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DNA of Avian Myeloblastosis-Associated Virus Type 2 Integrates at Multiple Sites in the Chicken Genome

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The cellular sites of integration of the avian myeloblastosis-associated virus type 2 (MAV-2) DNA have been examined by Southern blot analysis of cellular DNA from infected cloned and uncloned chicken embryonic fibroblasts. Provirus-cell juncture fragments were not detected in restriction enzyme digests of DNA from MAV-2-infected uncloned cells. However, each MAV-2-infected cell clone examined produced a unique set of juncture bands. These findings indicate that multiple sites of integration exist for MAV-2 proviruses in cellular DNA.

MAV-2, one of the avian myeloblastosis-associated viruses, causes nephroblastomas, osteogenic osteoblastomas, and visceral leukosis in chickens (14). MAV-2 can productively infect chicken embryonic fibroblasts (CEF) in vitro, but does not transform them morphologically.

Recently, we have shown that the unintegrated linear DNA intermediate of MAV-2 has a mass of 5.3 megadaltons (Md) and contains terminal directly repeated sequences of approximately 0.3 Md (4). It was also shown that, in infected-cell DNA, MAV-2 proviruses are integrated colinearly with respect to the unintegrated linear viral DNA (4). In those earlier studies employing mass cultures of infected cells, we did not detect DNA juncture fragments containing proviral sequences and flanking host cell DNA sequences. It was presumed from these results that the MAV DNA had integrated at multiple sites in the host cell genomes (4). Analogous findings have been reported for other retroviruses (1, 5, 10, 12, 13). Some of these authors also suggested that certain sites of integration may regulate the expression of the integrated provirus (2, 3, 11). By restriction endonuclease and Southern blot analyses of cellular DNA from cloned and uncloned MAV-2-infected CEF, we have confirmed that MAV-2 integrates at multiple sites in the chicken genome.

MAV-2 unintegrated linear viral DNA has a mass of 5.3 Md and is cleaved twice by *Eco*RI to produce fragments of 2.6, 1.8, and 0.9 Md (4). The *Eco*RI fragments are placed in the relative order (5'-1.8-2.6-0.9-3') with respect to the 3' terminus of the viral RNA. An additional 4.4-Md fragment of uncertain origin is present in variable amount in *Eco*RI-digested unintegrated DNA from avian retroviruses (4). Also, MAV-2 proviruses are integrated colinearly with respect to the unintegrated viral DNA (4). Therefore, *Eco*RI is a useful restriction endonuclease to study integrated DNA as two relatively large terminal viral fragments are attached to adjacent cellular DNA sequences.

As a second enzyme it would have been preferable to use one which does not cleave MAV-2. However, all of the seven enzymes tested (*Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, *Bam*HI, *Xba*I, and *Sac*I) cleave MAV-2 DNA at least once (unpublished data). *Kpn*I cleaves MAV-2 linear DNA once to produce two large fragments of 3.6 and 1.7 Md (Fig. 1a). If blots of *Kpn*I-digested MAV-2 viral DNA were hybridized with a 12 to 16S standard avian myeloblastosis virus complex (AMV-S) RNA probe specific for the 3' terminus, the 1.7-Md fragment hybridized more intensely than the 3.6-Md fragment (Fig. 1b). The limited homology of the 3.6-Md *Kpn*I fragment to the 3' terminus of the viral RNA is consistent with the presence of a large, directly repeated terminal genomic sequence (4) although slight contamination of the 3' probe with 5' sequences cannot be excluded. The recognition sites for *Hin*dIII, *Eco*RI, and *Kpn*I in the MAV-2 genome are mapped in Fig. 2.

CEF cultures from a single 11-day-old C/E chicken embryo (Spafas, Inc.) negative for group-specific antigen and for chicken helper factor were infected with MAV-2 at a multiplicity of 1 to 5 infectious units per cell. High-molecular-weight ($\geq 3 \times 10^7$) cellular DNA from the MAV-2-infected CEF was extracted, precipitated by the method of Hirt, and purified as previously described (4, 6, 7, 9).

