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Active viral genes in transformed cells lie close to the nuclear cage

P.R. Cook*, J. Lang, A. Hayday[1], L. Lania[1,2], M. Fried[1], D.J. Chiswell[1], and J.A. Wyke[1]

Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, and [1]Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX, UK

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Nuclear DNA is looped by attachment to a matrix or cage. Using nine different lines transformed by polyoma or avian sarcoma virus, we have mapped viral sequences integrated within these loops. In all lines that contain high concentrations of viral transcripts and express the transformed phenotype, the integrated viral genes lie close to the points of attachment to the cage. Integration of polyoma DNA induces outlying cellular sequences to become closely associated with the cage. The strength of this correlation between gene activity and proximity to the cage was examined using sub-clones of one avian sarcoma virus transformant. Proviral sequences are closely associated with the cage in this transformant, much less so in two untransformed 'flat revertants' which contain no detectable viral transcripts but regain their close association with the cage in two retransformed derivatives.
Key words: nuclear cage/transforming viral genes/active genes

Introduction

Unlike many bacteriophages that integrate at a unique site in the bacterial chromosome, the transforming viruses of higher cells show no such site specificity: the integrated virus is found at different sites in different transformants (Hughes *et al.*, 1978; Lania *et al.*, 1979). As nuclear DNA is looped (Cook and Brazell, 1975; Igo-Kemenes and Zachau, 1977; Paulson and Laemmli, 1977; McCready *et al.*, 1979) by attachment to a subnuclear structure (Agutter and Richardson, 1980) called variously the nuclear 'envelope' (Franke, 1974), 'matrix' (Berezney and Coffey, 1974), 'lamina-pore complex' (Aaronson and Blobell, 1975), 'ghost' (Riley *et al.*, 1975) or 'cage' (Cook and Brazell, 1975), we might expect the virus to be integrated at random within the loops. However, it has been suggested that the cage is the site of transcription (Jackson *et al.*, 1981) and as viral sequences are expressed in transformed cells, we would predict that they must lie close to the cage. Therefore, we mapped their positions in cells transformed by polyoma and avian sarcoma viruses and confirm that they lie close to the cage.

We chose to study viral sequences of polyoma and avian sarcoma virus (ASV) integrated into the cellular DNA of transformed rat cells since the appropriate cells and probes were available and, more importantly, since these cells yield robust cages that protect the fragile DNA from non-specific shearing. Two clonal series of transformed cells, each containing viral sequences integrated at one different cellular site, were obtained by transformation of Rat-1 cells (Mishra and Ryan, 1973) with polyoma (Lania *et al.*, 1979, 1980) or ASV (Wyke and Quade, 1980; Chiswell *et al.*, 1982; Varmus *et al.*, 1981). One mouse line transformed by polyoma (i.e., Tsa 3T3) was also included (Lania *et al.*, 1979, 1980). All express a transformed phenotype and contain high concentrations of viral transcripts (Chiswell *et al.*, 1982; Kamen *et al.*, 1979). As a control we mapped the unexpressed albumin gene.

Sequences are mapped relative to their point of attachment to the cage using our established procedure (Cook and Brazell, 1980). Living cells are lysed in a non-ionic detergent and 2 M salt to release structures that resemble nuclei (Cook and Brazell, 1975; McCready *et al.*, 1979; Cook *et al.*, 1976). These nucleoids are especially suitable for structural studies on the higher order folding of DNA since the cage protects the now naked and histone-free DNA from breakage: as a result nucleoids contain all the nuclear DNA. Furthermore, as it is supercoiled, it must be intact. Nucleoids are partially digested with a restriction endonuclease: then cages – and any associated DNA – sedimented free of detached DNA. The cage-associated DNA is purified and completely redigested using the same restriction endonuclease. Equal weights of this DNA are resolved into discrete fragments by gel electrophoresis and these are transferred to a filter and the relative amounts of any sequence on the filter determined by autoradiography after hybridization with the appropriate probes. Following the first partial digestion, sequences lying close to the point of attachment will tend to co-sediment with the cages and so will be present in relatively greater abundance on the filter: therefore, they yield bands of greater intensity on autoradiography. The degree of enrichment is determined by reference to known amounts of total DNA run in adjacent channels in the gel.

