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Transcriptional 'enhancers' from SV40 and polyoma virus show a cell type preference

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ABSTRACT

Transcriptional "enhancers" have been identified in both the monkey virus SV40 and in the mouse polyoma virus. Here we report that these enhancers show a cell type preference. This was done, (i) by assaying for T antigen expression and viral DNA replication of polyoma DNA which contains its own enhancer or the enhancer of SV40 virus, and (ii) by linking either of the two enhancer elements to the rabbit β1-globin gene and measuring transient globin expression in mouse and primate cells. The results were consistent in all the assays: In mouse cells the polyoma enhancer is slightly more effective than the SV40 enhancer. However, in primate cells the SV40 enhancer induces a four to six fold higher level of gene expression than does the polyoma enhancer.

INTRODUCTION

Polyoma virus and simian virus 40 (SV40) are two closely related, but host cell specific, DNA tumor viruses. The former grows in mouse cells whereas SV40 grows in monkey cells, and also human cells albeit with lower efficiency. In addition, the two viruses share the ability to infect and transform non-permissive cell lines (for review see ref.1).

Both viral genomes have a regulatory region between their early and late coding regions which contains all the signals, so far identified, that govern the initiation of transcription of the early genes. Also within this region are located the binding sites for the early gene product, T antigen, and the origin of viral DNA replication (for review see ref.1). DNA sequences around the HpaII 3/5 junction of polyoma virus are commonly referred to as the origin of replication because they are both required for viral replication and they share significant homology with the origin regions of both SV40 and

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BKV (reviewed by Griffin; 1). It has however been shown that non-coding sequences to the late side of this loosely defined "origin" are essential in cis for early gene expression and also for replication (2). These latter essential upstream sequences fall within the DNA fragment which we have shown causes an enhancement, by about two orders of magnitude, in the level of expression of a linked rabbit β-globin gene in a transient expression assay (3). Within the analogous region of SV40 are also sequences required for early gene transcription (4,5) and which can enhance the expression of linked, non-viral genes over large distances (6,7). Somewhat surprisingly, however, despite the functional similarities between the enhancer regions of the two related viruses, there is very little significant primary sequence homology, to which some of these functions can be correlated.

These experiments were performed to determine whether the enhancer elements of the two viruses show a cell type preference. This was done by analyzing the functions of a number of recombinants, in which the late non-coding sequences of polyoma virus were replaced by various fragments of DNA from the enhancer region of SV40. We further analyzed and quantitated this effect in a simpler system, where enhancers from either one of the two viruses were linked to the rabbit β-globin gene. These DNA's were used to transfect human, monkey or mouse cell cultures, by the calcium phosphate coprecipitation technique (8), and the transient expression of the globin gene was assayed. The results confirm that the viral enhancers exhibit a cell type preference, the implications of which are discussed.

MATERIALS AND METHODS

Plasmid construction

The constructions of the recombinant plasmids used in these experiments are outlined schematically in figures 1 and 4. Standard recombinant DNA techniques, described previously (3), were used and all work involving recombinant plasmids was done under conditions conforming to the standards outlined in the U.S. National Institutes of Health Guidelines for

Recombinant DNA Research.

Cell growth and transfection

The human carcinoma cell line HeLa, the monkey kidney line CV-1 and the mouse 3T6 fibroblast cells were gifts from U. Pettersson of Uppsala, P. Berg of Stanford and W. Topp of Cold Spring Harbor, respectively. For most of the experiments HeLa and 3T6 cells were chosen because, in our collection of primate and rodent cell lines, they are respectively the most amenable to DNA transfection. They were grown in Dulbecco's modified Eagle's minimal medium (GIBCO), containing 2,5% fetal calf serum, 2,5% calf serum, 100 units of penicillin per ml and 100µg of streptomycin per ml (GIBCO). Plasmid DNA's were purified prior to transfection of the cells by CsCl density gradient centrifugation. The calcium phosphate transfections were performed with modifications as described previously (6).

