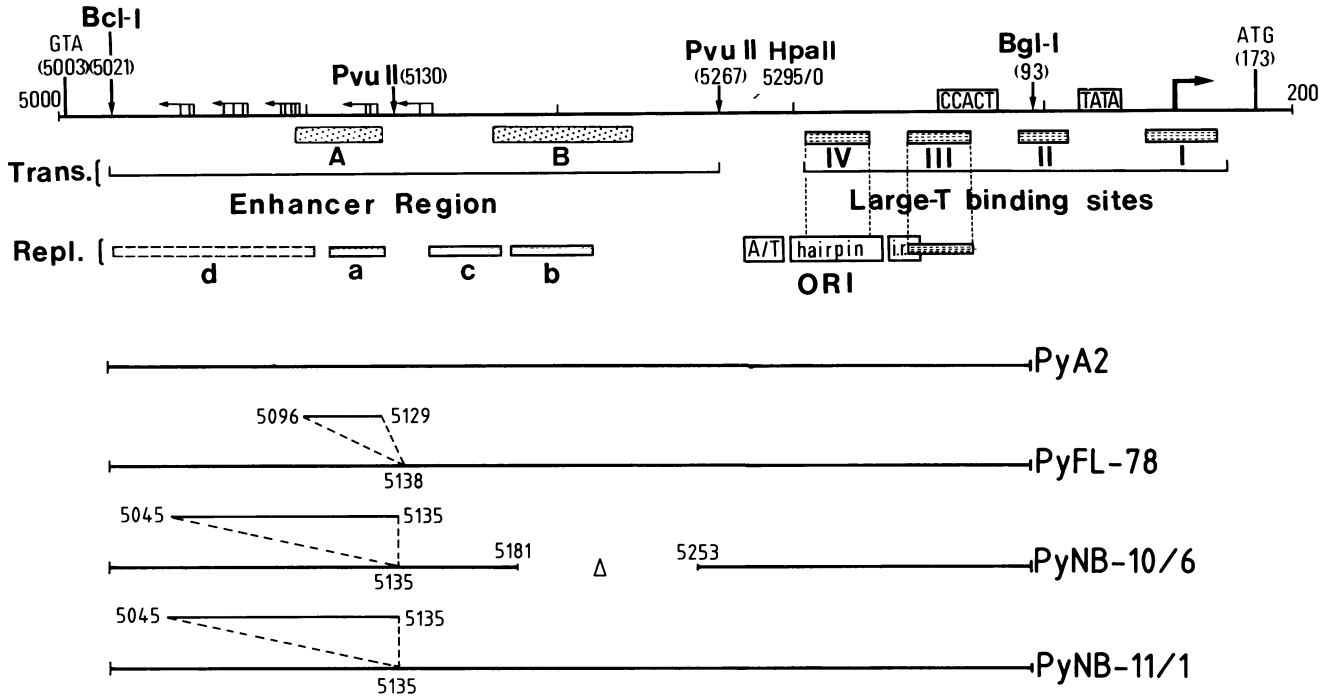

FIG. 1. Schematic representation of the relevant features of the Py regulatory region. In the top lines are reported: the locations of the initiation codons and the transcriptional starts (arrows) of both early (right) and late (left) coding regions (8); the enhancer region (23) with the domains defined by Herbomel et al. (11); the sites of large-T binding (4); and the elements necessary for replication as defined by Veldman et al. (24). The positions of the restriction enzyme recognition sequences are numbered according to Ruley and Fried (21). Below are shown the rearrangements, as duplicated or deleted sequences, of the Py mutants.

Construction of virus-plasmid recombinants able to replicate. From all the virus mutants tested, the genome segments spanning from the *Bcl*I site (nucleotide 5021) to the *Hin*cII site (nucleotide 2962) were cloned between the *Bam*HI and the *Hin*cII sites of the pUC19 plasmid vector (25). This viral region genome segment contains the complete Py regulatory and early coding regions which enable the recombinant plasmids to replicate in Py-permissive cells.

The DNA concentrations of the virus-plasmid recombinants were determined by measurement of optical density and by ethidium bromide staining of DNA run on agarose gels.

The mixed transfections were done with 1 μg of each recombinant DNA per 10-cm-diameter petri dish of cells plus 20 μg of pUC19 DNA as carrier, by using the $CaPO_4$ precipitation method (27). The background effect of the unreplicated input plasmid DNA was eliminated by using the *Bcl*I restriction enzyme, which is unable to digest DNA that has been methylated during growth in *Escherichia coli*.