Others have applied an essentially similar approach to map sequences remaining attached to another sub-nuclear structure, the matrix (Jeppesen and Bankier, 1979; Razin *et al.*, 1979; Nelkin *et al.*, 1980; Pardoll and Vogelstein 1980; Bowen, 1981; Basler *et al.*, 1981; Matsumoto, 1981; Robinson *et al.*, 1982). These results have been conflicting, the enrichments being unconvincing or undemonstrable. In contrast to nucleoids, most nuclear DNA is lost from the matrix during isolation and we believe that this uncontrolled detachment underlies the variable enrichments obtained.

Results

Integrated polyoma sequences

Figure 1a illustrates the mapping of polyoma sequences integrated into cellular DNA in one transformed line, 82. Control DNA, undigested during the first partial digestion, yields three bands when subsequently digested completely with *Eco*RI and hybridized with a polyoma probe. (Figure 1a, channel 2; i.e., 100% remaining). These correspond to the bands seen by Lania *et al.* (1980) and correspond to the left- and right-hand arms of the integrated virus – which also

[2]Present addresss: Istituto di Biologia Generale e Genetica, University of Naples, Naples, Italy.

*To whom reprint requests should be sent.

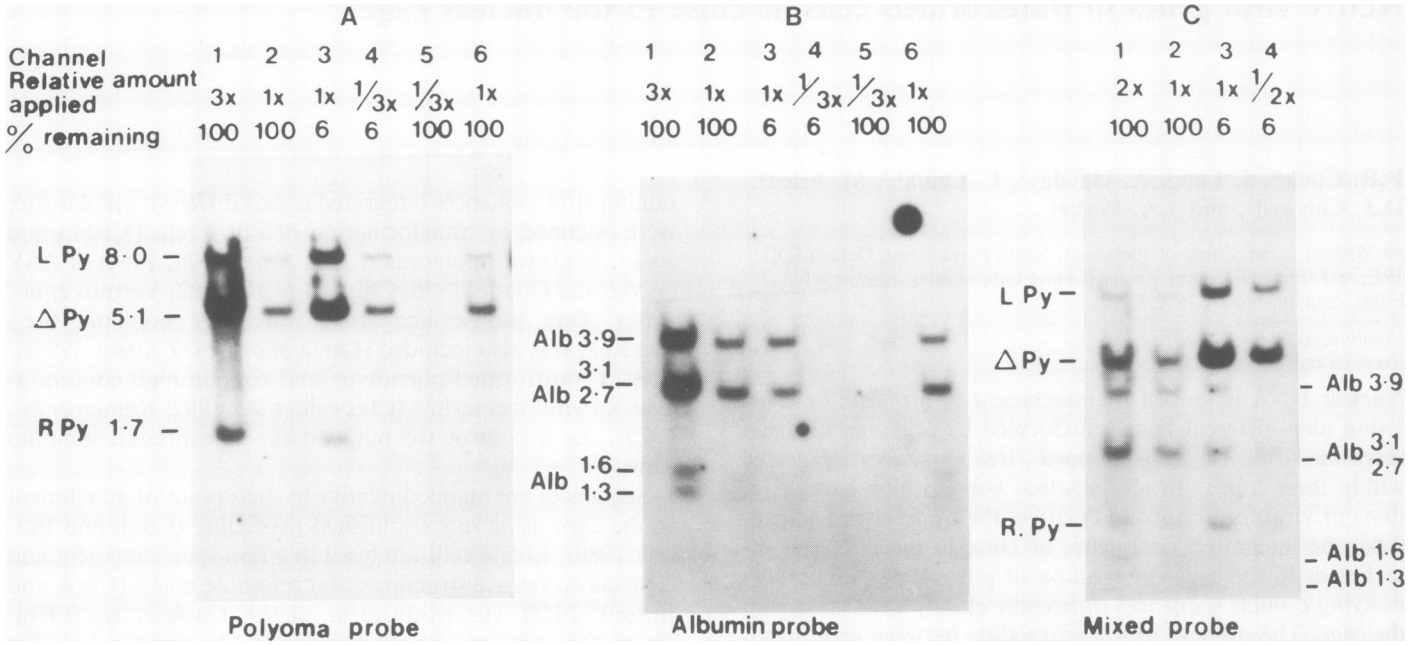

Fig. 1. Detachment mapping albumin and polyoma viral sequences in the polyoma transformant, 82. Samples of total DNA (100% remaining) and DNA that resists detachment by *Eco*RI (6% remaining) were completely digested with *Eco*RI and various amounts applied to three gels. After electrophoresis, blotting, and hybridization with polyoma or albumin probes, autoradiographs were prepared and photographed. The sizes of the three polyoma bands (LPy, △Py, and RPy corresponding to the left-hand, internal, and right-hand fragments respectively) and the five albumin bands (two are not clearly resolved under these conditions) are given in kilobases. The polyoma, but not the albumin, sequences are enriched in the nucleoid samples that retained 6% of the total DNA.

contain cellular sequences – and to an internal, and purely viral sequence. [In all cases, fragment sizes detected by hybridization agreed with the established restriction enzyme maps (Lania *et al.*, 1980; Wyke and Quade, 1980; Sargent *et al.*, 1979; Kioussis *et al.*, 1981)]. When 3 x, or 1/3 x, this amount of DNA is applied to the gel the bands are correspondingly stronger or weaker (Figure 1a, channels 1 and 4). The duplicate channels (2 and 6) of 1 x the control amount of DNA – which are included in all experiments – illustrate the uniformity of blotting and hybridization. If these nucleoids are partially digested with *Eco*RI to leave only 6% of their DNA remaining attached to the cage and then their DNA is purified and completely fragmented with *Eco*RI, the bands obtained subsequently are ~3 x more intense than those obtained with an equal weight of control