Induction of Nephroblastoma by Myeloblastosis-Associated Virus Type 1: State of Proviral DNAs in Tumor Cells

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Myeloblastosis-associated virus type 1 (MAV1) derived from a molecular clone of infectious proviral DNA (B. Perbal, J. S. Lipsick, J. Svoboda, R. F. Silva, and M. A. Baluda, J. Virol. 56:240-244, 1985) was shown to specifically induce nephroblastoma in chickens and therefore belongs to the MAV-N class. We show that nephroblastomas are polyclonal tumors containing rearranged proviral genomes. Rearrangements occur preferentially in the *gag-pol* region of the MAV1 proviral genome, and similar rearrangements can be detected in well-developed independent tumors.

Although there is much evidence which supports the induction of tumors by retroviruses lacking oncogenes via insertional activation of proto-oncogenes (3, 7, 10, 11, 21), this situation does not seem to account for the neoplasms related to myeloblastosis-associated virus (MAV) (2, 17, 25).

MAV is a replication-competent retrovirus which induces predominantly nephroblastoma and osteopetrosis and, less frequently, lymphatic leukemia and sarcoma in chickens (1, 8, 18, 19, 22). Several different strains of MAV (MAV type 1 [MAV1], MAV2, MAV2-O, and MAV2-N) have been iso-

involved in the induction of nephroblastoma by a genetically pure MAV1 (15).

Tumorigenic potential of cloned MAV1. Of 88 day-old chickens (55 Brown Leghorn, Edinburgh strain [*gs*⁺ *chf*⁺ V⁻] from the Institut National de la Recherche Agronomique, Nouzilly, France, and 33 White Leghorn Spafas from Spafas Inc., Storrs, Conn.) inoculated intraperitoneally with 10⁶ PFU of infectious cloned MAV1, 100% developed only nephroblastomas with a latent period ranging from 8 to 13 weeks. When injected intravenously in 12-day-old Brown

TABLE 1. Features of the nephroblastomas analyzed in this study

Nephroblastoma	Days after injection	Macroscopical features	Histological type ^a
776 AN	90	Caudal part of the kidney; no gross evidence of lesions	B
528	74	Hypertrophy of the whole kidney without nodular lesions	—
501D	74	Pink nodules protruding on the surface of the kidney	—
520	74	Lobular tumor 3 to 4 cm in diameter; greyish-pink; soft	C
501	74	Lobular tumor 5 to 6 cm in diameter; greyish-pink; fleshlike; soft	—
725	80	Massive tumor 8 to 10 cm in diameter; greyish-pink; fleshlike; soft	—
776	90	Hard yellowish-white tumor with occurrences of cartilage; 8 to 10 cm in diameter	D

^a B, C, and D, Fig. 1B, C, and D; —, no corresponding visual type in Fig. 1.

lated (1, 9, 12, 19). The identification of two genetically distinct MAV2 responsible for nephroblastoma (MAV2-N) and osteopetrosis (MAV2-O) was based on differences in T1-resistant oligonucleotide maps of viral RNA from both strains (23). However, plaque-purified MAV2-N and MAV2-O still induce 30% osteopetrosis and 20% nephroblastoma, respectively, in infected chickens (1, 18, 19). A high incidence of nephroblastoma occurs after infection of strain GB1 chickens with MAV2-O (2), and host susceptibilities in response to MAV infection were reported to range from 70 to 100% depending on the origin of the chicken strain used (25).

As a first step in our approach to understanding the molecular basis of the MAV pluripotent tumorigenic potential, we have undertaken a study of the molecular events

Leghorn embryos, MAV1 induced only nephroblastomas (study performed on 23 birds). Therefore, our cloned MAV1 belongs to the N class. These results and the dual tumorigenic potential of MAV2 strains suggest that plaque-purified isolates may not be genetically pure, or that MAV-O and MAV-N can derive from each other. Our preliminary observations indicate that the cloned MAV1-N is relatively stable, since it has been passaged in our laboratory for several years without giving rise to biologically active MAV1-O. A third possibility is that osteopetrosis induction may be modulated by the *env* genes.

Since nephroblastomas obtained 60 days postinjection were found to differ quite significantly in size and macroscopical aspect, no obvious relationship could be established between tumor size and the period of time elapsed after injection. On the basis of histological analysis and preliminary results of nephroblastoma development studies (V. Maloisel et al., manuscript in preparation), we selected seven tumors representing most stages of tumor progression

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FIG. 1. Origin of tumors representing different stages of nephroblastoma development; histological types. [A] Renal cortex of an 80-day-old noninfected Brown Leghorn chicken. Normal convoluted tubule sections and glomeruli. Abbreviations: Gm, mammalian type; Gr, reptilian type. [B] Tumor lesions in macroscopically normal kidney. Abbreviations: C, enlarged cystic tubule; Ba, blastema with tubular differentiation (T); IC, inflammatory mononuclear cells. Note the basophilic aspect (B) of the tumor tissue compared with acidophilic (A) normal cortical renal tissue. [C] Early progressive nephroblastoma emerging from one renal tubule. Blastemal cell islets (Ba) with irregular tubular differentiation (T) separated by narrow stromal septa (S). Tumoral tissue is poorly limited by a thin stroma reaction (arrows) from normal tissue (N). [D] Tumor exhibiting epithelial differentiation with basaloid features (E). Tumoral cells are arranged in nests and cords within narrow stromal septa (S). Tubular arrangements are occasionally seen (arrows). Magnification, $\times 792$.