RESULTS

Permissivity of cell lines to Py. The permissivity to Py wt (A2 strain) of several stabilized in vitro cell lines was assessed by measuring cell death, DNA replication, and viral infective particle production. Results (summarized in Table 1) show that the 3T3 and 3T6 fibroblasts and the FL cells were highly susceptible to Py. NB cells in either growth condition—suspension, corresponding to a less-differentiated stage, or monolayer, corresponding to a more-differentiated stage—were always only slightly permissive (semipermissive), as was previously reported (3); the EC cells were not permissive to Py growth unless differentiation was induced, whereupon they became semipermissive. We call semipermissive those cell lines in which Py replicates less and produces fewer viral particles than in permissive cells (at least 1,000-fold less) without any evident cell lysis, and we call nonpermissive those in which Py does not replicate at all.

Some differences in the growth abilities of different Py strains in different cell lines have been observed, but they are difficult to quantify. To assess such differences quantitatively and to correlate the various growth abilities to a cell-specific selective advantage of Py regulatory region mutants, we devised an assay based on the competitive growth of Py mutants in cell lines with mixed infection.

Mixed infection of permissive cell lines. A first set of experiments was done with 3T3 (data not shown because identical to results obtained with 3T6 cells), 3T6, and FL cell lines. All these cell lines were permissive to Py (Table 1). However, a surviving cell population recovered from the infected cultures creates the conditions for persistent infection, giving rise to semipermissive cells (6, 20).

Mixed infections of both the 3T3 and 3T6 cell lines (Fig. 3; Table 2) showed that at the very early stages of Py replication (3 and 6 days) there is no significant modification of the initial 1:1 ratio of PyA2 wt to the mutant genomes. In contrast, in FL cells the PyFL78N mutant genome shows a very clear-cut growth advantage over PyA2 (7) and over two PyNB mutants as well. It is noteworthy that PyNV11/1, whose duplicated region includes the sequence duplicated in pYFL78N, did not exhibit a growth advantage in FL cells.

Results obtained 12 days after infection were considerably different. All mutants showed a growth advantage over the PyA2 strain in all cell lines; in almost all cases, the PyA2 genome was reduced to only a small fraction of the total viral

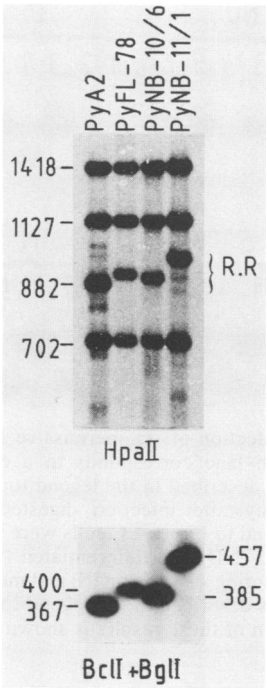

FIG. 2. Restriction enzyme patterns of Py regulatory region mutants. Southern blot (22) hybridization patterns of *Hpa*II- or *Bcl*I plus *Bgl*I-restricted Py DNA of mutants and wt viral strains. The upper panel shows the *Hpa*II digestion of viral DNA extracted from 3T6 cells 3 days after infection and probed with whole cloned Py genome. R.R., Restriction fragments containing the virus regulatory region. The lower panel shows the same virus DNAs digested with *Bgl*I-*Bcl*I restriction endonucleases and hybridized to a probe containing the regulatory region only (nucleotides 5021 to 93). Fragment lengths in base pairs are indicated.

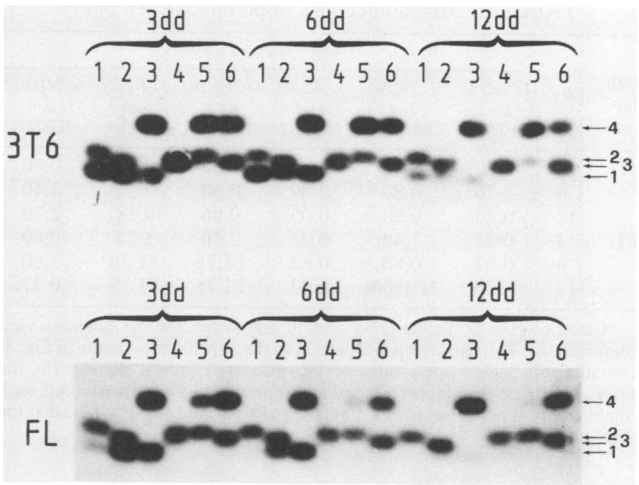

FIG. 3. Mixed infection of permissive 3T6 and FL cell lines. Each lane corresponds to a combination of two different viruses (see Materials and Methods). Viral DNAs were extracted at 3, 6, and 12 days after infection. Lanes: 1, PyA2 plus PyFL78; 2, PyA2 plus PyNB10/6; 3, PyA2 plus PyNB11/1; 4, PyFL78 plus PyNB10/6; 5, PyFL78 plus PyNB11/1; 6, PyNB10/6 plus PyNB11/1. DNAs were digested by *Bcl*I plus *Bgl*I and hybridized to a probe containing the regulatory region only. The arrows indicate the positions of the *Bcl*I-*Bgl*I fragment of: 1, PyA2; 2, PyFL78; 3, PyNB10/6; and 4, PyNB11/1. The optical density quantification of these results is shown in Table 2.

