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Expression of integrated Rous sarcoma viruses: DNA rearrangements 5′ to the provirus are common in transformed rat cells but not seen in infected but untransformed cells

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The study of Rous sarcoma virus (RSV)-infected rat cell clones offers a novel approach to unravelling the mechanisms controlling eukaryotic gene expression. RSV-transformed rat cell clones frequently contain duplicated proviral sequences immediately upstream of an intact provirus. This category of proviral rearrangement is not seen in cells that remain untransformed after RSV infection nor in subsequently segregating transformants. These results suggest that such rearrangements occur during or soon after proviral integration, and that they may favour early proviral expression.

Key words: Rous sarcoma virus/integration/upstream sequence/rearrangement

Introduction

Clonal rat cell lines, each derived from an individual cell containing a single integrated copy of Rous sarcoma virus (RSV), provide a powerful tool with which to study the effect of chromosomal environment on gene expression. Rat cells are non-permissive for RSV replication and so an integrated provirus remains a single genetic unit. Since integration is random and proviral expression results in a readily discernible phenotypic alteration (cellular transformation), the effect of cellular regulatory influences active at multiple loci can be investigated.

Transformation of mammalian cells by RSV is a relatively rare event, the provirus remaining transcriptionally silent in the majority of infected cells (Boettiger, 1974a; Varmus *et al.*, 1975). This observation is particularly intriguing in view of evidence that infected but untransformed cells often contain an intact proviral transcriptional unit with a functional *src* gene, yet they can be superinfected with exogenous RSV (Boettiger, 1974b; Chiswell *et al.*, 1982). Furthermore, the RSV LTR acts as a potent promoter and enhancer in transient expression assays in several different types of mammalian cells (Gorman *et al.*, 1982; Luciw *et al.*, 1983). *Cis*-acting cellular negative regulatory influences affecting the integrated provirus have been inferred from these findings (Gillespie *et al.*, 1985) and this hypothesis is consistent with previous suggestions that chromosomal location plays an important role in determining proviral expression (Wyke and Quade, 1980; Jaenisch *et al.*, 1981; Feinstein *et al.*, 1982).

We have previously shown that 6/8 transformed rat cell lines contain DNA rearrangements at the proviral locus and we have suggested that such rearrangements may be causally related to proviral expression and hence cell transformation, perhaps by alleviating *cis*-acting inhibition (Gillespie *et al.*, 1985; Levantis *et al.*, in press). This interpretation was based largely on data from cell clones obtained in a single series of rat cell infections (Wyke and Quade, 1980) and, since other workers had not reported such genomic rearrangements, we were concerned that the clones we had studied were atypical for some unknown reason. Moreover, our previous results, although highly suggestive, did not demonstrate a specific association between the observed rearrangements and proviral expression, since we were not able to compare our transformed clones with a comparable series of infected but untransformed clones that harboured an inactive provirus. An additional point of interest was raised by the knowledge that infected but untransformed clones occasionally segregate transformed progeny (Boettiger, 1974a; Turek and Opperman, 1980), but that the fate of the proviral locus during such segregation was unknown. We therefore designed a series of experiments to address these points and in this paper we show that (i) proviral rearrangements are common in a new series of transformed clones, (ii) they are not seen in cells harbouring an inactive provirus and (iii) they do not appear in the transformed derivatives that segregate from such clones.

Results

Rat-1 cells were exposed to filtered B77 strain RSV at a multiplicity of infection (MOI) of 0.01 (AA series) or 0.1 (BB series) chick focus-forming units per cell and seven transformed clones were isolated. A *Hin*dIII digest of genomic DNA from these clones was hybridized with a probe made from pSRA-2 (DeLorbe *et al.*, 1980) which contains a DNA copy of the entire RSV genome. B77 proviral DNA contains two *Hin*dIII sites in close proximity (Figure 2A) and so the two virus-cell junction fragments detected in each clone indicate that the proviral sequences are present at a single locus (Figure 1A). *Eco*RI cleaves within the proviral LTR and at two other sites within the provirus. Thus, if there are no rearrangements three characteristic internal fragments should be generated from an intact provirus (Figure 2A). An *Eco*RI-digest of DNA from the seven clones hybridized with pSRA-2 revealed the three internal fragments in each case, but additional bands containing proviral sequences were seen in three clones (Figure 1B). Subgenomic RSV probes (Figure 2A) were then used to construct restriction maps of the proviral integration sites in these three clones. Two contained restriction fragments immediately upstream of the 5′ LTR that hybridized to proviral probes (Figure 2B), but in the third case the extra bands seen in the *Eco*RI digest did not contain any of the proviral sequences present in our subgenomic probes.

