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Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication

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Two mouse neuroblastoma cell lines were analyzed for their permissivity for polyoma virus growth. One (N18) is fully permissive for polyoma replication, the other (41A3) shows limited permissivity and the viral genome persists, without noticeable cell death. Virus persistence does not seem to alter the cells' ability to differentiate *in vitro* and leads to selection of viral mutants altered in the untranscribed regulatory region of the genome. The mutant types obtained appear to be related to the degree of host cell differentiation. Nucleotide sequence analysis of the restriction fragment covering the regulatory region shows that duplications are present in all mutants, while deletions in the non-duplicated segment are only present in mutants selected from less differentiated cells. These alterations involve both domains of the regulatory region that are considered to be essential for DNA replication and for enhancer activity. Mixed infections with polyoma wild type show that the selected mutants have *cis*-advantage in replication in neuroblastoma cells and not in 3T6 cells. Mutants carrying the deletion in the non-duplicated segment of the enhancer show a selective advantage in replication over the undeleted one in mixed infection. This advantage is much stronger in neuroblastoma cells in suspension (less-differentiated stage) than in monolayer cells (more-differentiated stage). An interpretation of the overall structure of the regulatory enhancer region, based on the observed differences between the mutants selected at different stages of differentiation in neuroblastoma and previously described mutants selected in undifferentiated cells, is discussed.

Key words: mouse neuroblastoma/cell differentiation/polyoma persistence/viral mutants

Introduction

Virus infection of eukaryotic cells may provide a useful experimental system for gaining insight into the complex regulation of gene expression. In many systems the expression of the viral genome is dependent on the state of differentiation of the host cells, as has been recently reviewed (Maltzman and Levine, 1981). Therefore, the study of host-virus interaction may contribute significantly to the understanding of the molecular events occurring during cell differentiation.

Of particular interest in these studies are findings obtained after the infection of mouse undifferentiated cell lines, such as embryonal carcinoma cells (EC), with wild type polyoma (Py) virus. It has been shown that only differentiated cells support viral replication, while undifferentiated cells are not permissive (Kelly and Condamine, 1982). The lack of virus growth in undifferentiated cells has been related to a block in early viral mRNA synthesis. However, Py mutants have been obtained which are also able to grow in undifferentiated EC cells and in trophoblast-derived cells (Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Fujimura *et al.*, 1981; Sekikawa and Levine, 1981; Tanaka *et al.*, 1982).

In semi-permissive Friend erythroleukemic (FL) cells, the induction of differentiation does not modify response to Py infection, which usually results in the selection of persistently infected cells (Delli Bovi *et al.*, 1984). This state of persistent infection, however, does not appear to interfere with the expression of differentiated cell functions (Coraggio *et al.*, 1980) and leads to the selection of viral variants which exhibit a host-cell-specific *cis*-dominant growth advantage (De Simone *et al.*, 1985).

On the basis of the results obtained with EC and FL cells, it seemed worth extending this type of analysis to other cell lines, such as neuroblastoma (NB) cells, in which a modulation of the differentiation stage is possible.

Several clones derived from C1300 mouse neuroblastoma respond to various changes in culture conditions by increasing their ability to express specific properties of neurons. One procedure capable of inducing a more advanced differentiation in NB cells is the transfer of cells from suspension to monolayer cultures. The most obvious marker of differentiation in monolayer cultures is the formation of fibres (Augusti-Tocco *et al.*, 1970). This morphological event depends on the appearance of a microtubule-associated protein which is responsible for microtubule assembly (Olmsted and Lyon, 1981). Other molecular events which occur concomitantly with fibre formation are an increase in ribosomal RNA content (Casola *et al.*, 1974) and in protein synthesis (Zucco *et al.*, 1975), as well as modifications of surface proteins (Schubert and Jacob, 1970) and ion channels responsible for electrical activity (Miyake, 1978). Although some of these events have been shown to be dependent on post-translational control (Casola *et al.*, 1974; Zucco *et al.*, 1975), the appearance of new transcripts in differentiated NB cells grown in monolayers has also been reported (Grouse *et al.*, 1980).

Here we describe the different response to Py infection of two NB cell lines and the establishment of a persistent infection in one of them. The state of persistent infection selects for viral variants with alterations in the regulatory untranscribed region of the viral genome. The mutants obtained from suspension or monolayer cultures are characterized by different rearrangements in the regulatory region.

Results

Permissivity for polyoma growth

Two NB lines were used in this study, N18 and 41A3. These lines were independently isolated (August-Tocco *et al.*, 1970; Seeds *et al.*, 1970), and exhibit similar properties in culture,

although some differentiation markers are absent in N18 (Denis-Donini and Augusti-Tocco, 1980). However, their response to Py infection is considerably different. Line N18 is fully permissive, as shown by the high viral titer ($\sim 10^8$ p.f.u./ml) and massive cell death after infection in both suspension or monolayer cultures (data not shown). Cell death is due to a secondary infection, as has been shown for FL cells (Delli-Bovi *et al.*, 1984); in fact it is strongly reduced (to 10–20%) when cells are grown in agar after infection.

Clone 41A3 behaves quite differently in that it does not show any significant cell death after Py infection under any of the tested conditions. In fact, cells grown in monolayer or suspension, pretreated or not with prostaglandin (see Materials and methods) and grown after infection in either monolayer, suspension or agar, did not differ in survival compared with the mock-infected control. Therefore no effect of viral infection was detectable even in conditions allowing for secondary infection (growth in monolayer or suspension).