(Fig. 1 and Table 1) and addressed the question of whether the development stage of the different nephroblastomas could be associated with a particular proviral integration pattern in the tumor cell DNA.

Nephroblastomas are polyclonal tumors. DNA was obtained from freshly explanted nephroblastomas (taken within an hour after sacrifice of the infected birds by intracardiac puncture). All procedures related to purification of high-molecular-weight DNA, endonuclease digestion, electrophoresis, Southern blotting, and hybridization were performed as described by Perbal (14).

The identification of *Eco*RI 5'-proximal junction fragments was performed by hybridizing the blots with probes: XH specific for the U3 region of avian myeloblastosis virus (25), B3 (U5 and leader sequences), and B2 (*gag* sequences). The identification of *Eco*RI 3'-proximal junction fragments relied on the use of XH, B3, and EXE probes, the latter being specific for *env* sequences (Fig. 2). Because the pattern of endogenous sequences varies slightly from one animal to another (20), and since all tissues of tumor-bearing animals contain viral particles (24), we used fibroblast DNA from each infected chicken as controls.

After hybridization of fibroblast DNA with the XH probe, all tumor DNAs gave rise to a positive signal ranging from a

smear in tumor 776 AN to a clear pattern of nine discrete bands in the most developed tumor (tumor 776). Intermediate stages were found to contain various amounts of junction fragments giving a smearing pattern whose intensity decreased as the intensity of the discrete bands increased (Fig. 2A).

The results obtained permitted us to draw an inverse relationship between the stage of tumor development and the complexity of the junction fragments pattern. A scanning of the autoradiograms (data not shown) revealed that the relative intensity of junction fragments may vary from a 3:1 ratio in tumor 776 (7.8- and 20.0-kilobase-pair [kb] fragments) to a 10:1 ratio in nephroblastoma 520 (3.5- and 1.4-kb fragments). Since the XH probe contains only 300 base pairs of viral sequences, these variations are unlikely to result from different amounts of homologous sequences being represented in the various DNA fragments. Similar results were observed after hybridization of tumor DNA with the B2 and EXE probes, whose use allowed us to distinguish between 5'- and 3'-proximal junction fragments (Fig. 2B and C). Unlike in the previously reported study by Boni-Schnetzler et al. (2), none of the tumor DNAs analyzed in the course of our study was found to contain unintegrated MAV1 proviral genomes.

FIG. 2. Characterization of junction fragments in tumoral DNA. (A through C) High-molecular-weight DNA (15 μ g) from tumoral (776 AN, 528, 501D, 520, 501, 725, and 776) and corresponding control tissues was digested with *Eco*RI endonuclease, electrophoresed in 0.8% agarose gels, and hybridized to the XH (U3) (A), B2 (B2H193, *gag*) (B), or EXE (*env*) (C) 32 P-labeled probe. C1, C2, and C3 are representative of the three hybridization patterns obtained for control tissues. A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174RF DNA was run as size markers (numbers on the left, in kilobase pairs). (D) Diagram showing probes used to identify 5'- and 3'-proximal *Eco*RI junction fragments.

TABLE 2. Occurrence of MAV1-rearranged sequences in well-developed tumors

Tumor	No. of bands (sizes in kilobase pairs) ^a detected with probe:			
	B2 (<i>gag</i>)	EBG (<i>gag-pol</i>)	BGE (<i>pol-env</i>)	EXE (<i>env</i>)
501	1 (5.1)	1 (4.0)	4 (5.1, 4.0, 2.7, 1.6)	1 (1.5)
725	3 (5.1, 3.5, 2.05)	2 (3.5, 2.05)	3 (12.0, 5.1, 3.5)	2 (2.7, 2.0)
776	2 (2.1, 1.7)	2 (2.1, 1.7)	5 (7.6, 7.0, 2.9, 2.7, 2.5)	1 (3.3)

^a Bands which correspond to new *Hind*III MAV1-related fragments.

Since hybridization of *Eco*RI-digested DNA from MAV1-infected fibroblasts with the same probes did not lead to the detection of a smear even after an extended exposure time of the autoradiograms (data not shown), the smear observed with samples of less developed tumors is not the result of chronic infection and random integration of MAV.