Taken in conjunction with the eight clones previously reported (Gillespie *et al.*, 1985), nine out of a total of 15 transformed clones analysed, derived from three distinct series of infections, contain proviral rearrangements and these have been shown to be immediately upstream of the integrated provirus in all six clones for which sufficient data are available. To assess the significance and timing of such rearrangements we decided to

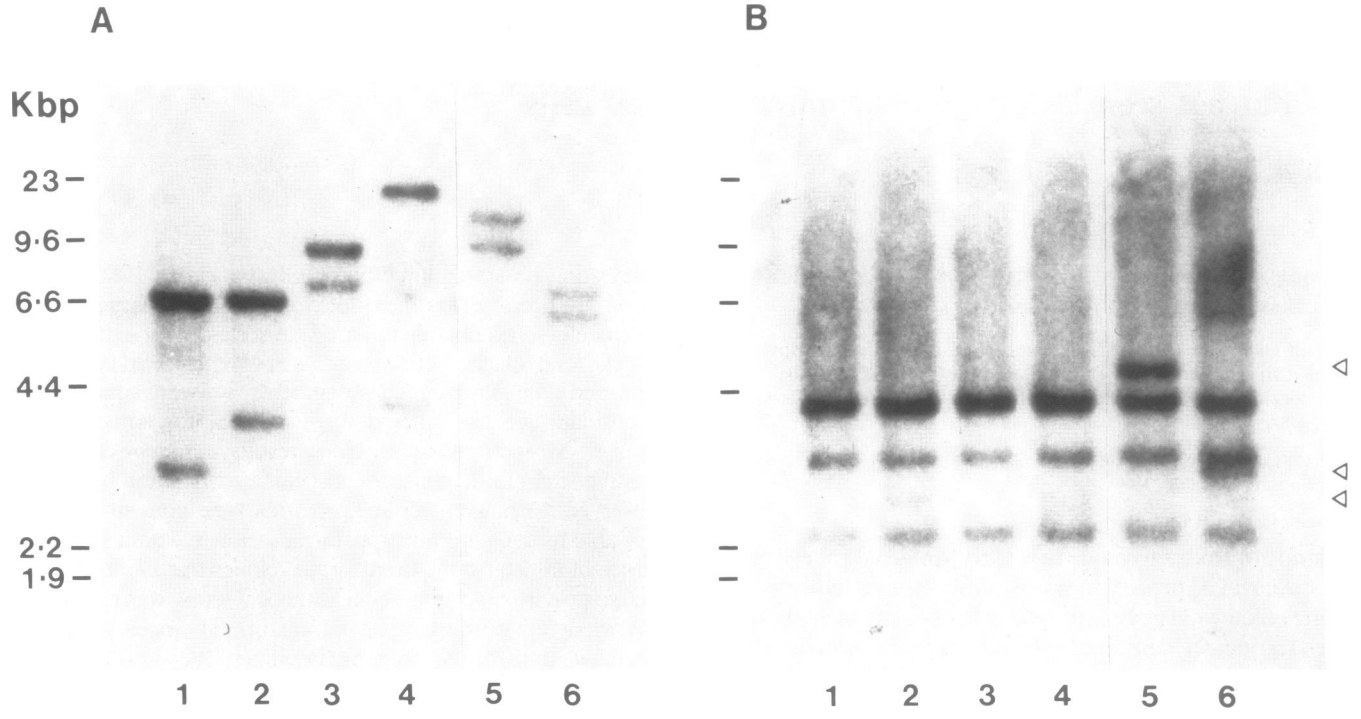

Fig. 1. Southern blotting analysis of rat cell clones transformed by RSV. Ten-microgram quantities of genomic DNA isolated from the various cell clones were digested with *Hin*dIII (**panel A**) or *Eco*RI (**panel B**). The resultant restriction fragments were separated by electrophoresis in a 0.8% w/v agarose gel and transferred to nitrocellulose. The filters were hybridized at 65°C with a ^{32}P-labelled probe, representing the entire RSV genome. The final wash was 0.1 × SSC at 65°C. The open arrows in **panel B** indicate the aberrant *Eco*RI fragments present in lanes 2, 5 and 6. Lane 1, BB1; lane 2, BB3; lane 3, BB16; lane 4, BB17; lane 5, BB30; lane 6, BB31.