Control of Py absorption indicated a regular viral infection. Py titration in monolayer or suspension cultures demonstrated that limited virus growth and maturation takes place, even after a prolonged interval (>1 month). Viral titers varied between 10^4 and 10^5 p.f.u./ml, suggesting limited permissivity of the host cells.

Effect of Py persistence on the expression of differentiated functions and on viral structure

To examine the effects of Py persistence on the expression of differentiated functions, clones were isolated from 41A3 cells infected in either monolayer or suspension cultures. No detectable differences were found between the ability of several clones to express specific differentiated functions, such as fiber growth and acetylcholinesterase (AchE) synthesis, and that of mock-infected cells (data not shown). Most of the clones showed limited virus production, as measured by spot test on 3T3 cells, without significant cell death and modification of growth rate.

Viral DNA was extracted from the clones by the Hirt procedure (Hirt, 1967) and Py DNA presence was estimated by Southern blotting. Free unintegrated viral DNA was found in seven out of nine clones isolated from cells infected in monolayer culture, which allows for the expression of a more differentiated state, and in six out of 13 clones isolated from cells infected in suspension culture, where cells are less differentiated. Blotting analysis of Py after digestion with *Hpa*II restriction endonuclease showed genome alterations in nearly all cases (Figure 1). These alterations vary in different clones and always involve the *Hpa*II/3 fragment which includes the regulatory region of the viral genome (Tooze, 1981).

Effect of the degree of cell differentiation on the viral genome

To investigate the possible difference in selective pressure on the viral genome structure between the cells grown at the different stages of differentiation, a Py persistently infected clone (NB-Py) was grown in monolayer for 45 days and then either maintained under the same growth conditions or transferred to suspension culture for an additional 20 days. Viral DNA was then extracted by the Hirt procedure (Hirt, 1967) from both cultures and cloned in the *Bam*HI site of the pAT 153 plasmid. To confine further analysis only to viable mutants, viral genomes were excised from recombinant Py-plasmid by *Bam*HI digestion and tested for viability by transfection in the standard 3T3 permissive host cells. More than 30 clones for each of the two growing conditions were assayed and <15% were found unable to replicate. The non-viable variants were mainly defective in late

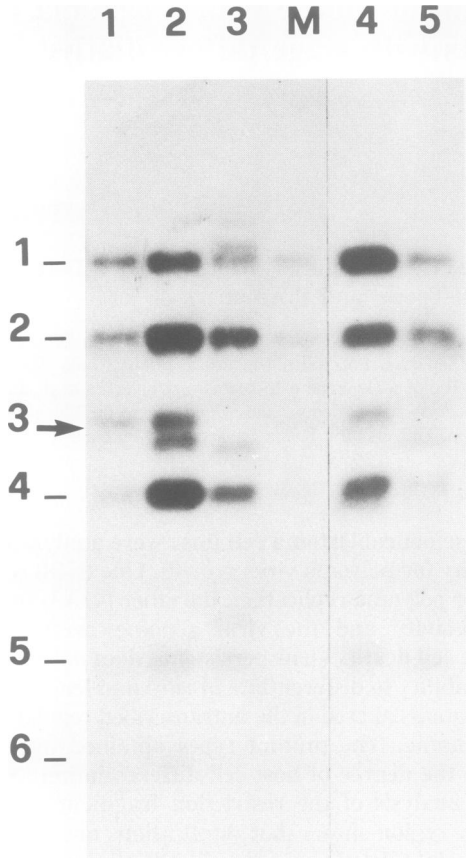

Fig. 1. Southern blot analysis of Py DNA extracted from some NB41A3-Py clones. DNA from Hirt extracts was digested with *Hpa*II endonuclease. **Tracks 1–3:** cell clones grown in suspension, **4** and **5:** cell clones grown in monolayer; M.Py wild-type DNA. Arrow indicates the *Hpa*II/3 fragment of the wild-type.

functions and a few showed considerably rearranged genomes (data not shown).

Eighteen viable cloned genomes from both monolayer and suspension cultures were digested with *Bam*HI and *Sst*I restriction endonucleases. Figure 2 shows that the genomes arising from the monolayer culture are different from Py A2 (wild type) and that they can be grouped into two groups (I and II) on the basis of their fragment size. The pattern obtained from the suspension culture shows the appearance of two additional groups (III and IV) of altered genomes, which represent about one third of the analyzed genomes. No wild-type A2 genome was found in the restriction mapping. Further analysis of representative genomes of the four mutant groups was carried out as shown in Figure 3: in all cases the genome rearrangement involved the fragment covering the regulatory region.

Sequence analysis of the Py mutants' regulatory region

The nucleotide sequence of the *Bam*HI-*Hpa*II fragments (nucleotides 4632–5295) of the four groups of Py mutants was carried out by the Sanger method (Sanger *et al.*, 1977). The results are shown in Figure 4: in all cases major rearrangements are present with no base substitutions compared with the parental Py A2 strain.