DNA (compare channels 2 with 3, and 4 with 5). In contrast, hybridization of an albumin probe to the DNA of nucleoids that retain 6% of the total DNA yields four bands, none of which are more intense than those obtained with the control DNA (Figure 1b: compare channels 2 with 3, and 4 with 5; one band is probably a doublet). We conclude that there are fewer restriction sites between the integrated viral sequences and the point of attachment to the cage than there are between the albumin gene and its adjacent attachment sequence, i.e., the viral sequences lie 'closer' to the cage.

The enrichment of the viral, but depletion of the albumin, sequences can be highlighted by hybridizing a mixture of the two probes to the same filter (Figure 1c). With the sample that retained 6% of the total DNA, the viral bands are darker than the equivalent controls and the albumin bands are slightly fainter. Such an enrichment and depletion of sequences within the same channel rules out the trivial objection that we are not applying the correct weights of DNA to the gel; this is in any case routinely checked by ethidium staining following electrophoresis.

The results obtained with the other polyoma-transformed lines are summarized in Table I. (Of course, comparisons between different cells should be made at the same levels of detachment). In no case is the concentration of albumin sequences in the DNA which is closely associated with cages richer than that in the control. In every case the integrated viral sequences are enriched in the fraction of DNA that pellets with the cages. Line 82 was analysed more extensively. In general, detaching more DNA from the nucleoids, whether with *Eco*RI or *Bam*HI, enriches the viral sequences to a greater extent (Table II). The extent of enrichment at high levels of detachment is greater than that we have seen with any of the 30 or so cellular sequences that we have now studied (Cook and Brazell, 1980; unpublished data). We can assign the point of attachment to the left or right side of the viral sequence in 82 by reference to the relative enrichments of each of the three viral bands. The left-hand junction sequence, which contains both cellular flanking sequences and viral sequences, is enriched more than the internal, and purely viral sequence: both are enriched more than the right-hand junction sequence (Table II and Figure 2; compare channel 5 with the others). These differences increase as more DNA is detached. We interpret this as indicating that the left-hand fragment is closest to the attachment site or is attached the strongest (but see later). We note that the left-hand, internal, and right-hand fragments contain 2, 1, and 0 active transcription units, respectively. [The left-hand fragment contains an inversion so that the published map (Kamen *et al.*, 1979) has been revised (Hayday *et al.*, 1982; H.E.Ruley *et al.*, in preparation)].