DNA (from 10 to 1%). There is also an evident hierarchy in replication efficiency within the mutant group: PyNB10/6 > PyNB11/1 > PyFL78N in 3T3 and 3T6 cells and PyFL78 > PyNB10/6 > PyNB11/1 in FL cells.

Mixed infection of semipermissive and nonpermissive cell lines able to undergo in vitro differentiation. Our previous data (16) indicate that the relative growth abilities of the PyNB10/6 and PyNB11/1 mutants change when further in vitro differentiation is induced in the NB host cells. To analyze the relationships among in vitro differentiation, permissivity to Py growth, and rearrangements in the Py regulatory region, we performed mixed-infection experiments with two of three murine cell lines which allow very limited Py growth and are able to undergo in vitro differentiation. The cell lines used were the semipermissive NB41A3 (16) and the nonpermissive EC F9 (14). Upon in vitro differentiation, the latter cell line partially lost its restriction to Py growth. As in previous experiments, cells were infected by the six viral mixtures and viral DNA was extracted at 3, 6, and 12 days after infection. Each cell line was infected at both a less-advanced and a more-advanced differentiation stage. Results of these experiments are reported in Fig. 4 and Table 3 and can be summarized as follows.

(i) All Py mutants grew better than the PyA2 wt in all cell lines tested, and this growth advantage seems to be more pronounced at the less-differentiated stage of the cells.

(ii) In differentiated NB cells (NB-m), the order of replication efficiency was PyNB10/6 > PyNB11/1 > PyFL78. This result is similar to those obtained with permissive cell lines after longer infection times (Fig. 3).

(iii) According to our previous data (16), in nondifferentiated NB cells, there is a very marked growth advantage of PyNB10/6 over PyNB11/1.

(iv) In nondifferentiated F9 cells, the only Py strain which was able to grow was the PyNB11/1 mutant. No other viral strain grew. When differentiation was induced in the F9 cells, the other Py strains also grew. In this case, the hierarchy in replication efficiency was PyNB11/1 > PyNB10/6 > PyA2 > PyFL78.

Mixed transfections. To rule out all possibility of outside effects during the infection procedure, i.e., differences among the various viral strains in adsorption or uncoating, etc., nearly all the described competition experiments were repeated by using a mixed-transfection procedure. For this purpose, DNA fragments containing the regulatory and the early coding regions of the four Py strains used were cloned in a pUC19 plasmid vector. These plasmids are able to replicate and to express the Py early functions but not the

TABLE 1. Permissivity to Py growth of different cell lines[a]

Cell line	% Cell death[b]	DNA replication[c]	Viral yield (PFU/ml)[d]	Py growth class
3T3	99	+ + + + +	10^8	Permissive
3T6	99	+ + + + +	10^9	Permissive
FL	90	+ + + + +	10^8	Permissive
NB-s	<1	+	10^3	Semipermissive
NB-m	<1	+ +	10^4	Semipermissive
EC-nd	<1			Nonpermissive
EC-d	<1	+	10^3	Semipermissive

[a] All measurements were done 6 days after PyA2 infection.
[b] Measured as percentage of dead cells, by trypan blue staining.
[c] Expressed in arbitrary units as estimated by Southern blot hybridization.
[d] Viral infective particles yield measured as PFU/milliliter in cell lysates.

TABLE 2. Mixed infections of permissive cell lines[a]

Cell line	Day	Mixture no. and viruses					
		1. A2 wt and FL78	2. A2 wt and NB10/6	3. A2 wt and NB11/1	4. FL78 and NB10/6	5. FL78 and NB11/1	6. NB10/6 and NB11/1
3T6	3	1.80	1.40	0.55	1.00	0.53	0.70
	6	2.20	0.80	0.80	0.50	0.36	1.40
	12	0.20	0.08	0.05	0.06	0.18	2.30
FL	3	0.22	1.40	0.75	2.10	1.24	0.60
	6	0.03	0.80	0.62	FL78	FL78	1.00
	12	FL78	NB10/6	0.02	FL78	FL78	0.77

[a] Quantification of the relative growth advantage for the different viral combinations. The measurements pertain to the experiments shown in Fig. 3. The numbers indicate the ratio of the measured optical density for the autoradiographic bands corresponding to the two viral strains for each combination. When, instead of a number, a virus is indicated it means that the other parental virus was not detectable.

late functions and, therefore, they do not produce infective particles.