The detection of several junction fragments in the DNA of tumor cells is not sufficient per se to demonstrate the polyclonal origin of the nephroblastomas, since transformed cells may contain more than one proviral genome and since chromosomal abnormalities may occur during tumor development. In the first case, all junction fragments would be represented in a molar ratio to each other, and in the second case, most junction fragments detected in the parental cell would also be detected in daughter cells. The great variation in the size and in the intensity of hybridization patterns obtained for the different junction fragments with the same probe suggests that nephroblastomas are polyclonal tumors. Furthermore, the less complicated patterns obtained in the case of well-developed tumors suggest that a selection of some cellular clones occurs during tumoral multiplication.

Identification of rearranged MAV1 proviruses in nephroblastoma DNA. To determine whether rearranged MAV1 sequences were integrated in tumor DNA, we took advantage of the *Hind*III restriction sites previously mapped on the cloned MAV1 genome (15). DNA samples from tumor cells and control fibroblasts were hybridized to MAV1 probes representing nearly all the MAV1 proviral genome.

The ratio of MAV-specific DNA fragments to endogenous DNA fragments in the control tissues was found to be lower than in fibroblasts infected in culture (20) and was considerably increased in tumor DNA, as shown by the heavy bands obtained with *gag* and with *env* probes (Fig. 3A and B).

New MAV1-related DNA fragments were detected in tumor cell DNA after hybridization with these probes. Only a few additional light bands were detected in diffuse tumors such as 776 AN, 528, 501D, and 520, while more intense discrete bands were detected in well-developed tumors (501, 725, and 776) (Table 2). Similar results were obtained with the U3- and U5-specific probes (not shown). New MAV1-related fragments were also detected after hybridization with the EBG (*gag-pol*) and BGE (*pol-env*) probes (Fig. 3C and D), in addition to the normal MAV1-specific fragments (2.8 and 3.4 kb for EBG; 3.4 kb for BGE). The more intense bands were detected in well-developed tumors. Interestingly, the number of additional MAV1-related fragments detected with the BGE probe was found to be larger than that revealed by the EBG probe (Table 2), suggesting that deletions of MAV1 sequences predominantly occur in the vicinity of the *gag-pol* genes junction.

FIG. 3. Rearranged MAV1 proviral genomes in nephroblastomas. (A through D) High-molecular-weight DNA (15 μ g) from tumoral and corresponding control tissues (C1, C2, C3) was digested with *Hind*III endonuclease, electrophoresed in 0.8% agarose gels, and hybridized to the B2 (B2H193, *gag*) (A), EXE (*env*) (B), EBG (*gag-pol*) (C), and BGE (*pol-env*) (D) 32 P-labeled probes. A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174RF DNA was run as size markers (numbers on the left, in kilobase pairs). The two heavy bands (8.4 and 4.8 kb) detected in nephroblastomas 528 and 520 with all probes containing vector sequences were also detected by pBR322 32 P-labeled DNA and therefore do not correspond to chicken or viral DNA. Similar findings were previously described (2). (E) Diagram showing probes used and the positions of *Hind*III sites (H) on the MAV1 proviral genome.

The results obtained with all probes allowed us to determine the putative structure of several rearranged fragments (Fig. 4). The results obtained in the analysis of the junction fragments and rearranged MAV1 sequences strongly suggest that DNA of well-developed tumors (725-776) contain at least 50% of altered MAV1 genomes.

RNA species corresponding to the new MAV1-related sequences were expressed in tumor cells (data not shown). In contrast to previously reported results (2, 25), the nephroblastoma cells did not express chimeric RNAs composed of MAV and *c-Ha-ras* sequences or high levels of *c-myc* RNA species (data not shown).

To explain our observations, we propose that nephroblastoma development in chickens is the result of a two-step process. The first step would correspond to a virally induced proliferation of infected cells after integration of MAV in a particular region of the chicken genome. This would be visualized by the smear of junction fragments which is

FIG. 4. Schematic drawing of rearranged MAV1 proviral sequences in nephroblastomas. Open boxes delineate the proviral sequences which are not detected in the new *Hind*III MAV1-related fragments; dashed lines indicate that the extent of the deletion is not precisely established. The proviral sequences contained in the new MAV1-related DNA fragments are deduced from the viral sequences represented in the probes which detected these fragments.

observed in beginning tumors (Fig. 2). Because the MAV genome is structurally very similar to the yeast Ty elements (5), the integration site in the chromosome could be a transcriptionally active region (for a review, see reference 26). The second step would involve the deletion of the MAV1 genome in the *gag-pol* region and result in the expression of a tumorigenic product triggering clonal multiplication of tumor cells. This would lead to the detection of a limited number of junction fragments and well-defined rearranged genomes in nephroblastomas.

On the other hand, it is also tempting to compare this situation with that of the human Wilms' tumor, in which a recessive alteration of two alleles seems required (4, 6, 13, 16). The two-step induction process of avian nephroblastoma would then correspond to the integration of the MAV genome in one allele of a particular chromosome followed by specific rearrangements at this locus (possibly induced by MAV) resulting in the alteration of the other allele.

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