Since MAV-2 proviruses integrate in a unique colinear fashion with respect to their unintegrated linear DNA, juncture bands representing terminal proviral fragments attached to flanking host sequences should be detectable in Southern blots if MAV-2 integrates at a specific site, or at

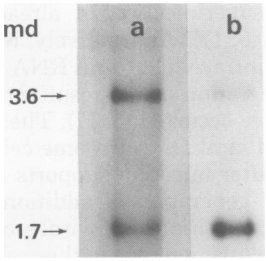

FIG. 1. *KpnI cleavage of MAV-2 unintegrated linear DNA. Unintegrated MAV-2 viral DNA was purified, digested with restriction endonuclease KpnI, electrophoresed in 0.7% agarose gels, by the Southern method, and hybridized with ^{125}I-labeled AMV-S RNA as described previously (4, 15, 16). (a) KpnI-digested MAV-2 DNA hybridized to an ^{125}I-labeled AMV-S RNA probe representative of the entire viral genome; (b) same as (a) but hybridized to 12 to 16S polyadenylic acid-containing ^{125}I-labeled AMV-S RNA (3' probe).*

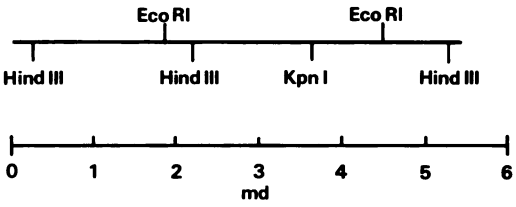

FIG. 2. *Restriction endonuclease cleavage sites in MAV-2 linear viral DNA. Right-hand side of cleavage map corresponds to 3' side of viral RNA. The EcoRI and HindIII cleavage sites represent data published earlier (4).*

very few sites, in cellular DNA. However, if MAV-2 proviruses integrate at many sites, only the endogenous sequences and internal MAV-2 proviral DNA fragments would be visible.

After *Eco*RI (Fig. 3b) or *Kpn*I (Fig. 4b) digestion of DNA from MAV-2-infected CEF, only the endogenous bands (Fig. 3a and 4a) were detectable in Southern blots. The internal 2.6-Md *Eco*RI fragment of MAV-2 DNA comigrated with the endogenous fragment of the same size. The presence of integrated MAV-2 DNA was demonstrable by *Hind*III digestion of the DNA from these same cells which generated a 3.1-Md internal fragment (4). These results suggest that MAV-2 DNA is inserted at numerous sites within the host genome.

To conclusively establish that MAV-2 integrates at multiple sites, we analyzed DNA from colonies arising from individual MAV-2-infected cells. In DNA from MAV-2-infected cloned cells, juncture bands should be detectable regardless of the number of integration sites. Because a relatively small number (0.5×10^7 to 1.0×10^7) of cells from each clone was available for DNA analysis, the Hirt precipitation step (9) used in our analysis of mass cultures was omitted. Thus, unintegrated viral DNA was not excluded in DNA preparations from cloned cells. Also, the amount of DNA available from each clone was sufficient for only one or two enzyme analyses with either *Eco*RI or *Kpn*I or both (Fig. 3 and 4).

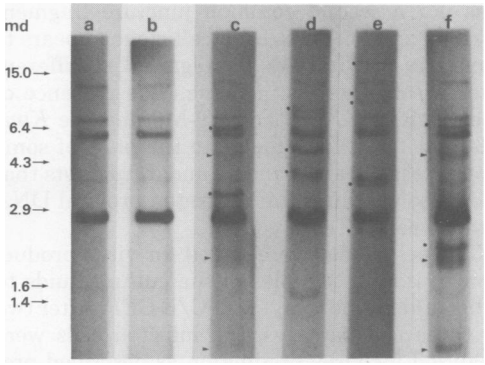

FIG. 3. *Autoradiographs of Southern blots of EcoRI-cleaved DNA from uninfected or MAV-2-infected CEF. High-molecular-weight cellular DNA was digested with EcoRI, electrophoresed in 0.7% agarose gels, blotted, and hybridized with ^{125}I-labeled AMV-S RNA as described previously (4, 15, 16). (a) DNA from uninfected cells; (b) DNA from MAV-2-infected but uncloned cells; (c through f) DNA from MAV-2-infected cloned cells: (c) clone 1, (d) clone 3, (e) clone 4, and (f) clone 5. Dots indicate bands believed to represent proviral-cellular DNA juncture fragments, and arrows indicate bands representative of unintegrated viral DNA. HindIII-digested phage λ DNA was used as molecular mass markers.*