We used these DNAs in mixed-transfection experiments according to the same rationale as in the infection experiments. All cell lines used previously (except the FL cells) were transfected. The DNA from the transfected cells was extracted after 3, 6, and 12 days; the viral DNA in the plasmids was analyzed by using the same procedure as after infection.

The results of these experiments (Table 4) fit the data obtained with mixed infections, demonstrating that the cell line-specific patterns of growth advantage described above are not the result of any side effect associated with the infection procedure and that rearrangements of the regulatory region alone are responsible for the observed growth differences.

DISCUSSION

We have already shown that in FL cells a specific duplication of the A enhancer domain gives the virus a *cis*-advantage in replication (7) and that in NB cells selected constitutions show the same advantage (16). These findings are in accordance with the earlier observation that in PCC4 EC cells the viable mutant virus is unable to rescue the unviable Py wt genome (9).

Our present results provide definitive, confirming evidence that the ability of Py to replicate in restricted hosts is a noncomplementable property and, therefore, must be

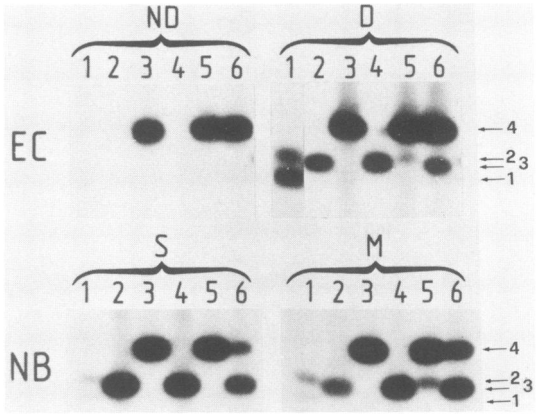

FIG. 4. Mixed infection of semipermissive NB and nonpermissive EC cells. Each lane corresponds to a combination of two different viruses, as described in the legend to Fig. 3. Viral DNAs were extracted 12 days after infection, digested, and hybridized as described in the legend to Fig. 3. EC cells were infected both during the nondifferentiated (ND) and differentiated (D) stages; NB cells were infected both during suspension (S) and monolayer (M) stages. Arrows 1 to 4 are as described in the legend to Fig. 3. The optical density quantification of these results is shown in Table 3.

mediated by the interaction of cell factors with specific *cis*-acting DNA sequences. This *cis*-acting interaction has been the subject of recent studies in several laboratories and has been reviewed in reference 10.

Moreover, our results show that any given regulatory region can behave as favorable, neutral, or unfavorable relative to others when the comparison is made in a cell-specific manner. PyFL78N appears to be the most cell-specific mutant because it enjoys a very strong growth advantage in the cell line it was selected from (FL cells), it has only a moderate advantage (if any) over the PyA2 in other, semipermissive cells, and it even suffers a disadvantage with respect to PyA2 in F9-differentiated cells. By comparing the growth efficiency of PyNB11/1 with that of PyFL78 (the duplication of the first comprising that of the second), it appears that these growth characteristics are not simply caused by a duplication of one or more signals but rather by a more complicated pattern of rearrangement among cooperating domains, which probably gives rise to new signals that play a role in virus-host specificity. In this context it has been reported that the duplication of so-called

TABLE 3. Mixed infections of semipermissive and nonpermissive cell lines[a]

Cell line	Days	Mixture no. and viruses					
		1. A2 wt and FL78	2. A2 wt and NB10/6	3. A2 wt and NB11/1	4. FL78 and NB10/6	5. FL78 and NB11/1	6. NB10/6 and NB11/1
NB-m	3	1.60	0.16	0.20	0.26	0.30	0.48
	6	0.43	NB10/6	0.02	NB10/6	0.10	0.83
	12	0.19	NB10/6	NB11/1	NB10/6	0.06	1.60
NB-s	3	1.20	0.08	0.05	0.25	0.14	0.42
	6	0.17	NB10/6	NB11/1	NB10/6	0.03	2.60
	12	0.13	NB10/6	NB11/1	NB10/6	NB11/1	6.10
EC-d	3	1.50	0.12	0.03	0.21	0.06	0.28
	6	1.53	NB10/6	NB11/1	0.27	0.02	0.50
	12	2.06	NB10/6	NB11/1	0.11	0.02	0.20
EC-nd	3			NB11/1		NB11/1	NB11/1
	6			NB11/1		NB11/1	NB11/1
	12			NB11/1		NB11/1	NB11/1

[a] See Table 2, footnote *a*. The measurements of the 12-day samples pertain to the experiments shown in Fig. 4.