Group I mutants, present in both the monolayer and the suspension cultures, show a duplication at the late side from the *Pvu*II/4 fragment, which includes the A domain of the enhancer region

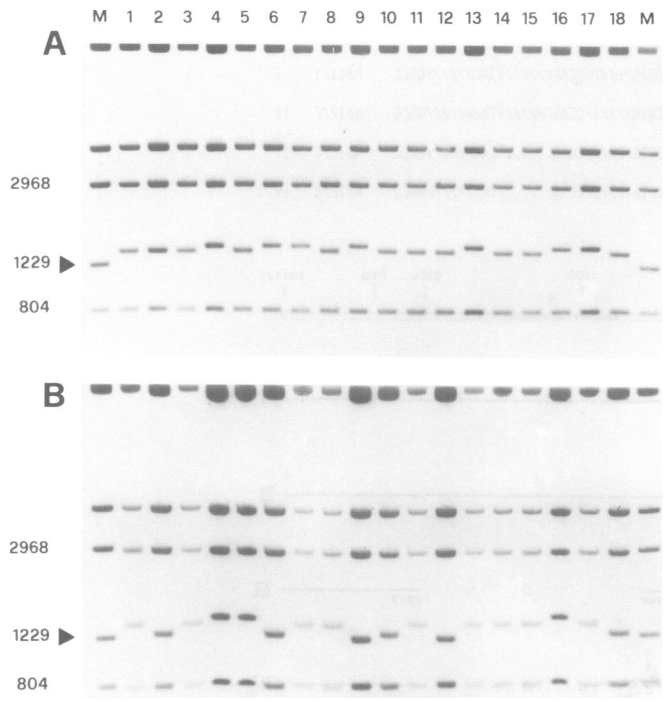

Fig. 2. Ethidium-stained agarose gel electrophoresis of cloned Py genomes in pAT153 plasmid. Viral genomes from Hirt-extracted DNA of a NB-Py clone grown in monolayer for 45 days and maintained under the same culture conditions (**A**) or transferred to suspension culture for an additional 20 days (**B**). The DNAs were digested with *Bam*HI-*Sst*I. The arrow indicates the *Bam*HI-*Sst*I fragments comprising the regulatory region (nucleotides 4632 – 569). The shortest *Sst*I band (291 nucleotides) ran out of the gel.

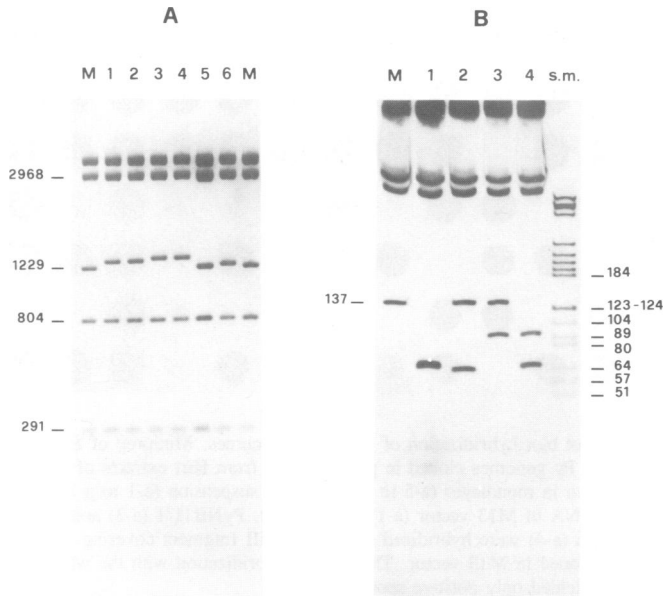

Fig. 3. Restriction enzyme mapping of purified Py DNA cloned in pAT 153. Ethidium-stained gels. **(A)** Agarose gel electrophoresis after *Bam*HI-*Sst*I digestion. **1** and **3:** group II and group I Py mutants from NB-Py cells grown in monolayer. **2, 4, 5** and **6** groups II, I, IV and III Py mutants from NB-Py cells grown in suspension. M: PyA2 marker DNA. **(B)** Acrylamide gel electrophoresis after *Pvu*II digestion of the four groups of Py mutants selected in suspension growth. M: PyA2 marker DNA; **1:** Group IV; **2:** Group II; **3:** Group I; **4:** Group III. sm: pBR322 digested with *Hae*III as size marker.

as defined by Herbomel *et al.* (1984). It is likely that group I is the progenitor of the three other groups since the same duplication boundaries are always present. In group II mutants, a 27 bp deletion is present in the duplicated segment. Group III and group IV mutants, which appear when the culture is switched to suspension growth, both display an identical deletion of a segment including the B domain of the enhancer region. Group IV mutants are characterized by an additional 24 bp deletion in the duplicated fragment and appear to be derived from group III mutants, since the deleted fragment in the duplication is different in size and position from that of group II mutants.

The notion of a drastic difference in selective pressure on the B domain between the NB differentiative stages finds support in the analysis of 94 cloned genomes in pAT. The genomes, not selected as virus-yielding molecules, were hybridized with a probe that carries only the B domain sequence (*Hae*III fragment from nucleotides 5174 – 5229 cloned in M13). Results of the dot blot of these genomes (Figure 5) show that in monolayer cell growth nearly all genomes carry this sequence (39/44) while in suspension growth ~ 1/2 of the genomes do not hybridize with the probe (22/48). The kinetics of the appearance of the B-deleted form in suspension growth, measured by dot blot analysis, show a progression from 25% after 5 days to 38% after 10 days (data not shown). The lack of B^-Py mutants in the first 18 clones previously analyzed from cells grown in monolayer may be due to the selection of virus-yielding genomes.

Tissue and cell differentiative stage specificity of the selected mutants

To test the specificity of the mutants selected in NB cells at both stages of differentiation, cells grown in suspension (less-differentiated stage) or in monolayer (more-differentiated stage) were doubly infected with mutants Py NB 11/1 (group I), Py NB 10/6 (group III) and Py A2 (moi = 50 + 50). Parallel infection of 3T6 and 3T3 cells was performed as control. After 3 and 6 days of infection for all cells, and 9 and 12 days for Py NB mixed infected cells, viral DNAs from Hirt extracts were analyzed by restriction enzyme digestion. Results reported in Figure 6A show that both Py NB mutants have a *cis*-advantage of replication over parental Py A2 in NB cells under both growth conditions, but not in 3T6 or 3T3 cells (data not shown). This advantage of growth in NB cells is likely to be due to the duplication that includes the A domain, and clearly supports the tissue specificity of the Py NB mutants since all of them carry such duplication (Figure 4).

