HRAS, EGFR, MET, and RON Genes Are Recurrently Activated by Provirus Insertion in Liver Tumors Induced by the Retrovirus Myeloblastosis-Associated Virus 2

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ABSTRACT Myeloblastosis-associated virus 2 (MAV-2) is a highly tumorigenic simple avian retrovirus. Chickens infected *in ovo* with MAV-2 develop tumors in the kidneys, lungs, and liver with a short latency, less than 8 weeks. Here we report the results of molecular analyses of MAV-2-induced liver tumors that fall into three classes: hepatic hemangiosarcomas (HHSs), intrahepatic cholangiocarcinomas (ICCs), and hepatocellular carcinomas (HCCs). Comprehensive inverse PCR-based screening of 92 chicken liver tumors revealed that in ca. 86% of these tumors, MAV-2 provirus had integrated into one of four gene loci: *HRAS*, *EGFR*, *MET*, and *RON*. Insertionally mutated genes correlated with tumor type: *HRAS* was hit in HHSs, *MET* in ICCs, *RON* mostly in ICCs, and *EGFR* mostly in HCCs. The provirus insertions led to the overexpression of the affected genes and, in the case of *EGFR* and *RON*, also to the truncation of exons encoding the extracellular ligand-binding domains of these transmembrane receptors. The structures of truncated *EGFR* and *RON* closely mimic the structures of oncogenic variants of these genes frequently found in human tumors (*EGFRvIII* and *sFRON*).

IMPORTANCE These data describe the mechanisms of oncogenesis induced in chickens by the MAV-2 retrovirus. They also show that molecular processes converting cellular regulatory genes to cancer genes may be remarkably similar in chickens and humans. We suggest that the MAV-2 retrovirus-based model can complement experiments performed using mouse models and provide data that could translate to human medicine.

KEYWORDS avian retroviruses, insertional mutagenesis, retroviral oncogenesis

Myeloblastosis-associated virus 2 (MAV-2) is a replication-competent virus of the avian leukosis virus (ALV) group from the *Alpharetrovirus* genus. It has been discovered as a natural helper of avian myeloblastosis virus (AMV), a defective retrovirus transducing the *v-myc* oncogene (1, 2). Though MAV-2 does not contain a transduced oncogene, it is strongly tumorigenic, with a latency of tumor appearance of less than 2 months. In contrast to the majority of other simple retroviruses that give rise predominantly to hematopoietic malignancies, MAV-2 induces only nonhematopoietic tumors. In chickens infected with MAV-2 at the 12-day embryonic stage, the tumors emerge in three organs: the kidneys (nephroblastomas), lungs (hemangiosarcomas), and liver (3, 4).

Retroviruses not carrying oncogenes cause neoplasia by a mechanism known as oncogenesis through insertional mutagenesis (5). Due to the very efficient retrovirus dissemination throughout the organism and the quasirandom choice of integration

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sites, most loci of a host genome are hit by a provirus insertion in some cells of an infected tissue. Some of these insertions land inside or in the neighborhood of potential cancer genes. This may either destroy these genes or, conversely, boost their activity through the very strong promoters and enhancers contained in provirus long terminal repeats (LTRs). If the inflicted changes lead to cell transformation, the affected cell clone may overgrow the other cells and give rise to a tumor. The responsible genes can be easily identified since they are tagged by integrated proviral sequences. Recurrent provirus integration into the same locus in independent tumors (known as a common site of integration [CIS]) indicates the presence of a cancer gene in the locus.

The foundations of this field were laid down by analyses of ALV-induced chicken B-cell lymphomas, where the cellular oncogene *MYC* was found to be recurrently activated by provirus integration (6). Subsequent studies identified further CISs in chicken B-cell lymphomas (*MYB*, *MIR-155*, and *TERT* [7, 8]), in chicken erythroblastosis (*EGFR* [9]), and in chicken myeloid leukosis (*MYC*, *TERT*, and *ZIC1* [10]). Many cancer genes were also discovered in mouse T-cell, B-cell, and myeloid lymphomas, erythroid leukemias, and mammary carcinomas (11). Before the advent of the high-throughput sequencing of human cancer genomes, insertional mutagenesis screening was the most efficient instrument in the quest for cancer genes (12).

In previous studies, we analyzed MAV-2-induced nephroblastomas and lung hemangiosarcomas and confirmed insertional mutagenesis as the mechanism of oncogenesis. We have found cancer genes that are recurrently activated by provirus insertion in nephroblastomas (*FOXP1*, *PLAG1*, *TWIST*, and *HRAS*) and in lung hemangiosarcomas (*FRK*) (3, 4, 13). In our model we have also made the original observation that nontumor cells injected into the blood circulation and passing further into tissues strongly promote the formation of some tumor types from cells primed by MAV-2 insertional mutagenesis. Based on this observation, we proposed the concept of industasis: the promotion of a fully malignant phenotype of an incipient tumor cell by a stray nontumorigenic cell through the disruption of local homeostasis (3).