Viral integration induces attachment of outlying cellular sequences

Does the virus integrate selectively in sequences lying close to the cage or does it integrate randomly, inducing new at-

Table I. Detachment mapping albumin, viral, and junction sequences in various cell lines

Cell	Percentage DNA remaining (relative enrichment)			
	Albumin	Polyoma	ASV	Polyoma junctions
Parent				
Rat-1	4% (1 x)	100% (no bands)	100% (no bands)	9% (0.8 x)[82J1] 4% (1.6 x)[53C1] 9% (1.0 x)[7TL] 4% (1.2 x)[7TR]
Polyoma-transformed				
82	6% (0.9 x)	14% (1.7 x) 6% (3.5 x) 4% (4.6 x)[b] 1% (6.7 x) 0.8% (18.0 x)		
53		6% (2.3 x)		
7axT	4% (0.9 x)	4% (4.0 x)		
Tsa 3T3	5% (0.7 x)	5% (3.0 x)		
ASV-transformed				
A + 11	13% (0.6 x)		13% (2.1 x)	
A + 22	3% (0.8 x)		3% (3.9 x)	
A 23	6% (0.5 x)		6% (>3.0 x)	6% (0.6 x)[7TR]
B 31	5% (0.6 x)		5% (3.9 x)	5% (0.5 x)[7TL]
A11 VIT	14% (0.8 x) 2% (1.0 x)		17% (2.1 x)[a] 14% (2.0 x) 7% (3.0 x) 5% (3.1 x)[a] 5% (>9.0 x)[b] 4% (7 x) 2% (>9.0 x)	
Flat revertants of A11 VIT				
13N			3% (1.4 x)	
21N	10% (1 x)		17% (1.0 x)[b] 10% (0.9 x) 10% (1.5 x) 4% (0.8 x) 4% (2 x)	
Aza-cytidine selected retransformants				
21 aza-C trans 1			8% (5.6 x) 7% (5.1 x)	
21 aza-C trans 3			8% (7.7 x) 7% (6.0 x) 6% (4.5 x)	

Autoradiographs like those illustrated in Figure 1 were prepared for each cell-line, and scanned using a microdensitometer and peak heights measured. The relative intensities of one of the strongest bands were determined by reference to similar bands obtained with varying weights of total DNA.

[a]Nucleoids were incubated with ribonuclease to remove all but 4% or less of the RNA labelled in 15 min with [^{3}H]uridine (10 μCi/ml) prior to the first *Eco*RI digestion.

[b]*Bam*HI was used instead of *Eco*RI in both digestions.

tachments? Various viral sequences and contiguous cellular sequences have been cloned (Hayday *et al.*, 1982; H.E.Ruley *et al.*, in preparation), therefore we can test these possibilities by seeing whether cellular sequences which flank the inserted virus lie close to the cage in the parental Rat-1 cells (Table I). Cellular sequences homologous to all four such junction probes tested (i.e., 82J1, from the left side of the integrated virus in 82; 53C1, from the right side in 53; 7TL and 7TR from the left and right sides, respectively, in 7axT) are readily detached from untransformed Rat-1 cages and cages prepared from ASV-transformants (i.e., the relative enrichments are ≤ 1.6 (Table I). By contrast, in the polyoma transformants these cellular sequences are attached to the integrated viral DNA and so are clearly associated with the cage. The attachment of outlying cellular sequences induced by viral integration can be highlighted as follows. The junction probe from the right side of the virus in 7axT (7TR) hybridizes with one major *Eco*RI fragment of 5.0 kb from parental Rat-1 cells (A.Hayday *et al.*, in preparation). When the virus integrates, it does so into only one of the two homologous chromosomes, so that the junction probe now hybridizes to two fragments from the transformant – one of 5.0 kb from the unaffected chromosome and another of 5.1 kb, which contains viral sequences. With total DNA, the 5.0-kb band is the more intense (Figure 3; channels 1, 2, and 5); however, when all but 4% of the DNA is detached from 7axT nucleoids, the band intensities are reversed (channels 3 and 4). The purely cellular 5.0-kb band is depleted whilst the viral 5.1-kb band is enriched. A