Mixed infection with Py NB 11/1 (group I) and Py NB 10/6 (group III) of NB cells grown in suspension (less-differentiated stage) shows (Figure 6B) a significant advantage in replication of Py NB 10/6. In fact, 12 days after infection the ratio of fragment *Hpa*II/3 from Py NB 10/6 to PyNB 11/1 is ~ 20/1. In NB cells grown in monolayer, Py NB 10/6 also shows an advantage in replication from the sixth to the twelfth day, but the final ratio between the two *Hpa*II/3 fragments is ~ 4/1.

Differentiative stage selective pressure for deleted B^- mutants

To determine the stage differentiation specificity of mutant groups III and IV, deleted in the B domain (B^-) cells grown in suspension or monolayer, were infected with Py NB 11/1. At 1, 4, 8, 12 and 18 days after infection, the genetic constitution of the enhancer region was analyzed by blot hybridization with the entire Py genome or with the probe for the B domain. Results reported in Figure 7 shows that in more-differentiated cells the B-deleted form after 18 days constitutes ~ 5 – 10% of the whole

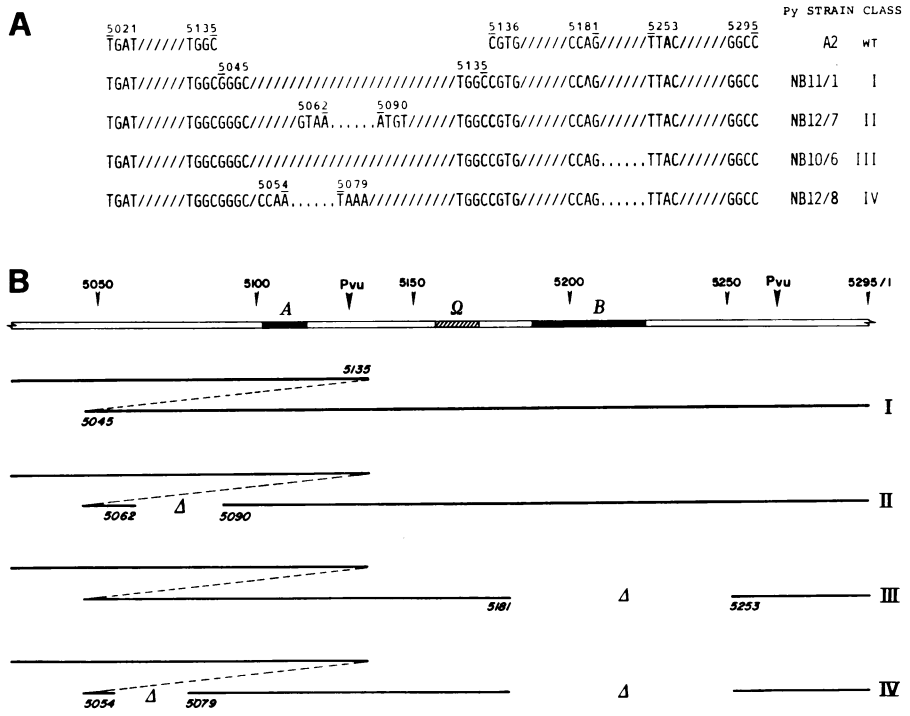

Fig. 4. Nucleotide sequence of Py-NB mutants. **(A)** Comparison between PyA2 wild-type and mutant sequences. Sequence and numbering are those of Ruley and Fried (1983). Nucleotides at rearrangement junctions are specified. Bars represent unaltered nucleotide segments; dots are deleted segments. **(B)** Schematic representation of the sequences reported in **A**. A and B domains are indicated as defined by Herbomel *et al.* (1984). Ω domain is defined in the text. Cleavage sites for *Pvu*II are indicated.

and that it is detectable after 12 days. In cells grown in the less-differentiated stage the B-deleted form is readily detectable after 4 days and the final ratio after 18 days is ~50–60%. Remarkably, these results are in agreement with those obtained during selection in the persistently infected NB-Py clone previously analyzed.

Discussion

The interaction of Py virus with NB cells has been investigated as an approach to understanding the changes in the expression of the cellular genome which occur during the differentiation process. The two cell lines behave quite differently in their interaction with Py. Line N18 is fully permissive for Py growth, while line 41A3 poorly supports viral growth, reaching a carrier state condition for Py similar to, but with a higher frequency than, that described for FL cells (Delli-Bovi *et al.*, 1984). In both the latter cases, cell survival after infection is high, clones which produce virus can be isolated and viral mutants carrying alterations in the regulatory region of the genome can be selected. Similarly to FL cells, the response of 41A3 NB cells to Py infection is not notably affected by the differentiation state at the time of infection.