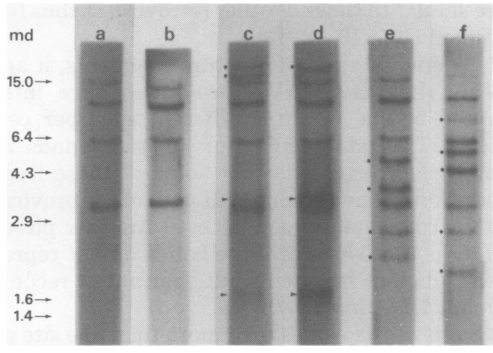

FIG. 4. *Autoradiographs of Southern blots of KpnI-cleaved DNA from uninfected or MAV-2-infected CEF. High-molecular-weight DNA was digested with KpnI and analyzed as in Fig. 3. (a) DNA from uninfected cells; (b) DNA from MAV-2-infected but uncloned cells; (c through f) DNA from MAV-2-infected cloned cells: (c) clone 1, (d) clone 6, (e) clone 3, and (f) clone 8. Dots indicate bands believed to represent proviral-cellular DNA juncture fragments, and arrows indicate bands representative of unintegrated viral DNA. HindIII-digested phage λ DNA was used as molecular mass markers.*

*Eco*RI or *Kpn*I digestion of DNA from various cell clones generated juncture fragments not previously observed with uninfected or infected but uncloned cells. Most of the DNA fragments had different masses. The 2.5-Md band which appeared in *Kpn*I digestion of DNA from clones 3 and 8 probably does not reflect integration of the proviruses at an identical site since there was not a second common juncture fragment (Fig. 4e and f). Thus, each cell clone appears to have had proviral DNA integrated at different sites within the host genome. The presence of the *Eco*RI 4.4-, 1.8-, and 0.9-Md and the *Kpn*I 3.6- and 1.7-Md fragments in the blots of some clones (Fig. 3c and f and 4c and d) suggests that they also contained unintegrated proviral DNA 3 to 4 weeks after infection.

The cell clones were tested for virus production by adding samples of the culture fluids to subconfluent monolayers of C/E CEF. After two or three cell passages, the infected cells were analyzed for virus production as described previously (8). All 16 MAV-2-infected CEF clones tested were virus producers, as evidenced by the banding of radioactively labeled virions in the 1.16-g/ml region of sucrose density gradients (data not shown).

These studies indicate that multiple sites are available in chicken DNA for the integration of MAV-2 DNA. This finding contrasts with the existence of a specific site for integration in the provirus which is integrated colinearly with respect to the unintegrated linear viral DNA (4). Therefore, these aspects of the integration of the MAV-2 viral DNA intermediate in chicken cells are similar to those of other retroviruses thus far studied (1, 5, 10, 12, 13).

By enumeration of juncture fragments, it appears that the MAV-2 proviruses were integrated at one to three different sites per cell clone. The odd number of juncture bands in some clones suggests that some of these fragments may have comigrated with other proviral DNA fragments of similar size. Another possibility is that some of these bands do not represent juncture fragments but aberrant or recombinant DNA intermediates.

Some of the clones with more than one site of proviral DNA integration could have arisen from two or three cells or from multiple first-round integrations. However, the variation in the intensity of some of the juncture fragments suggests that they may have resulted from proviruses being integrated during the outgrowth of the cell colonies. Reinfection of already infected surrounding cells by subgroup B viruses and massive synthesis of viral DNA which persists for several weeks have been reported recently (17). Thus, additional proviral integrations may arise after superinfection of already infected cloned cells (5, 17). Alternatively, reverse transcription of intracellular viral RNA with subsequent reintegration of the resultant viral DNA may also have occurred (5, 17). The presence of unintegrated viral DNA in some cell colonies 3 to 4 weeks after infection supports these possibilities. The generation of additional juncture bands by transposition of proviral sequences (13) within the cellular genome in these clones seems unlikely in light of recent evidence (5, 10).

All clones produced infectious virus independently of the location of the integration site within the cell genome. In clones containing more than one provirus, we have not been able to exclude the possibility that one or more of the proviruses were actually nonfunctional. However, 5 of the 16 virus-producing clones appeared to contain a single provirus integrated at different sites. Thus, most MAV-2 provirus integration sites appear to be compatible with viral gene expression and virus synthesis.

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