Table II. The left-hand *Eco*RI fragments of the integrated virus in 82 lie closest to the cage

Percentage DNA remaining	Relative enrichment of various fragments		
	Left	Internal	Right
14	1.7 x	1.7 x	1.6 x
14	2.0 x	1.4 x	1.0 x
6	3.5 x	3.0 x	2.2 x
1	6.7 x	5.5 x	
0.8	18.0 x	13.7x	10.0 x

Band intensities in autoradiographs prepared like those in Figures 1 and 2 were measured and the relative enrichments determined. The left-hand, internal, and right-hand fragments contain 2, 1, and 0 active transcription units, respectively.

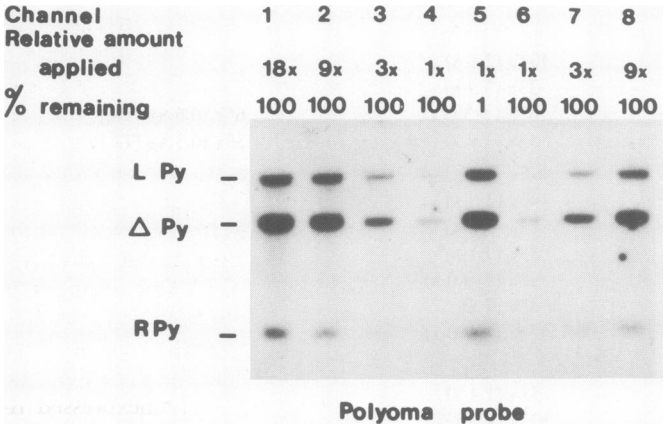

Fig. 2. Mapping the polyoma proviral sequence in 82 with high levels of detachment. Various amounts (from 1 to 18 x) of total DNA (100% remaining) or which resisted detachment by *Eco*RI (1% remaining) were applied to the gel as indicated and an autoradiograph prepared using the polyoma probe. The left-hand polyoma band (LPy) is enriched more than the right-hand band (RPy).

similar enrichment of the viral bands but depletion of the purely cellular band is obtained when the junction probe 82J1 is used with 82 nucleoids (i.e., when 2% of the DNA remained, the LPy and cellular bands are enriched >9 x and <1 x, respectively).

ASV transformants

A typical experiment like that illustrated in Figure 1 using A11 VIT, a Rat-1 cell transformed by ASV is illustrated in Figure 4. After digestion with *Eco*RI, three bands are detected by the ASV probe, the top one being a doublet (Wyke and Quade, 1980). The bands obtained with the DNA purified from cages that retained only 4% of the total are at least 3 x more intense than the control bands (Figure 4, compare channels 3 and 4 with 1, 2, and 9–11). Further experiments on A11 VIT and similar experiments on the other ASV-transformants are summarized in Table I. In each case the ASV sequences, which are known to be expressed (Varmus *et al.*, 1981; Chiswell *et al.*, 1982 and D.J.Chiswell, unpublished data), are enriched in the pelleted fraction whereas the albumin sequences are depleted.