In this respect the response of 41A3 NB cells and FL cells is definitely different from that of embryonal carcinoma cells, where induction of differentiation causes a dramatic change in the sensitivity to Py. This is not surprising in view of the characteristics of the system. In fact, EC cells represent stem cells, which in culture can undergo various developmental programs; in the case of NB, as in FL cells (Delli-Bovi *et al.*, 1984), we are analyzing the modulation of a terminal stage of differentiation in cells that are already committed to a definite developmental pathway. Therefore it is to be expected that the extent of variation of genome expression (regulation factors) occurring when

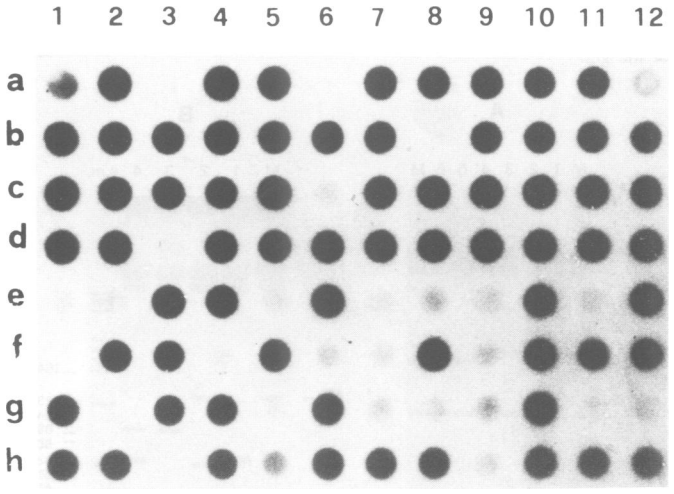

Fig. 5. Dot blot hybridization of cloned Py genomes. Miniprep of *E. coli* harboring Py genomes cloned in pAT plasmid from Hirt extracts of NB cells grown in monolayer (a-5 to d-12) and in suspension (e-1 to g-12) as well as DNA of M13 vector (a-1), PyA2 (a-2), PyNB11/1 (a-3) and PyNB10/6 (a-4) were hybridized with Py *Hae*III fragment covering the B domain cloned in M13 vector. The control hybridization with the whole Py genome yielded only positive spots.

the cells are induced to a more differentiated state is much more limited than in EC cells. It is, therefore, significant that in spite of these differences, the viral mutants selected in the three systems always involve the same region of the viral genome.

The mutations of the Py regulatory region obtained in EC cells appear to differ depending on the cell line in which they were selected. The major rearrangements selected in EC F9 cells consist of duplications in the early side of the *Pvu*II/4 fragment which includes the B domain (Katinka *et al.*, 1981; Fujimura *et al.*,