In this paper, we present molecular analyses of chicken liver lesions induced by MAV-2. These analyses identified *HRAS*, *EGFR*, *MET*, and *RON* as insertionally mutagenized driver genes and characterized the mechanisms of their tumorigenic activation.

RESULTS

Tumor induction. Altogether, 92 tumors from 53 liver tumor-positive animals were collected. Cell promotion increased the incidence of liver tumors from ca. 15% to ca. 35%; when cell and CCl₄ promotions were combined, the incidence increased to ca. 75%. We identified three tumor types: hepatic hemangiosarcomas (HHSs), intrahepatic cholangiocarcinomas (ICCs; in avian veterinary medicine called bile duct carcinomas [14]), and hepatocellular carcinomas (HCCs), plus rare cases of mixed HCC/ICC histotypes. Detailed pathology and tumor histology as well as details of tumor promotion effects are available (V. Karafiat, P. Pajer, V. Pecenka, and M. Dvorak, unpublished data).

Clonal status of the tumors and intraorgan metastasis. To carry out a high-throughput search for viral integration sites (VISs), we optimized the inverse PCR (iPCR) technique so that for each tumor we could obtain a great majority (86%) of all clonal junction fragments in a single PCR (Fig. 1). This technique provided a quite realistic picture of clonality, including a semiquantitative assessment of the proportion of eventual subclones or minor independent clones with better sensitivity and resolution than Southern blots (Fig. 1C). As judged from the uniform band intensity on iPCR gels, a majority of lesions were formed by a single clone, with no signs of acquiring additional proviruses during tumor growth; low-abundance extra clones or subclones could be seen in only some samples. Identical iPCR patterns in DNAs isolated from distant parts of a nodule also demonstrated the clonal homogeneity of several lesions tested this way (see Fig. 1B, tumor 6781Li1, samples a and b, as an example). In contrast, iPCR patterns in discrete liver tumors as well as tumors of the lungs and kidneys from the same animal were completely diverse, implying independently originating tumors.

FIG 1 Procedure for identifying VISs. (A) Scheme of iPCR. LTRs are shown as rectangles divided into U3, R, and U5 segments. UT5 and UT3 are untranslated parts of the provirus adjacent to 5' and 3' LTRs, respectively. Wavy lines represent chicken genomic sequences. iPCR fragments corresponding to left (5') and right (3') provirus/host junctions were produced by combined BstYI plus BclI digestion, circularization by self-ligation, linearization with ApaLI, Eco81I, or SphI, and, finally, amplification by PCR. (B) Examples of iPCR products. Tumor samples are marked with four-digit chicken number, organ specification (Li, liver; Ki, kidney; Lu, lungs), and tumor serial number. Three iPCRs were carried out for each sample, providing for left junctions (primers L and 5' [lanes L]), right junctions (primers R and 3' [lanes R]), or both (primers L and R [lanes C]). A typical VIS is represented by a pair of iPCR bands in lane C plus lane L or R; fragments in lanes L and R are shorter by 59 bp and 87 bp, respectively. The junction fragment labeling system is shown in the lanes for samples 6239Li2, 6781Li1b, and 6789Li1. Exceptions reflecting the existence of defective proviruses occurred frequently. Proviruses with deleted/mutated sequences complementary to primers 5' or 3' (e.g., solo LTRs) provided a fragment only in lane C (e.g., fragment C1). Rearranged proviruses in which an LTR was adjoining both UT3 and UT5 sequences produced iPCR fragments in all three lanes (e.g., fragment X1). A similar arrangement is also present in unintegrated circular proviruses containing one or two LTRs in tandem; corresponding fragments of ca. 1 kb with variable (usually low) intensity were seen in all three lanes for many samples (fragments U). Samples 6781Li1a and -Li1b are distant parts of the same tumor. Samples 6239Li2 and -Li3 are clonally related; fragments unique to 6239Li3 are indicated. The negative gel image is shown for better resolution. Lane M, molecular size markers. (C) Comparison of resolutions for iPCR gel and Southern blot. iPCR lanes (right) show both left and right flanks (corresponding to lanes C in panel B). The Southern blot (left) was prepared using the ApaLI restriction enzyme and MAV-specific U3 probe; it displays only left flanks and a 7.33-kb internal ApaLI fragment common for all MAV-2 nondefective proviruses.

In a few animals, however, two liver nodules displayed identical patterns, indicating intrahepatic metastasizing; all these nodules fell into the HHS group. In such cases only one nodule was further analyzed, with the single exception of samples 6239Li2 and 6239Li3 (Fig. 1B), where clonally related nodules carried unique extra insertions.