Sub-clones of A11 VIT present us with an opportunity to test the strength of this correlation between gene activity and

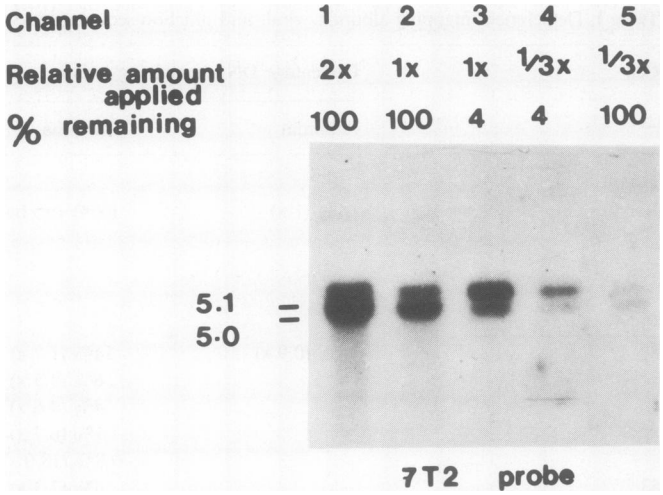

Fig. 3. Detachment mapping a junction sequence (7TR) in 7axT. Various amounts of total DNA or DNA which resisted detachment by *Eco*RI (4% remaining associated with cages) were applied to the gel. Autoradiographs were prepared using the junction probe 7TR. The 5.1-kb and 5.0-kb bands are enriched 2.4 x and 0.6 x respectively.

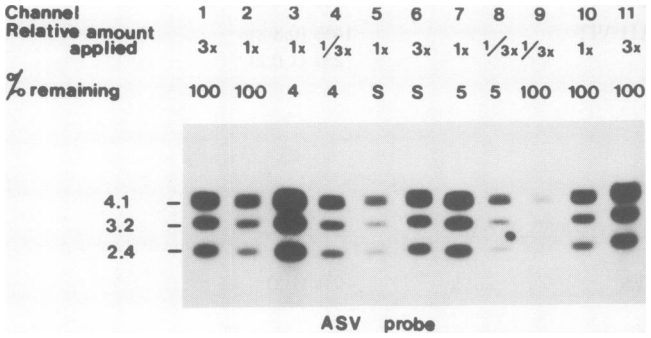

Fig. 4. Detachment mapping ASV sequences in the ASV transformant, A11 VIT. Various amounts (3 x, 1 x, or 1/3 x) of total DNA (**channels 1, 2, 9, 10, 11**), DNA which was detached by *Eco*RI (**channels 5, 6**), or which resisted detachment (**channels 3, 4, 7, 8**) were applied to the gel. The percentage DNA remaining associated with cages is indicated. Autoradiographs were prepared using the ASV probe. The detached DNA used in **channels 5** and **6** was purified from the supernatant (S) above the pellets used for **channels 3** and **4** and redigested with *Eco*RI. **Channels 7, 8**: nucleoids were pre-treated with ribonuclease (20 μg/ml; 10 min at 37°C) prior to the first digestion with *Eco*RI. Parallel experiments showed that the ribonuclease detached >96% of the label from nucleoids after pulse-labelling cells with [^{3}H]uridine (10 μCi/ml) 15 min immediately prior to the preparation of nucleoids.

proximity to the cage. Two sub-clones (i.e., 13N and 21N) have lost the transformed phenotype and contain no detectable viral transcripts (Chiswell *et al.*, 1982). When these 'flat revertants' are treated with the antimetabolite, aza-cytidine (aza-C), and recloned, transformed colonies containing viral transcripts emerge at a high frequency (D.J.Chiswell and J.A.Wyke, in preparation). Two such clones derived from 21N (i.e., 21 aza-C trans 1 and 3) were analysed. As far as can be judged by restriction enzyme mapping, all cells in this series contain unchanged proviral sequences inserted in the same cellular sequence. However, they differ in whether or not the proviral sequence is expressed. Detachment mapping indicates that the ASV sequences, which are closely associated with the cage in the transformed VIT, are much less so in the untransformed 'flat revertants' (13N and 21N) but have re-

gained their close association with the cage in the aza-C treated derivatives (Table I). For example, when all but 5% or less of the DNA is detached, the ASV sequences are enriched >3 x in the transformed lines (i.e., A11 VIT, 21 aza-C trans 1 and 3), but <2 x in the 'flat revertants'. Although further work is still required to confirm whether the attachments of the proviral region in the transformed cells and the 'flat revertants' are different and not due to some unforeseen consequence of expression (see below), it is clear that expressed proviral genes are always close to the cage.