Immunofluorescence, S1 nuclease hybridization assays and MboI replication assays

48 hours after transfection, the cells were fixed with methanol and stained with antibodies (gifts of K.D. Smith, Baltimore and R. Kamen, London) to reveal the presence of rabbit β-globin in the cytoplasm of the cells, or large T antigen in the nuclei, as described (6). The S1 mapping (9,10), using a single-stranded, end-labelled DNA probe, was done essentially as described by Favaloro et al. (11). The MboI assay, for DNA which has been replicated in the transfected cells, was performed according to O'Hare (12).

RESULTS

Early gene expression and replication of polyoma-SV40 hybrid recombinants

We have used two polyoma-SV40 hybrid recombinants, in which the enhancer sequences of a polyoma-plasmid recombinant have been replaced by SV40 enhancer-bearing sequences (figure 1). Mouse 3T6, human HeLa and in some cases monkey CV-1 cells were transfected with wild-type polyoma plasmid DNA or polyoma-SV40 hybrid recombinants in parallel, after which we assayed for transient polyoma large T antigen expression by indirect immunofluorescence. In each experiment the DNA's were

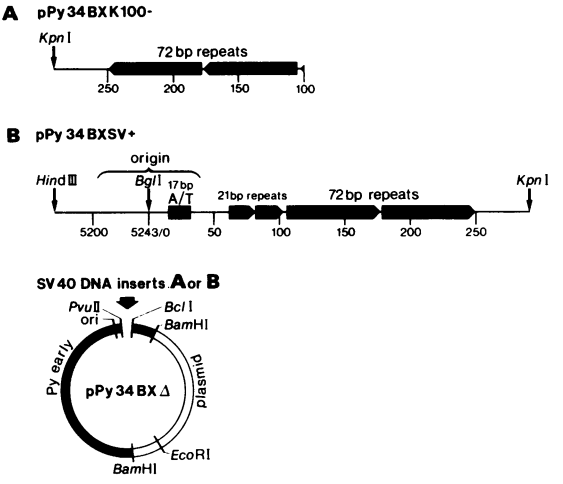

Fig. 1. Construction of the hybrid polyoma recombinants, bearing SV40 enhancer sequences.
pPy34BXΔ is a polyoma-plasmid recombinant from which 244 bp of non-coding viral DNA, bearing the enhancer as well as essential sequences required in cis for early gene expression and replication, have been deleted and replaced by synthetic XhoI linkers. SV40 DNA fragments were inserted into this unique site of pPy34BXΔ. Insertion of the 196 bp SV40 enhancer fragment (A), in an inverted orientation as indicated, generated the hybrid recombinant pPy34BXK100-. Insertion of the 366 bp SV40 fragment bearing both the enhancer and the origin of DNA replication (B), in the analogous orientation, generated pPy34BXSV+.

coprecipitated together with calcium phosphate and then split into equal fractions. These portions of the same precipitate were then used to transfect the different cell lines, as described (6). Table 1 summarises the results obtained in two such experiments. In the mouse cells the polyoma T antigen gene is expressed better (although not very significantly; 15-60%) when it is linked to the polyoma enhancer. In contrast, in the primate cells, T antigen expression is about 400% more efficient when the gene is linked to the SV40 enhancer. This dramatic increase in T antigen expression in the primate cells is discernable as both an increased intensity of nuclear fluorescence (Figure 2) as well as a higher proportion of positively staining cells (Table 1). S1 nuclease analysis of the 5' termini of the early mRNA's in 3T6 and HeLa cells revealed that in all cases the polyoma major early cap sites

Table 1. Immunofluorescence assay for transfected cells producing T antigen

Cells/transfecting DNA	Total cells[1]	Cells T antigen +ve	SV40/Py enhancer
Experiment 1:			
3T6/Py-SV40 hybrid[2]	44000	1720 (3,9%)	0,63 (3T6)
3T6/Py wild type[3]	36000	2220 (6,2%)	
HeLa/Py-SV40 hybrid	42000	8660 (20,6%)	3,2 (HeLa)
HeLa/Py wild type	48000	3060 (6,4%)	
CV-1/Py-SV40 hybrid	23000	1780 (7,7%)	3,6 (CV-1)
CV-1/Py wild type	20000	430 (2,2%)	
Experiment 2:			
3T6/Py-SV40 hybrid[2]	40000	520 (1,3%)	0,86 (3T6)
3T6/Py wild type[3]	50000	760 (1,5%)	
HeLa/Py-SV40 hybrid	43000	2580 (6,0%)	5,0 (HeLa)
HeLa/Py wild type	41000	500 (1,2%)	
CV-1/Py-SV40 hybrid	20000	848 (4,2%)	3,8 (CV-1)
CV-1/Py wild type	21000	232 (1,1%)	