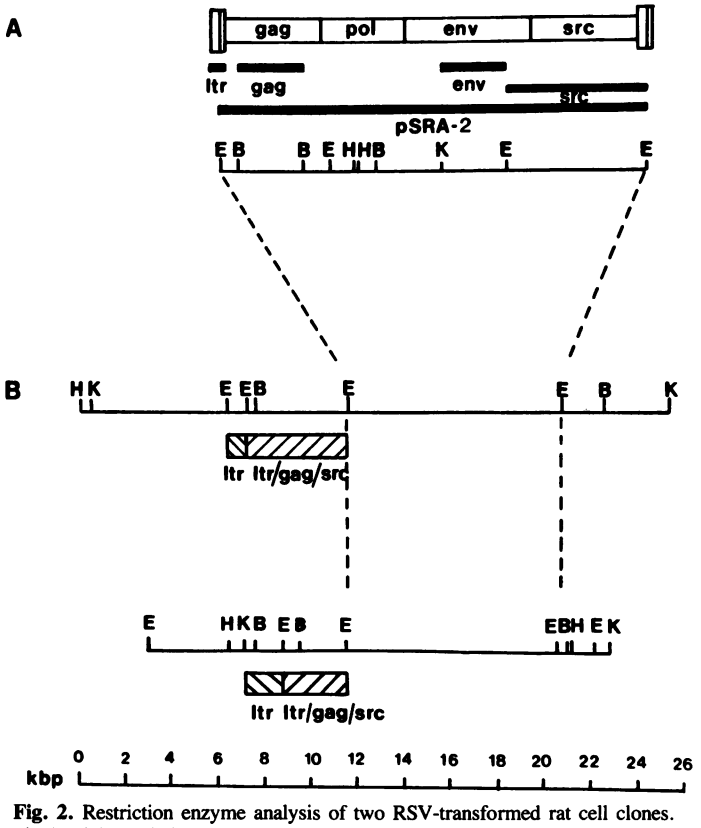

Fig. 2. Restriction enzyme analysis of two RSV-transformed rat cell clones. (**A**) Partial restriction enzyme map of the B77 provirus. The probes used in Southern hybridization were prepared from pSRA-2 (DeLorbe *et al.*, 1980) by gel purification of the relevant restriction fragments. (**B**) Partial restriction enzyme maps of the sequences flanking the integrated provirus in BB30 (upper map) and BB31 (lower map). B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. Hatched bars represent restriction fragments detected by hybridization with proviral subgenomic probes.

obtain and analyse a number of infected but untransformed rat cell clones.

Rat-1 cells were exposed to filtered RSV at a MOI of 0.8 chick focus-forming units per cell and 48 h later 104 single-cell clones (CC series) were obtained at random by micromanipulation. The clones were derived from two sets of infections, either with or without 10^{-7} M dexamethasone in the medium. We had previously shown that dexamethasone enhances the frequency of transformation by RSV (Wyke and Quade, 1980; unpublished data) and we were interested in examining whether its presence influenced the frequency and pattern of proviral integration. Fifty-three clones (numbers CC 1–53) were studied from the infections lacking dexamethasone and 51 (numbers CC 101–151) from the infections where it was present. None of the clones was initially transformed, an expected finding since a simultaneous assay of the input virus showed that its transformation efficiency on Rat-1 cells was only 0.1% of that on chick cells.

Genomic DNA from these clones was digested with *Hin*dIII and Southern blots hybridized with a probe representing the *gag* region of the RSV genome. Under these conditions a single band representing the 5′ virus–cell junction fragment will be seen for each intact provirus present in a given clone. *Gag*-related sequences were detected in 35/95 clones for which adequate Southern blots were obtained. These provirus-containing clones were distributed approximately equally between the dexamethasone-containing and dexamethasone-free infections. Thus, dexamethasone does not greatly enhance proviral integration, although infections in its presence showed a slightly greater percentage of clones bearing more than one provirus (Table I). Four of the clones contained three or more bands that hybridised with the *gag* probe and were not studied further. The other 31 clones, containing either one or two *gag*-positive *Hin*dIII-restriction fragments, were characterized in more detail (Table I).