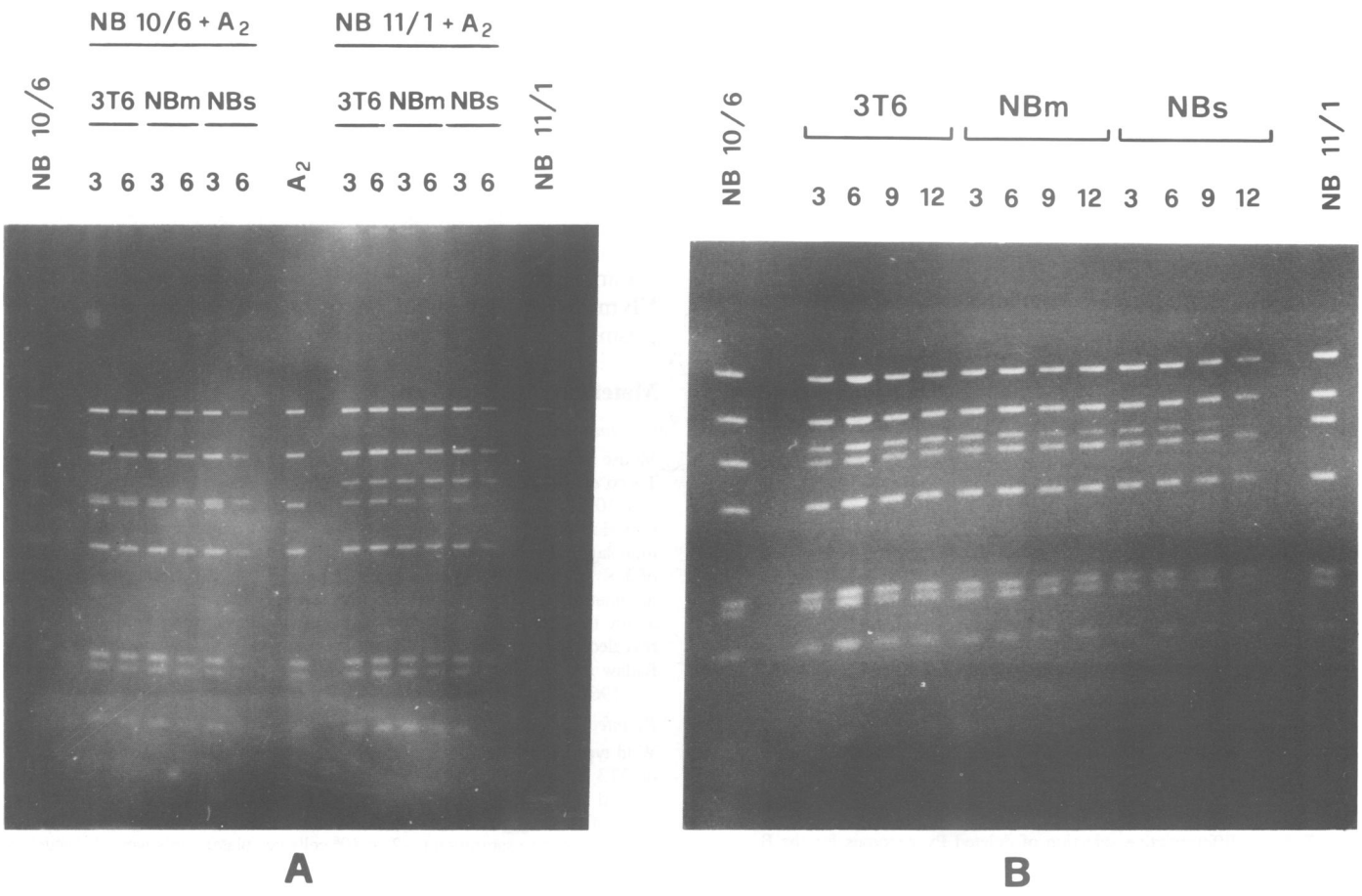

Fig. 6. Competitive growth of PyA2 and PyNB mutants in 3T6 and NB cells at different stages of differentation. Ethidium-stained gels of *Hpa*II digested Hirt extracts of **(A)** cells at 3 and 6 days after infection with PyA2 and PyNB 11/1 or PyNB 10/6 and **(B)** cells at 3, 6, 9 and 12 days after infection with PyNB 11/1 and PyNB 10/6. Numbers denote days after infection, NBm and NBs are NB cells grown in monolayer or suspension. Markers are from 3T6 cells infected with a single Py type.

1981; Sekikawa and Levine, 1981), whereas the mutants selected in EC PCC4 cells display duplications starting at the boundary of the *Pvu*II site at nucleotide 5130 and extending towards the late side, over the A domain, together with deletions covering the B domain (Vasseur *et al.*, 1980; Katinka *et al.*, 1980).

In the NB cell system, we observed that a change in the differentiative stage of the cells is followed by modifications in the Py regulatory region. All viable viral mutants selected in the more-differentiated NB cells (monolayer culture) exhibit a duplication on the left side from the *Pvu*II/4 fragment covering the A domain (Figure 4, group I and group II mutants). When cells are switched to a less-differentiated state (suspension culture) for 20 days, approximately one third of the viral genomes acquire, in addition to the duplication, a deletion on the early side of the *Pvu*II/4 fragment (nucleotides 5181 – 5253) extending over the B domain (Figure 4, groups III and IV mutants).

We found that in mixed infection of Py NB mutants (group I common to both culture conditions or group III selected in suspension growth) with Py A2, the former displays a net *cis*-advantage of replication in NB cells at both stages of differentiation, but not in 3T6 or 3T3 cells. This observation supports the likelihood that the A domain duplication is tissue specific. Moreover, we observed a much greater *cis*-advantage in the replication of Py NB group III mutants in NB cells grown in suspension than in NB cells grown in monolayer. Furthermore, we observed different ratios of appearance of B-domain-deleted forms in NB cells infected with Py group I mutants in the two stages of NB differentiation. These results support the notion that the origin of mutant groups III and IV during the persistent infection of NB cells in the less-differentiated stage is due to a consistent disadvantage of viruses carrying the B domain. Since in monolayer growth there is always a small fraction of unattached cells, it is likely that the limited selection of B^-Py genomes is assigned to this cell fraction of the more-differentiated culture.

The striking regularity of the deleted B^- forms selected deserves some comment. In Hirt extracts of 3T6 cells infected with Py NB 11/1, but not with Py NB 10/6, we find that ~2 – 3% of viral genome molecules contain a rearranged regulatory region. The majority of the altered genomes had lost either the A or the B domain of the enhancer, in addition to the more rearranged molecules involving the regulatory region (unpublished observations). Moreover, two major hypersensitive sites for DNase I in Py chromatin have been identified (Herbomel *et al.*, 1981) which correspond to the A and B domains. These sites are supposed to be nucleosome free and therefore preferentially exposed to the interaction with various enzymes, some of which might very well be involved with crossing over. The possibility that the instability of Py NB 11/1 and the regularity of the rearranged molecules may be related to the chromatin structure, is presently being investigated.

From a comparative analysis of our NB mutants and those known to display major rearrangements in EC and trophoblast cells, we can further define another domain in the regulatory region which we will refer to as Ω (see Figure 4). This region

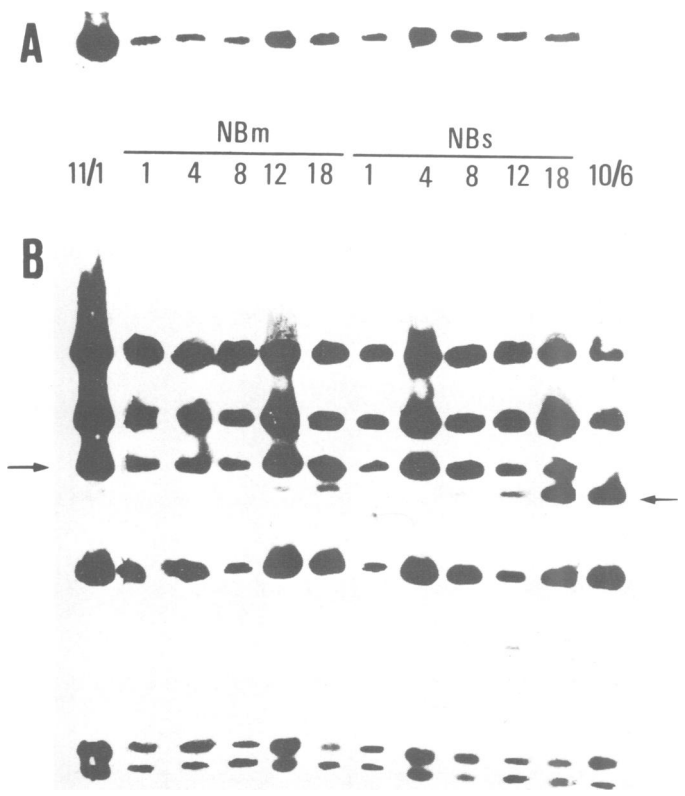

Fig. 7. Stage-differentiative selection of deleted Py genomes for the B domain. NB cells grown in monolayer (NBm) or suspension (NBs) were infected with PyNB1/1 at moi=50 and at different times the Hirt extracts were digested with *Hpa*II restriction endonuclease and analyzed by Southern blot in consecutive hybridization with the *Hae*III fragment covering the B domain **(A)** and the whole Py **(B)**. The marker is from a Hirt extract of 3T6 cells infected by PyNB11/1 or PyNB10/6. Numbers denote days after infection. In **A** only the *Hpa*II/3 band is reported since it is the only one to hybridize with the probe used.