1) The total number of cells was extrapolated from counting areas of 0.145mm^2.
2) Two different Polyoma-SV40 hybrids were used in experiments 1 and 2. In experiment 2 the hybrid recombinant pPy34BXK100- (Fig.1) was used whereas a very similar hybrid, with an SV40 enhancer fragment K164- (from the KpnI site of SV40 to nucleotide 164) inserted in the same orientation into the same polyoma-plasmid recombinant was used for experiment 1.
3)Likewise two different wild type polyoma-plasmid recombinants were used for the two experiments. These differed only by the presence of a synthetic XhoI linker inserted at the PvuII site at 70 map units of the recombinant used in experiment 1.

(2) were used (data not shown). In the case of the hybrid, pPy34BXSV+, where the major early cap sites of SV40 are present and are colinear with the polyoma early coding region, it is interesting to note that both sets of cap sites were used to express the polyoma early genes (not shown).

An indirect way to detect viral early gene expression is to assay for viral DNA replication, which depends upon efficient T antigen synthesis. We assayed the replication of the transfected DNA's in both 3T6 and HeLa cells (figure 3). Again in the mouse cells we find almost equivalent replication of both of the polyoma-SV40 hybrids and wild-type polyoma plasmid, in agreement with the data on T antigen production. Replication of wild-type polyoma plasmid DNA is almost absent