Identification of insertionally mutated genes. To identify VISs, 547 iPCR-produced MAV-2 junction fragments from 92 liver tumors were gel isolated, PCR reamplified, and directly sequenced, and sequences were aligned to the chicken genome. Thirty-eight fragments contained only sequences of rearranged MAV-2 proviruses. The remaining fragments represented 344 unique VISs (165 of them yielded iPCR products from both ends of the provirus). Thus, there were 3.7 clonal proviruses per tumor on average,

TABLE 1 List of CIS-associated genes

CIS/gene	No. of hits	Cluster size (kb)	P value ^a	Provirus		Gene product description
				Position ^b	Orientation ^c	
<i>EGFR</i>	26	2.8	$5.10^{-104}/1.10^{-100}$	In	Bidirectional	Transmembrane tyrosine-protein kinase
<i>HRAS</i>	23	13.6	$3.10^{-75}/5.10^{-71}$	Up	Sense	Small regulatory GTP-binding protein
<i>MET</i>	21	16.1	$5.10^{-66}/8.10^{-62}$	Up	Sense	Transmembrane tyrosine-protein kinase
<i>RON</i>	9	0.9	$6.10^{-33}/5.10^{-30}$	In	Sense	Transmembrane tyrosine-protein kinase
<i>AKT1</i>	3	86.1	0.025/1	Up/in/down	Sense/antisense	Serine/threonine protein-kinase
<i>RASSF3</i>	2	7.2	0.25/1	In	Sense/antisense	Ras association domain-containing protein
<i>EPAS1</i>	2	17.4	0.51/1	Up	Antisense	Hypoxia-inducible factor 2-alpha
<i>HS1BP3</i>	2	32.2	0.73/1	In/down	Antisense	HCLS1-binding protein
<i>PTPN21</i>	2	46.5	0.85/1	Up/down	Antisense	Protein tyrosine phosphatase
<i>RIC1</i>	2	37.4	0.78/1	Up/in	Antisense	RAB GEF complex partner
<i>SLC27A1</i>	2	12.1	0.38/1	In/down	Sense	Fatty acid transporter

^aTwo approximate calculations were carried out representing lower/upper bounds.

^bIn, inside the gene; up, upstream of the gene; down, downstream of the gene.

^cBidirectional, proviruses with LTRs pointing in opposite direction due to provirus rearrangements. Slashes denote variance between individual tumors.

ranging from 1 to 11 proviruses per tumor. Positions in the chicken genome were reliably assigned for 325 VISs (see Table S1 in the supplemental material); sequences in the remaining 19 VISs were repetitive, too short, or missing in the chicken genome assembly. None of the VISs found corresponded to any of the VISs identified previously in virus-producing cell lines used for the induction of tumors.

Among VISs, we identified four major CISs with very high statistical significance (P values below 10^{-30}). Each major CIS was situated inside or just upstream of a well-known cancer gene: *HRAS*, *EGFR*, *MET*, or *RON*. These genes were hit in 16, 21, 20, and 7 independent tumors, respectively, with clusters of insertions ca. 14 kb, 3 kb, 1 kb, and 16 kb wide (Table 1 and Fig. 2; see also Table S1). Concurrent integration in two or more major CISs was not found in any tumor, and in 12 tumors (14%), no major CIS was found to be hit (Table 2; see also Table S2).

There was a clear (yet not absolute) correlation between the mutated gene and tumor type: *HRAS* mutation was found only in HHSs and *MET* mutation only in ICCs, while mutated *EGFR* and *RON* were found in HCCs, ICCs, or combined HCC/ICCs (Table 2; see also Table S2). It is clear that the percentage of both different tumor types and affected cancer genes is influenced by tumor promotion. The effect of repeated liver injury by CCl₄, which specifically boosts ICCs with provirus insertion in *MET*, is most evident (Table 2).

In addition to the four major CISs, we also identified one minor CIS hit in three tumors (*AKT1* locus), and six minor CISs hit twice in our set of tumors (Table 1; see also Table S1). However, the statistical significance of these recurrences was very low, even for the three hits into the *AKT1* locus. Additionally, there was no increase in *AKT1* mRNA levels in two of the tumors for which RNA was available (Fig. 3), implying that *AKT1* likely played no role in the pertinent tumors.

Modes of cancer gene deregulation: overexpression and gene truncation. Next, we examined the expression of the identified cancer genes using CIS-specific reverse transcription-PCR (RT-PCR) that detected either all gene-specific mRNAs or only hybrid mRNAs containing sequences of both the MAV-2 LTR and a candidate cancer gene. The RT-PCR confirmed recurrent provirus integration into the major CISs and demonstrated significantly increased expression of the candidate cancer genes from the affected loci (Fig. 4).