One trivial explanation of all these results is that nascent transcripts, which are presumably closely associated with their templates, prevent access of *Eco*RI to potential cutting sites in transcribing DNA. This possibility was eliminated in two ways. First, when *Bam*HI replaced *Eco*RI in both digestions, similar enrichments were seen (Table I). Second, detaching nascent RNA had no effect on the sensitivity of viral sequences to subsequent detachment by *Eco*RI. This was demonstrated by pulse-labelling cells with [^{3}H]uridine, preparing nucleoids and then pre-treating them with sufficient ribonuclease to detach >95% of the nascent RNA. When all but 5% of the DNA was detached with *Eco*RI, the viral sequences were still enriched in the pelleted fraction (Figure 4, channels 7 and 8) just like the samples that had not been pretreated with ribonuclease (Figure 4, channels 3, 4; Table I).

Discussion

Three types of attachment of DNA to the cage have been detected using different methods. One is structural, remaining throughout the cell-cycle (Warren and Cook, 1978), which fixes the DNA in loops so that sequences within them can be mapped. The other two are detected functionally and concern replicating (McCready *et al.*, 1980) or transcribing (Jackson *et al.*, 1981) sequences. These functional attachment sites must constantly change as DNA passes through them. Obviously, much concerning the relationship between these various attachments is obscure and this should be borne in mind in the discussion that follows.

Using a series of nine independent lines transformed by polyoma virus or ASV, we find that in all lines the integrated virus lies close to the cage. We feel that such a correlation is unlikely to be fortuitous. The virus cannot selectively integrate close to the cage since it induces outlying cellular sequences in the parental Rat-1 cells to become closely associated with the cage. How then is this association with the cage – and its transcription machinery – brought about? We can suggest two simple models, but as yet cannot decide between them. The first, and we think the likeliest, is that the virus might encode its own 'attachment sequence' for use during a replication cycle (Buckler-White *et al.*, 1980). 'Attachment sequences' should completely resist detachment and, after pelleting the cages, should only be found in the pellet, enriched by 100 x when 1% remains attached. However, we find some viral sequences in the supernatant (Figure 4, channels 5 and 6; similar results are obtained with 82) and in the pellet the enrichments are lower than expected. Nevertheless, we can reconcile this simple model with our results if the cages are imperfectly separated from detached DNA by pelleting. (We would expect replicating sequences and even some detached DNA to pellet with cages and some cages to break and therefore not pellet). A possible candidate for such an attachment site is the 'enhancer' sequences of polyoma and retroviruses. These *cis*-acting sequences promote the transcription of genes that may be many thousands of base pairs away (Moreau *et al.*, 1981; de Villiers and Schaffner, 1981; Levinson *et al.*, 1982). Their concentrations in the three viral fragments in Table II (i.e., 2:1:0) reflect the enrichments of the fragments in the pellet. Alternatively, the virus might induce an adjacent cellular sequence to attach, perhaps by some conformational re-arrangement (Cook, 1974). Such an attachment, close to, but not within, the viral sequence is consistent with the enrichments found and the presence of viral sequences in the supernatant. Whatever the mechanism, it is clear that viral integration induces flanking cellular sequences to become more closely associated with the cage and its transcription machinery. This may be one way that the virus switches on adjacent cellular genes that lie 'upstream' from the integrated virus (Payne *et al.*, 1982).