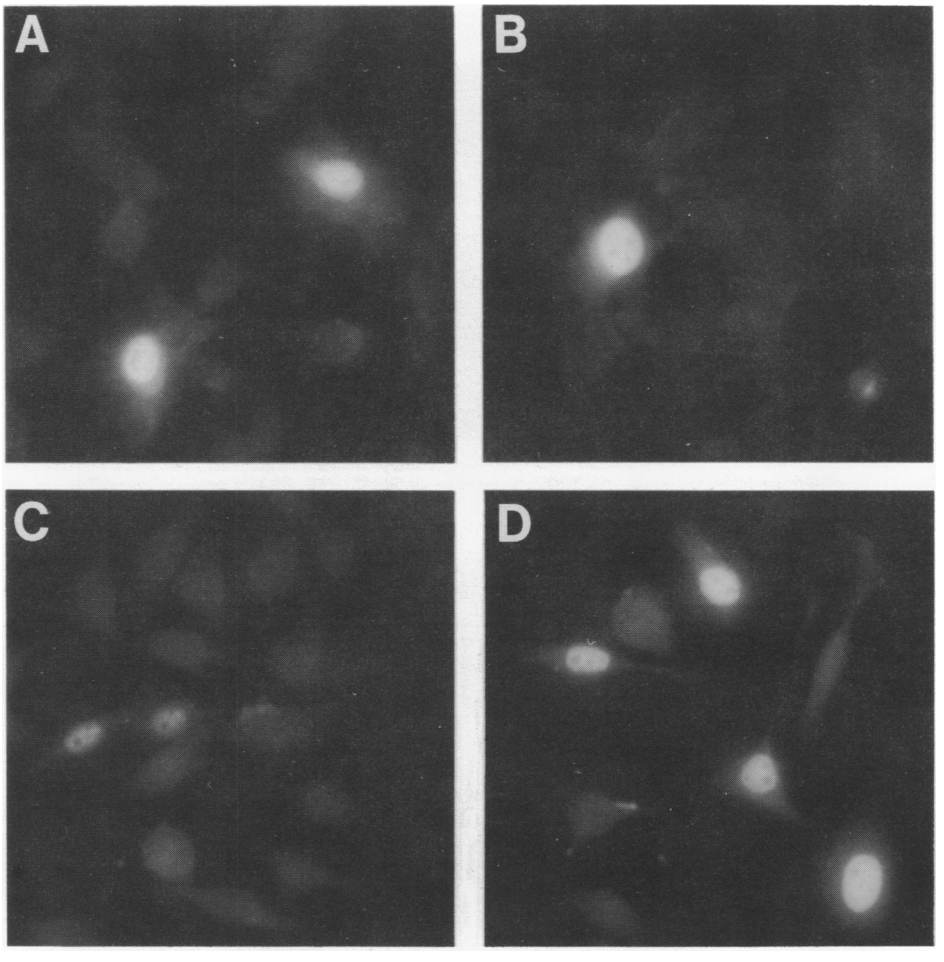

Fig. 2. Polyoma virus T antigen production in transfected mouse 3T6 and human HeLa cells.
Panels A-D are photomicrographs, showing a representative field at 360-fold magnification in each case, of transfected cells stained by indirect immunofluorescence for nuclear T antigen. Panels A and B show mouse 3T6 cells transfected with wild-type polyoma plasmid DNA and the hybrid recombinant pPy34BXK100- (figure 1A), respectively. Panel C is a photograph of human HeLa cells transfected with wild-type polyoma plasmid DNA, and D shows the visibly increased T antigen expression in HeLa cells transfected with pPy34BXK100-, the hybrid which has an SV40 enhancer replacing the polyoma enhancer.

in the primate cells (compare lanes 2 in figure 3A and 3B) but it is readily detectable with the hybrids, where an SV40 enhancer substitutes for the polyoma enhancer. Since both

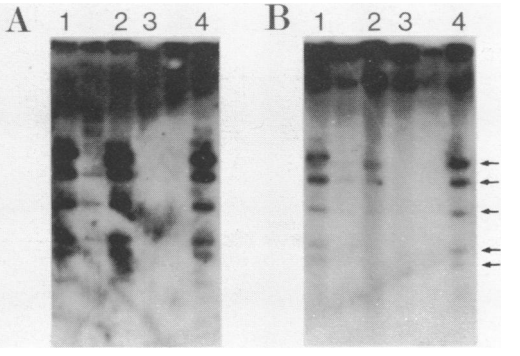

Fig. 3. DNA replication analyses of polyoma-SV40 hybrid recombinants. A and B show autoradiographs of MboI digested Hirt supernatant (24) DNA's from transfected mouse 3T6 and human HeLa cells, respectively (hybridized to nick-translated polyoma virus DNA). The arrows indicate specific digestion products which arise from DNA replicated within the transfected cells. MboI will not cleave the bacterially methylated input DNA. Lanes 1 and 4, DNA from cells transfected with the hybrid recombinants, pPy34BXSV+ (figure 1B) and pPy34BXK100- (figure 1A), respectively. Lane 2, transfection with wild-type polyoma plasmid pPy43.34.70 (3). Lane 3, negative control transfection with the polyoma recombinant, pPy34BXΔ, which lacks the polyoma enhancer sequences.

hybrid recombinants replicated equally well, the ability of the polyoma-SV40 hybrids to replicate in the HeLa cells does not depend on the presence of an SV40 origin of replication but only upon sequences of the 72 bp repeated region and adjacent late side sequences.

The expression of a rabbit β-globin gene, linked to the papovavirus enhancers, also shows host cell specificity.

Viral enhancers also exert a host range effect when they are detached from the viral background and linked to a rabbit β-globin gene. Two β-globin-enhancer plasmids were constructed, which are identical except that one contains the 196 bp SV40 enhancer DNA fragment (figure 4B) and the other contains the 244 bp of polyoma enhancer DNA (figure 4A). Calcium phosphate precipitates of each DNA were prepared and then split. Half of each precipitate was used to transfect human cells and the other half was used to transfect mouse cells. The expression of the β-globin gene was assayed by both immunofluorescence (see Table 2) and S1 nuclease mapping of the 5' termini of cytoplasmic RNA extracted from transfected cells (figure 5). It