Table I. Summary of the number and structure of the proviruses present in each of the RSV infected but untransformed rat cell clones

No. of proviruses	Clone designation	*Eco*RI restriction fragment pattern
≥2	CC3 CC120 CC138 CC139	ND
2	CC6 CC16 CC33 CC114 CC115 CC132 CC141 CC151	normal pattern + extra bands
	CC135	normal pattern
1	CC1 CC19 CC20 CC35 CC47 CC118 CC123	defective pattern
	CC32 CC42 CC112 CC113 CC143	ND
	CC2 CC17 CC22 CC26 CC31 CC103 CC105 CC116 CC119 CC130	normal pattern

The methods used to obtain RSV-infected but untransformed rat cell clones, together with those used to ascertain the number of proviruses present in each clone, are described in the text. *Eco*RI cleaves within the proviral LTR and twice again within the provirus, and thus the normal pattern refers to the three characteristic fragments generated from an intact provirus. When one or more of the internal fragments are replaced by aberrant bands the provirus is described as having a defective pattern. The presence of aberrant bands in addition to the three internal fragments is signified by normal pattern + extra bands.

Nine clones were found to contain four *Hin*dIII or *Bam*HI virus−cell junction fragments using either pSRA-2 or LTR probes, and are therefore likely to be doubly infected. In several cases two of the four bands were very faint (Figure 3A, lane 1, closed arrows) and we attribute this to the loss of one provirus from a subpopulation of cells. Direct evidence for this interpretation was obtained by deriving several single-cell subclones from the doubly infected clone CCl41. A *Hin*dIII digest of DNA from four of the subclones was hybridized with the *gag* probe. Three of the subclones contained the anticipated two bands (e.g. Figure 3B, lanes 2 and 3), each band representing a 5′ virus−cell junction fragment, but the fourth subclone contained only one of the bands (Figure 3B, lane 1), thus demonstrating the loss of proviral sequences from a subpopulation of cells in the parental clone. An *Eco*RI digest of all but one of the nine doubly infected parental clones revealed extra bands containing proviral sequences in addition to the three internal proviral fragments (Figure 3A, lane 4). This pattern suggests that at least one of the two proviruses present in each clone either has a defective structure or is intact but has an associated rearrangement.

Twenty-two clones contained a single *Hin*dIII fragment detected with a *gag* probe and are therefore likely to contain a single provirus. Further data are available on 17 of these. With the exception of CC118 all contained two *Hin*dIII virus cell fragments detected with the pSRA-2 probe (Figure 3C, lanes 1 and 3). Two patterns were obtained using the same probe following an *Eco*RI digest: in 10 clones three internal fragments were detected with no extra bands, indicating the presence of an intact provirus (Figure 3C, lane 4), whereas in seven clones one or more of