TABLE 4. Mixed transfections[a]

Cell line	Mixture no. and viral DNAs					
	1. A2 wt and FL78	2. A2 wt and NB10/6	3. A2 wt and NB11/1	4. FL78 and NB10/6	5. FL78 and NB11/1	6. NB10/6 and NB11/1
3T6	0.15	0.08	0.06	0.07	0.16	3.10
FL	FL78	NB10/6	NB11/1	FL78	FL78	2.30
NB-m	0.13	NB10/6	NB11/1	NB10/6	NB11/1	1.40
NB-s	0.11	NB10/6	NB11/1	NB10/6	NB11/1	4.90
EC-d	3.60	NB10/6	NB11/1	NB10/6	NB11/1	0.16
EC-nd			NB11/1		NB11/1	NB11/1

[a] See Table 2, footnote *a*. Only the data relative to plasmid DNA extracted 12 days after transfection are reported.

nonenhancer sequences of the untranscribed region of simian virus 40 can result in the creation of a new enhancer (26).

A more composite situation emerges from the outlined results with the NB mutants. In fact, PyNB10/6 and PyNB11/1 are identical except for the deletion of the β region in PyNB10/6. The relative growth advantage of these mutants depends once again on the cell lines in which they are assayed. There are no differences between the two mutants in permissive cells (3T3, 3T6, and FL). Conversely, a marked growth advantage of the PyNB10/6 mutant over the PyNB11/1 mutant reveals itself in nondifferentiated semipermissive NB cells, and the same effect (though much less evident) can be observed in differentiated NB cells and in 12-day-infected permissive cells. These findings indicate that in all of these cells a functioning β region results in a disadvantage in the replication of the Py genome. This β *cis*-acting disadvantage is partially released in NB cells when in vitro differentiation is allowed to take place.

The opposite occurs in the nonpermissive F9 cells, in which only PyNB11/1 can grow. In these cells, a functional β region is required along with the duplication of the α region for growth inhibition to be overcome. One should note that all the Py mutants selected in F9 cells that have been described up to now have point mutations or duplications (or both) of the β region (but never deletions). It can be concluded that the duplication present in PyNB11/1 is able to act as a functional analog of these β domain modifications, a fact which suggests the existence of a cooperative regulatory mechanism. Furthermore, in these cells the block to Py replication is partially released upon in vitro differentiation.

The overall picture that emerges from these data suggests that in the Py regulatory region a cooperative action of different host-regulatory factors takes place. Depending on the cell type, the β regulatory domain can act either as an inhibitor (NB) or as an activator of Py DNA replication. Moreover, no deletions of the β region have ever been found which were not associated with an α region duplication, whereas point mutations or rearrangements of the β region can have the same effect as the more extended α-containing duplication of PyNB11/1 (i.e., growth in F9 cells). A last point requiring consideration is the relationship of the regulation of Py DNA replication to that of early and late viral gene expression. As mentioned above, the α and β domains overlap physically with the A and B enhancer domains. Whether they are also functionally identical, i.e., whether the transcriptional enhancer is also a replicational enhancer, is not yet clear. If there is functional overlap, the cell specificity of Py mutant growth patterns could be explained by postulating that the same tissue-specific mechanism which regulates virus and host cell gene expression via enhancer activation or inhibition (or both) also modulates viral DNA replication.

It is worthwhile recalling that in permissive hosts, such as FL cells, the selection of such specific advantageous enhancer constitutions as PyFL78N, is a very rare event, since Py wt efficiently grows in those cells. To select viral mutants, persistently infected cells must be cultured for very long periods (6). However, if persistently infected PyFL wt cells are superinfected by PyFL78N, few generations are needed to substitute the previous resident virus, and the loss of the Py wt genome is caused by dilution out of the replicating pool (unpublished results). The simplest interpretation of such results is again that the enhancer constitution of PyFL78N is a better substrate than the enhancer of Py wt in binding a limiting cellular factor.

The results reported confirm the hypothesis previously advanced (1) that the Py regulatory region is a composite region with several units which differentially interact with cells of different tissue origins and at various differentiative stages. Therefore, the Py regulatory region may be used as a probe for the study of gene control during mouse cell differentiation.

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