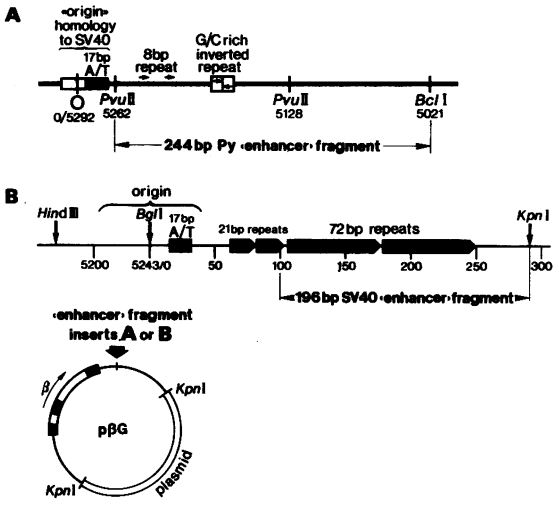

Fig. 4. Schematic representation of the enhancer regions from polyoma virus and SV40, and their positions of insertion into a β-globin plasmid recombinant.
pβG has the 4700 bp KpnI fragment, bearing the genomic rabbit β1-globin gene (kindly provided by T. Maniatis, Cambridge, Mass; 25,26), within a plasmid derivative of pAT153 (27). The β-globin-polyoma-enhancer recombinant pβGPyenhancer, has the 244 bp polyoma DNA fragment (A) inserted at a site about 1700 bp downstream of the β-globin gene cap site by means of synthetic linkers, as indicated. Similarly pβGSVenhancer has the SV40 196 bp enhancer fragment (B) inserted at the same site of pβG, in the same relative orientation.

is obvious from these data that there is little difference between the activities of the two enhancers in mouse cells, as in the previous experiments. In HeLa cells, however, the SV40 enhancer mediated a much higher level of β-globin gene

Table 2. Indirect immunofluorescence assay for transfected cells producing globin

Cells/transfecting DNA	Total cells*	Cells positive for β-globin
3T6/pβGPyenhancer	77000	65 (0,08%)
3T6/pβGSVenhancer	79000	55 (0,07%)
HeLa/pβGPyenhancer	15000	115 (0,8%)
HeLa/pβGSVenhancer	13000	584 (4,5%)

** The total number of cells was extrapolated from counting areas of 0.145mm^2 (at 400-fold magnification a total number of 170-205 cells were counted). The number of globin-positive cells was always counted in an entire 8x8mm field.*