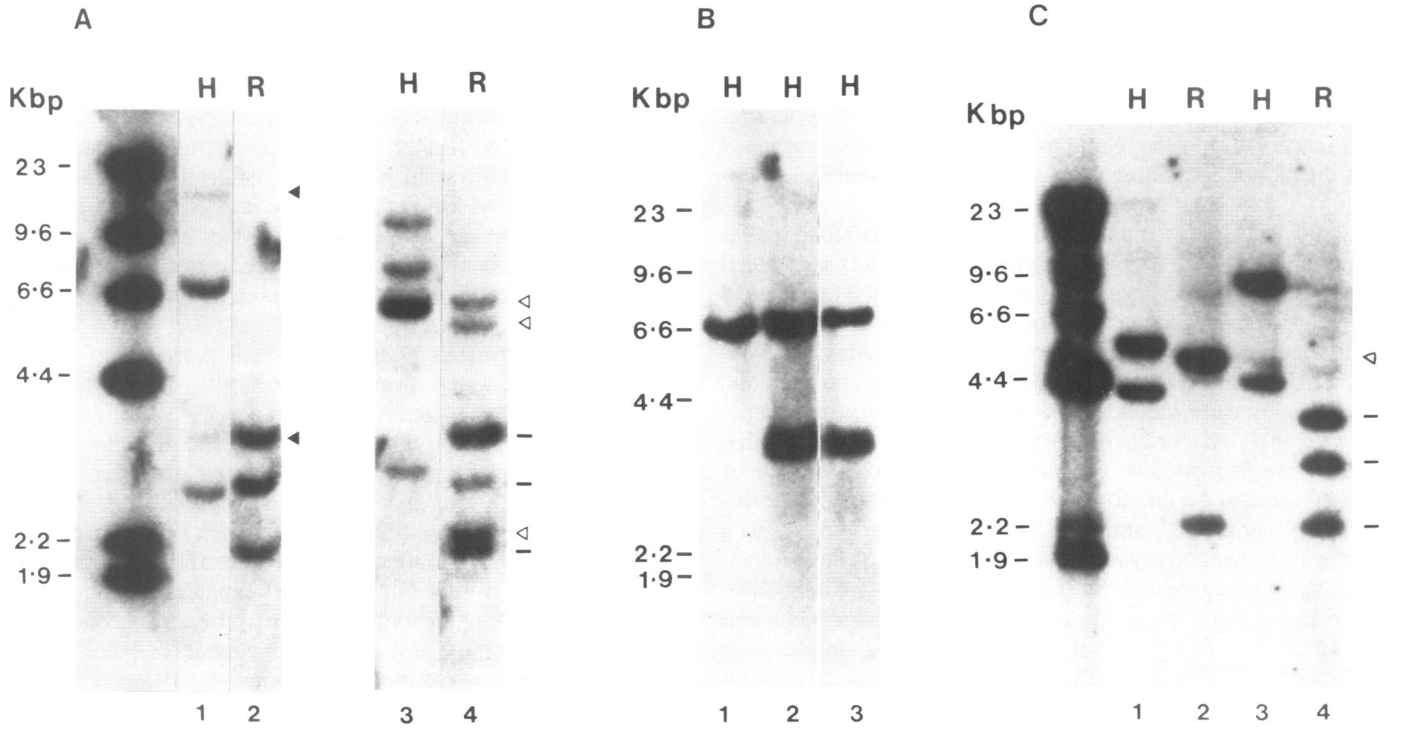

Fig. 3. Southern blotting analysis of rat cell clones infected but not transformed by RSV. Ten-microgram quantities of genomic DNA isolated from the various cell clones were digested with either *Hin*dIII (H) or *Eco*RI (R). The restriction fragments were separated by electrophoresis in a 0.8% w/v agarose gel and transferred to nitrocellulose. The filters were hybridized at 65°C with a ^{32}P-labelled probe representing either the entire RSV genome (**panels A** and **C**) or the *gag* region (**panel B**). The final wash was 0.1 × SSC at 65°C. (**A**) Examples of doubly infected clones. Lanes 1 and 2, CC135; lanes 3 and 4, CC132. Closed arrows indicate the presence of two weakly hybridizing bands in lane 1, bars indicate the normal *Eco*RI internal fragments in lanes 2 and 4 and open arrows indicate aberrant *Eco*RI fragments in lane 4. (**B**) Single-cell subclones derived from CC141. Lane 1, CC141/N1; lane 2, CC141/N2; lane 3, CC141/T7. (**C**) Examples of clones containing a single provirus. Lanes 1 and 2, CC19; lanes 3 and 4, CC31. Bars indicate the normal *Eco*RI internal fragments in lanes 2 and 4 and the open arrow indicates the aberrant *Eco*RI fragment in lane 2.