appears to be essential for viral growth since it is never lost in any Py-viable mutant. Its domain is defined by the deletion present in groups III and IV mutants on its early side (nucleotide 5181) and by the deletion present in trophoblast Py mutants Tr 91 and Tr 92 (Tanaka *et al.*, 1982) on its late side (nucleotide 5157).

The finding that specific modifications in the *Pvu*II/4 fragment (Figure 4) can be selected differentially by varying the degree of differentiation of 41A3 NB-Py cells allows us to postulate that specific alterations of the Py regulatory region can be used as indicators or probes of a cell-differentiative stage. These results are consistent with the notion that the interaction of specific host cell proteins with the viral regulatory region affects both transcription activation and viral DNA replication (Scholer and Gruss, 1984). These cell factors are thought to be elements regulating differentiation, as proposed by Gillies *et al.* (1983) and Banerji *et al.* (1983) for immunoglobulin gene expression. The ability of the Ig enhancer to substitute the polyoma regulatory region for viral growth in myeloma cells, but not in fibroblasts, further supports this interpretation (de Villiers *et al.*, 1984).

The two groups of mutants (groups III and IV) selected during suspension growth of the NB-Py clone have both lost the region that includes the B domain of the enhancer. This domain shares a sequence homology with the SV40, Ig and AdA2 enhancers. Deleted mutants were selected in PCC4 EC cells, suggesting that the deleted sequence might interact with a cell factor that is detrimental in *cis* for viral replication. This would imply that sequences of the Py enhancer may play not only a positive or netural, but also a negative role in a cell-specific way.

The finding (Borreli *et al.*, 1984) that the product of the Adeno *E1 A* gene represses Py enhancer activity, further supports the notion of sequences in the viral regulatory region that may have a negative control action in specific cellular conditions, and that this effect may be mediated by regulatory factor(s) that recognize Py as well as cellular sequences in gene-control regions.

Further work is in progress on the enhancing properties of Py-NB mutants regulatory regions on heterologous genes in chimeric plasmids assayed in NB cells at different stages of differentiation.

Materials and methods

Cell lines and culture conditions

Mouse neuroblastoma clonal lines N18 (Seeds *et al.*, 1970) and 41A3 (Augusti-Tocco *et al.*, 1970) were grown in Ham's F10 medium (Gibco) supplemented with 10% fetal calf serum in tissue culture dishes (for monolayer growth) or in bacterial dishes (for suspension growth). To maximize cell differentiation, monolayer cultures were treated with prostaglandin (Sigma) at a concentration of 3×10^{-6} M for 7 days prior to infection (Gilman and Nirenberg, 1971). Differentiation of neuroblastoma cells in monolayer cultures was monitored by the ability to grow fibers and by an increase of AchE activity. AchE activity was revealed either by a modification of the Kamovsky histochemical method (El-Badaw and Schenk, 1967) or by spectrophotometric determination (Ellman *et al.*, 1961).

Py infection and titration

Wild type Polyoma strain Py A2, kindly supplied by M.Fried, was propagated on 3T3 or 3T6 cells at low mutliplicity. NB cell infections were routinely performed at a multiplicity of 50. Virus production and titration was determined by the following procedure cells and culture medium were collected from NB culture grown to saturation ($\sim 2 \times 10^6$ cells per plate), cells were disrupted by freezing and thawing (three times), and plaque assays were carried out on 3T3 monolayers. Gross titers were obtained by the sheep red blood cell agglutination test (Tooze, 1981).

DNA extraction, restriction endonuclease digestion and blot hybridization

Viral DNAs were extracted from cell cultures by the Hirt selective procedure (Hirt, 1967). Viral DNA from different NB-Py clones or from Py genomes cloned in the pAT153 plasmid was digested with several restriction endonucleases under the conditions specified by the manufacturers (New England Biolabs, Beverly, MA).

Restriction DNA fragments were separated on 1.2% agarose gels run in Tris-borate electrophoresis buffer; DNA was transferred on cellulose filters (BA 85, S & S), and filter hybridization was carried out by the Southern procedure (Southern, 1975). P32-labeled DNA probes were prepared by the standard nick-translation procedure (Rigby *et al.*, 1977). Clone plasmid containing whole Py or thee *Hae*III fragment from nucleotides 5174–5229 (Herbomel *et al.*, 1984) were used as probes in blot hybridization experiments. For the dot blot hybridization of cloned genomes in pAT, both whole Py and the *Hae*III fragment were cloned in M13 vector. Polyacrylamide gel electrophoresis (10% w/w) was carried out according to Maniatis *et al.* (1975).