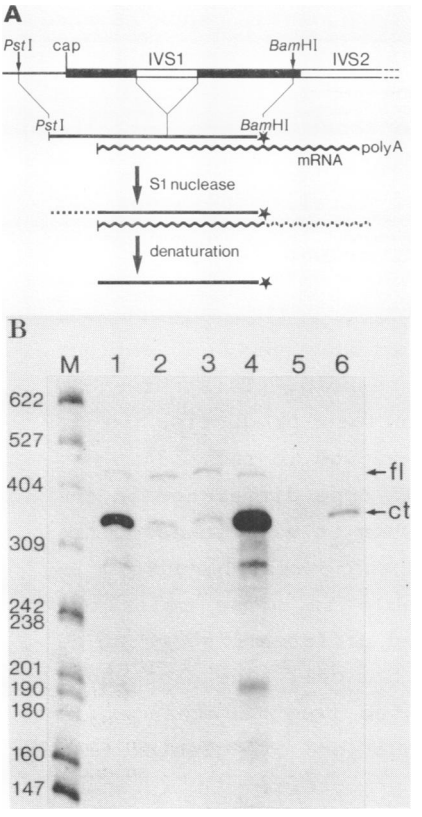

Fig. 5. S1 nuclease analysis of the 5' termini of β-globin-specific RNA, extracted from transfected mouse and primate cells.
Unlabelled cytoplasmic RNA was hybridized to an excess of end-labelled DNA probe (prepared from a globin gene clone lacking the first intervening sequence, IVS1; a gift from H. Weber, Zürich; 28), after which the hybrids were digested with S1 nuclease, denatured, fractionated by polyacrylamide gel electrophoresis and autoradiographed, as indicated schematically (A). fl, full length DNA probe; ct, correct terminus (a 354 nucleotide fragment is protected from S1 digestion, by mRNA correctly initiated at the globin gene cap site); M, marker DNA fragments of the indicated lengths. Lanes 5 and 6, probe fragment protected by two different concentrations of rabbit reticulocyte RNA. Lanes 1 and 4, transfection of HeLa cells (lane 1, clone with the polyoma enhancer; lane 4, clone with the SV40 enhancer). Lanes 2 and 3, transfection of the 3T6 cells (with the remaining halves of the DNA precipitates used to transfect the HeLa cells in lanes 1 and 4, respectively).

transcripts than did the polyoma enhancer. This difference was 4 and 5.6 fold when measured by Cerenkov counting, of the excised bands in the S1 nuclease assay (figure 5, lanes 1 and 4), or by counting the number of immunofluorescent cells (see Table 2), respectively. (The generally high level of globin-specific RNA obtained from HeLa cells reflects the extreme amenability of these cells to DNA transfection).

DISCUSSION

It has been shown previously that papovavirus enhancers can act in a number of different cell lines, derived from various species (3,6,7). Here we demonstrate that enhancers show a cell type preference in their activity.

Such a cell type preference of enhancer activity could at least in part be responsible for the host range of the viruses

although it is likely that other viral components, such as the origin of replication and viral proteins, have evolved to fully exploit their specific host cell. One could argue that the quantitative difference seen in the host ranges of the enhancers would not suffice to explain the clear cut differences in the viral host ranges. Viral host ranges can however be based on quantitative rather than qualitative properties: A. Grässmann and his colleagues have shown by means of microinjection experiments that mouse cells support one round of SV40 virus growth provided that SV40 T antigen production is raised above a certain threshold. This threshold is about threefold above maximal T antigen production in an SV40 virus infection of mouse cells (reviewed in ref. 13).

We have previously reported a cell type difference in the expression of a rabbit β-globin gene, when it was linked to the entire polyoma virus genome (3). With those recombinants, β-globin expression was readily detectable in mouse cells but hardly in primate cells. This pronounced difference seems to be a compound of two effects, namely, (i) the cell type preference of enhancers; i.e. low activity of the polyoma enhancer in primate cells, and (ii) the attenuation of globin gene expression by enhancer-proximal viral promoters. In a tandem array of promoters the one proximal to the enhancer has been found to be preferentially used (14,15) and we assume that the activity of the enhancer-proximal viral promoters depressed β-globin gene transcription to levels near the limits of detection in the HeLa cells.

Experiments with two other systems, namely retrovirus long terminal repeats and polyoma variants which grow in embryonal carcinoma cells, are also compatible with a cell specific host range of enhancer activity. G. Khoury and his colleagues have reported that a hybrid SV40 virus, with the reiterated 72 bp sequence from the long terminal repeat of mouse Moloney sarcoma virus replacing its own enhancers sequences (16), produced more T antigen in mouse cells than in primate cells (17; however see also ref.18). Unfortunately in the above experiments the three criteria commonly cited to distinguish papovavirus enhancers from other upstream promoter elements, namely orientation

independence, enhancing activity over very large distances and transcription initiation at the linked gene's own cap site(s), have not been addressed.

Mouse embryonal carcinoma cells are nonpermissive for the early gene expression and replication functions of polyoma virus. Polyoma virus variants which grow in these cells have been selected (19-22), and all of these were found to have sequence alterations within the 244 bp segment which bears the enhancer activity identified by us. There is an increase in T antigen expression by these mutants in embryonal carcinoma cells but it remains to be seen whether this increased early gene expression is cell type specific or general. It has also been shown that the sequence alterations are essential *in cis* for replication (23), which implies that they affect the viral origin of replication as well.

On the basis of the observed cell type preference, one may expect that enhancers specifically interact with host cell factors and that this affect may be exploited to isolate such a class of factors involved in tissue specific gene expression.

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