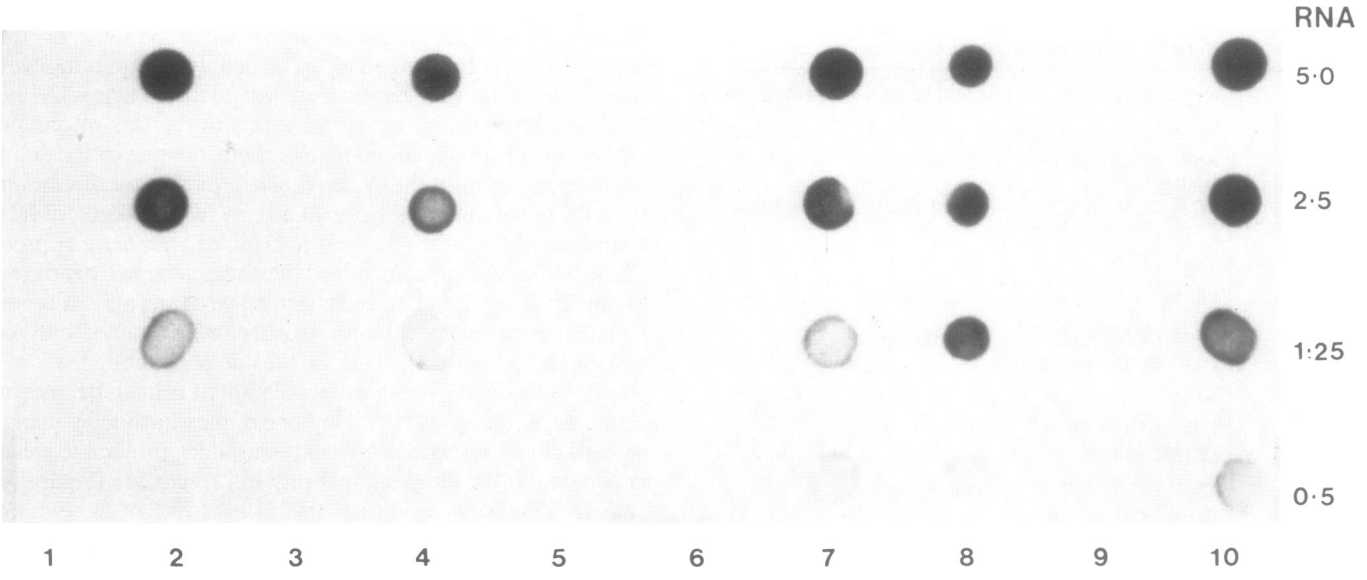

Fig. 4. RNA dot blot analysis of transformed segregants. Single-cell subclones with either a normal (N) or transformed (T) morphology were derived from two parental clones (each containing a single provirus) that subsequently segregated transformants. Rat-1 is the original uninfected rat cell line from which the infected clones were derived. VIT is an RSV-transformed rat cell clone and 21N a stable revertant clone derived from it, both described previously (Chiswell *et al.*, 1982). Four dilutions of total cellular RNA, extracted from the various cell clones by the method of White and Bancroft (1982), were spotted onto nitrocellulose and hybridized with the LTR probe. Relative amounts of RNA are indicated. Lane 1, Rat-1; lane 2, VIT; lane 3, 21N; lane, CC20; lane 5, CC20/N1; lane 6, CC20/N3; lane 7, CC20/T5; lane 8, CC130; lane 9, CC130/N1; lane 10, CC130/T11.

the internal fragments were replaced by aberrant bands, indicating the presence of a defective provirus (Figure 3B, lane 2). CC118 contained three *Hin*dIII fragments and a defective *Eco*RI pattern. Preliminary analysis suggests that the clone contains a provirus with a duplication at its 3′ end (data not shown).

Transforming virus could be rescued from all clones containing a single intact provirus (data not shown), thus confirming the presence of a potentially active proviral transcriptional unit and a functional *src* gene. No transforming virus could be rescued from the clones containing a defective provirus with the exception of CC118.

Five of the clones containing a single provirus (four with a normal and one with a defective proviral structure) segregated transformants after a varying number of passages. Transformed segregants were subcloned from four of these parental clones and *Hin*dIII and *Eco*RI digests of genomic DNA were hybridized with the pSRA-2 probe. The structure of the proviruses present in the transformants were identical to those of the proviruses present in their respective untransformed parental clones. To determine whether transformation was accompanied by RSV expression or whether it might be due to some other spontaneous transforming event, single-cell subclones with either a normal or a transformed phenotype were obtained from two of the parental clones that had segregated transformed cells. Total cellular RNA was extracted from the parent clones of mixed morphology and their subclones, and hybridized with the LTR probe. In each case LTR-specific RNA was readily detectable in the transformed but not the untransformed subclones (Figure 4).

Discussion

Rearranged proviral sequences adjacent to an intact provirus have been detected in 9/15 RSV-transformed rat cell clones, and in all six clones for which sufficient data are available the rearranged sequences lie immediately upstream of the integrated provirus. To obtain a comparable series of infected cells that had never exhibited transformation we examined 104 morphologically normal clones obtained after infection at a virus multiplicity calculated to give the maximum number of cells containing a single provirus. The distribution of the number of proviruses in individual cell clones accorded approximately to a Poisson distribution, showing that large clumps of virus did not contribute significantly to the infectious units. In 22 of these clones the provirus appeared to occupy a single locus, a situation comparable to that in the transformed clones we have studied, and 17 of these 22 were examined further. None contained rearrangements 5′ to the intact provirus, which are thus specifically associated with transformation and are not a frequent concomitant of proviral integration. However, seven of these clones did show proviral abnormalities: six contained defective proviruses (which are also the probable cause of the extra bands seen in most of the doubly infected cell clones; see Table I and Figure 3A) and in the case of CC118, a probable 3′ duplication was detected.