Cloning of Py mutants and selection of viable genomes

DNA from Hirt extracts of Py-infected NB cells was cloned in the *Bam*HI site of the pAT153 vector plasmid (Twigg and Sherrat, 1980). The cloning procedures were as described by Maniatis *et al.* (1982). Py genomes were recovered from recombinant Py plasmid DNA by *Bam*HI digestion. Purified Py DNA was transfected into 3T3 or 3T6 cells by the $CaPO_4$ method (Graham and Van der Eb, 1973).

DNA sequencing

The DNA fragments of the Py-NB mutants extending from the *Sau*3A1 to the *Hpa*II sites (nucleotides 5021–5295), filled in by Klenow polymerase (Maniatis *et al.*, 1982), were inserted in *Sma*I site of the M13 mp9 vector. Nucleotide sequencing in both directions was performed by the dideoxy-chain termination method (Sanger *et al.*, 1977). Some sequences were confirmed by the chemical cleavage method (Maxam and Gilbert, 1980).

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References

Augusti-Tocco,G., Sato,G.H., Claude,P. and Potter,D.D. (1970) *Symp. Int. Soc. Cell. Biol.*, **9**, 109-120.
Banerji,J., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729-740.
Borrelli,E., Hen,R. and Chambon,P. (1984) *Nature*, **312**, 608-613.
Casola,L., Romano,M., Di Matteo,G., Augusti-Tocco,G. and Estnoz,M. (1974) *Dev. Biol.*, **41**, 371-379.
Coraggio,I., De Simone,V., Lancieri,M., Revoltella,R. and Amati,P. (1980) in Rossi,G.B. (ed.), *In Vivo and In Vitro Erythropoiesis: The Friend System*, Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 423-437.
Delli-Bovi,P., De Simone,V., Giordano,R. and Amati,P. (1984) *J. Virol.*, **49**, 566-571.
Denis-Donini,S. and Augusti-Tocco,G. (1980) *Curr. Top. Dev. Biol.*, **16**, 323-348.
Denis-Donini,S., Estenoz,M. and Augusti-Tocco,G. (1978) *Cell Differ.*, **7**, 193-201.
De Simone,V., Lania,L., La Mantia,G. and Amati,P. (1985) *Mol. Cell. Biol.*, **5**, 2142-2146.
de Villiers,J., Shaffner,W., Lupton,S., Tyndall,C. and Kamen,R. (1984) *Nature*, **312**, 242-246.
Fujimura,F.K., Deininger,P.L., Friedman,T. and Linney,F. (1981) *Cell*, **23**, 809-817.
El-Badaw,A. and Schenk,E.A. (1967) *Histochem. Cytochem.*, **15**, 580-588.
Ellman,G.L., Courtney,K.D., Andres,V. and Featherstone,R.M. (1961) *Biochem. Pharmacol.*, **7**, 88-95.
Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) *Cell*, **33**, 717-728.
Gilman,A.G. and Nirenberg,M. (1971) *Nature*, **234**, 356-357.
Graham,F.L. and Van der Eb,A.J. (1973) *J. Virol.*, **52**, 456-464.
Grouse,L.D., Letendre,C.H., Zubaini,M.U. and Nelson,P.G. (1980) *J. Biol. Chem.*, **255**, 3871-3877.
Herbomel,P., Saragosti,S., Blangy,D. and Yaniv,M. (1981) *Cell*, **25**, 651-658.
Herbomel,P., Bourachot,P. and Yaniv,M. (1984) *Cell*, **39**, 653-662.
Hirt,B. (1967) *J. Mol. Biol.*, **26**, 365-369.
Katinka,M., Yaniv,M., Vasseur,M. and Blangy,D. (1980) *Cell*, **20**, 393-399.
Katinka,M., Vasseur,M., Montreau,N., Yaniv,M. and Blangy,D. (1981) *Nature*, **290**, 720-721.
Kelly,F. and Condamine,H. (1982) *Biochim. Biophys. Acta*, **611**, 105-141.
Maltzman,W. and Levine,A.J. (1981) *Adv. Virus Res.*, **26**, 65-116.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Maniatis,T., Jeffrey,A. and Van de Sande,M. (1975) *Bichemistry (Wash.)*, **14**, 3787-3791.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Miyake,M. (1978) *Brain Res.*, **143**, 349-354.
Olmsted,G.B. and Lyon,H.D. (1981) *J. Biol. Chem.*, **256**, 3507-3511.
Rigby,P.W.J., Dieckman,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-252.
Ruley,H.E. and Fried,M. (1983) *J. Virol.*, **47**, 233-237.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
Scholer,H.R. and Gruss,P. (1984) *Cell*, **36**, 403-411.
Schubert,D. and Jacob,F. (1970) *Proc. Natl. Acad. Sci. USA*, **67**, 247-254.
Seeds,N., Gilman,A.G., Ariano,T. and Nirenberg,M.N. (1970) *Proc. Natl. Acad. Sci. USA*, **66**, 160-167.
Sekikawa,K. and Levine,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1100-1106.
Southern,E. (1975) *J. Mol. Biol.*, **98**, 503-515.
Tanaka,K., Chowdhury,K., Chang,K.S.S., Israel,M. and Ito,Y. (1982) *EMBO J.*, **1**, 1521-1527.
Tooze,J. (ed.) (1981) *Molecular Biology of Tumor Viruses: DNA Tumor Viruses*, Cold Spring Harbor Laboratory Press, NY.
Twigg,A.J. and Sherrat,D. (1980) *Nature*, **283**, 216-218.
Vasseur,M., Kres,C., Montreau,N. and Blangy,D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1068-1072.
Zucco,F., Persico,M., Felsani,A., Metafora,S. and Augusti-Tocco,G. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 2289-2293.

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