Although a correlation between certain types of gross chromatin structure (e.g., heterochromatin) and gene function has been recognised for decades (Lewin, 1974), only recently have molecular correlates been uncovered. These include a sensitivity to digestion by deoxyribonuclease (Weintraub and Groudine, 1976), hypomethylation of the DNA (Ehrlich and Wang, 1981), and proximity of active genes to the cage (Jackson *et al.*, 1981; Robinson *et al.*, 1982). Since viral sequences in all nine primary transformants are expressed, the close association of viral genes with the cage strengthens this last correlation. Furthermore, expressed ASV sequences are close to the cage in A11 VIT and become less so when silent in the 'flat revertants', but then regain their close association when re-expressed after selection with aza-C. These changes in proximity to the cage mirror changes in hypomethylation and sensitivity to deoxyribonuclease (D.J.Chiswell and J.A.Wyke in preparation). Which, if any, of these is causative and which the effect of gene expression? The unexpressed α-globin gene in HeLa cells lies moderately close to the cage (i.e., enriched 4–6 x at high levels of detachment (Cook and Brazell, 1980)), so that proximity to the cage and its transcription machinery seems to be necessary, but not sufficient, for expression. It is easy to imagine how it might underlie both the gross structure of euchromatin and the fine structure of active nucleosomes. Perhaps, also, such attachments and detachments might change the developmental capacity of cells without inducing overt differentiation and so be involved in cell 'determination'.

Materials and methods

Cells

The derivation of the polyoma and ASV transformants has been described (Lania *et al.*, 1979, 1980; Wyke and Quade, 1980; Varmus *et al.*, 1981; Chiswell *et al.*, 1982).

Detachment mapping

The method for detachment mapping has been described (Cook and Brazell, 1980) and only variations are given here. Cells, labelled for 24 h with [methyl-^{3}H]thymidine, were lysed in 1.95 M NaCl, 0.1 M EDTA, 2 mM Tris (pH 8.0) and 0.5% Triton X100 and the released nucleoids (~10^8 in 10 ml) spun (Beckman SW 27 rotor; 7000–19 000 r.p.m.; 15–30 min; conditions depended on cell number and type) through 20 ml 15% sucrose on to a 7 ml shelf of 30% sucrose. The white aggregate of nucleoids was removed from the shelf, diluted and incubated with *Eco*RI or *Bam*HI. Cages were pelleted and the pellet dissolved in 1% sarcosyl, 100 mM EDTA and proteinase K (100 μg/ml) at 56°C for 1 h and a sample counted to determine the percentage of total DNA (prepared from undigested, unpelleted nucleoids) remaining associated with cages. This method gives a lower value than the filtration method used before (Cook and Brazell, 1980) for reasons given elsewhere (McCready *et al.*, 1980). DNA was purified, electrophoresed, 'blotted' in 10 x SSC and hybridized with the appropriate probes prepared by nick-translation.

Following hybridization, filters were washed four times (15 min each at

20°C) in 2 x SSC, 1 x Denhardt's solution, then 2 x (30 min at 55°C) in 0.1 x SSC, 0.1% SDS and finally once (30 min at 65°C) in the same solution. Autoradiographs were scanned using a microdensitometer and peak heights measured. The relative intensities of the bands were determined by reference to similar bands obtained with varying weights of total DNA. In some cases the probe was removed from the filters (McCrogan *et al.*, 1979) and the filters rehybridized.

Probes

The probes used were pRSA-8 (rat serum albumin; Sargent *et al.*, 1979; a gift from Dr.C.Szpirer), PyPAT (polyoma cloned in PAT 153), pSRA-2 (ASV; Delorbe *et al.*, 1980) and the polyoma junction sequences 82J1 (left side of the virus in 82), 53C1 (right side in 53), 7TL (left side in 7axT) and 7TR (right side in 7axT) (Hayday *et al.*, 1982; H.E.Ruley *et al.*, in preparation). The junction probes 53C1 and 7TR hybridize with a number of different bands – they contain repeated sequences – but the relative enrichments of the minor bands did not exceed those of the major band entered in Table I. The mouse albumin gene in Tsa3T3 was detected by cross-hybridization with the rat albumin probe.

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