This high frequency of aberrant proviruses in infected cells is not unexpected. Defective proviruses occur frequently in RSV-transformed rat cells (Hughes *et al.*, 1978) and Moloney murine sarcoma virus-transformed mouse cells (Shtivelman *et al.*, 1984). Moreover, 20−50% of mammalian cells infected with RSV are non-virogenic by means of fusion with chick cells (Steiner and Boettiger, 1977), a finding we confirm here. These latter results may reflect the presence of defective genomes in virus stocks, a well-known phenomenon, or they may be a consequence of events at or around the time of proviral integration. Indeed, these are not mutually exclusive explanations, for the mechanisms responsible for the generation of defective genomes in virus stocks are unknown. Our demonstration of proviral rearrangements adjacent to a complete provirus that may involve cell as well as proviral sequences strongly favours the occurrence of duplicative events associated with integration (Gillespie *et al.*, 1985), and it is conceivable that the mechanisms that engender duplications may also induce deletions.

The likely importance of events near the time of integration is supported by our finding that none of the transformants which

segregated from the untransformed clones containing a single provirus showed any alteration in their proviral structure. This suggests firstly that the upstream rearrangements are rare events that occur during or soon after integration, thereby promoting early transformation, and secondly that the anatomy of the provirus after integration is very stable. Our observation that the segregation of transformants is rare, occurring in only five clones after several months of culture, contrasts with the results of Turek and Opperman (1980). We suspect that the more frequent occurrence of transformation in their clones may reflect the fact that they were obtained after high-multiplicity infection and probably contained multiple proviruses.

This work and our earlier studies allow a description of the fate of individual RSV proviruses after integration into rat cells. The vast majority of these proviruses are inactive, as judged by the absence of cell transformation, and, where tested (Figure 4), the lack of viral RNA, yet most are not defective and can be rescued in heterokaryons with chick cells. The inefficient expression of these proviruses contrasts with their activity in chick cells. Moreover, since the RSV LTR functions effectively in transient expression assays in mammalian hosts (Gorman *et al.*, 1982) we conclude that these cells are not deficient in factors required for provirus expression, but that integration reduces its vigour by some negative influence. This accords with our earlier finding that the expression of distinct proviruses within the same cell could differ (Wyke *et al.*, 1980) and our hypothesis that proviral activity reflected integration at favoured sites (Wyke and Quade, 1980).

The results presented here suggest the existence of at least three different routes by which proviral expression may occur. Many transformed clones arising immediately after infection contain proviral 5′ rearrangements whereas the remainder show no abnormality (Gillespie *et al.*, 1985; *vide supra*). In addition late transformants, with no alteration in their proviral structure, become evident in a small number of initially untransformed clones after multiple passages. We have previously argued that the 5′ rearrangements may function to attenuate cellular negative regulatory influences (Levantis *et al.*, in press). If so, one must postulate that the rearrangements, although necessary in some clones for the establishment of proviral expression, are presumably not sufficient for its maintenance, since the rearrangements are unaltered in spontaneous revertants (Gillespie *et al.*, 1985) and cell hybrids (Dyson *et al.*, 1982) in which proviral expression is suppressed. This paper highlights the complexity of gene regulatory mechanisms operating in rat cells and demonstrates the feasibility of using integrated RSV proviruses as uniquely sensitive probes for detecting such regulation and dissecting its components.

Materials and methods

Cells

Culture conditions for the Rat-1 cell line (Mishra and Ryan, 1973) have been described by Wyke and Quade (1980). RSV-transformed foci were isolated by ring cloning and subsequently single-cell cloned by micromanipulation. The untransformed clones were obtained directly by micromanipulation of cells 48 h after exposure to RSV.

DNA analysis

DNA extraction, restriction enzyme digestion, gel electrophoresis, transfer of DNA to nitrocellulose, nucleic acid labelling and filter hybridization were performed using adaptations of established procedures (Maniatis *et al.*, 1981).

RNA analysis

Cytoplasmic RNA dot hybridization was performed according to the method described by White and Bancroft (1982).

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