The positions of VISs and the structure of provirus-induced transcripts, revealed by the sequencing of iPCR and RT-PCR products as well as further detailed mapping of the provirus structure (V. Pecenka, P. Pajer, V. Karafiat, and M. Dvorak, unpublished data), allowed us to propose detailed mechanisms for the activation of individual cancer genes (Fig. 2, 4, and 5). In the *HRAS* locus, all provirus insertions occurred inside introns upstream of the second or third (the first coding) exon. All closely analyzed activating proviruses were heavily rearranged, including large deletions, duplications, and inver-

FIG 2 Clusters of insertions in major CISs. The exon-intron structure of candidate cancer genes in major CISs and position of provirus insertions are shown. Open and solid rectangles represent noncoding and coding exons, respectively; transcriptional orientation is set from left to right. Bent arrows with tumor numbers indicate individual integration sites and orientation of the provirus LTR inferred from iPCR and RT-PCR results. Bidirectional arrows reflect the fact that some proviruses (nearly all in the *EGFR* locus) are rearranged and contain LTRs oriented in opposite directions. The arrow marked “ALV/erythroblastosis” in the *EGFR* locus shows a typical provirus integration site in RAV-1-induced chicken erythroblastosis (49). Asterisks mark the position of initiation codons; the asterisk in parentheses marks the *RON* internal AUG codon expected to be used as an alternative start of translation in the MAV-2/*RON* fusion mRNA. Sequences retained in retrovirally transduced oncogenes *v-erbB* and *v-sea* (41, 44) are indicated by gray rectangles above the genes. TM, transmembrane.

sions. Insertions resulted in the overexpression of hybrid MAV-2/*HRAS* mRNA containing the R-U5 part of the proviral 3' LTR and downstream host sequences. Commonly, a cryptic splice donor downstream of the LTR was used to adjoin the first downstream *HRAS* exon. Complete *HRAS* coding sequences, including the start codon, were preserved. No mutations were found in the entire coding sequences of several LTR-driven *HRAS* transcripts (data not shown). This suggests that normal HA-ras protein was overproduced in these tumors. The arrangement of activated *MET* resembled the situation in the *HRAS* locus: defective proviruses integrated within the promoter region or in the first intron, upstream of the second (the first coding) exon, and drove the overexpression of fusion mRNA encoding full-length *MET* protein.

A different mode of activation was found for the *EGFR* locus. All proviruses were rearranged similarly to those in the *HRAS* locus and had integrated within a narrow region in the middle of the gene. Insertions resulted in the truncation of *EGFR* exons coding for the N-terminal ligand-binding extracellular domain and triggered the high

TABLE 2 Correlation of tumor histotypes, insertionally mutated genes, and mode of tumor promotion

Mode of promotion ^a	Major CIS	No. of tumors ^b	Histotype
Virus only	<i>EGFR</i>	3/9	HCC
	<i>HRAS</i>	4/9	Not done
	<i>MET</i>	0/9	
	<i>RON</i>	0/9	
	None	2/9	Not done
Cells	<i>EGFR</i>	20/57	HCC
	<i>HRAS</i>	18/57	HHS
	<i>MET</i>	6/57	Not done
	<i>RON</i>	8/57	ICC + HCC/ICC
	None	5/57	HCC
Cells + CCl ₄	<i>EGFR</i>	3/26	ICC
	<i>HRAS</i>	0/26	
	<i>MET</i>	15/26	ICC
	<i>RON</i>	1/26	HCC
	None	6/26	ICC + HCC/ICC

^aBrown Leghorn and White Leghorn chickens were grouped together; the differences between the two strains (where comparison was possible) were not statistically significant. Similarly, tumors promoted by different types of cells were combined since the differences in outcome were not significant.
^bMost of the smallest nodules were analyzed either histologically or molecularly, not both; only molecularly analyzed samples are included.

expression of mRNA encoding only the C-terminal regions. Respective mRNAs were initiated at the proviral 5' LTR and processed by splicing between the MAV-2 donor site and the first downstream *EGFR* exon. The coding region of the spliced mRNAs consisted of the first six Gag codons (including the start codon) fused in-frame to sequences encoding transmembrane plus cytoplasmic EGFR domains.

The *RON* activation in some aspects paralleled the *EGFR* activation: the insertions of defective proviruses occurred in the middle of the gene and truncated the exons encoding the receptor's extracellular ligand-binding domain. However, the proviruses were mostly positioned inside exons (the chicken *RON* gene has uncommonly short introns), the mRNA expression was driven by the 3' LTR, and its translation rested on the internal in-frame *RON* AUG codon.

DISCUSSION

The strong oncogenicity of MAV-2. MAV-2 is a unique entity among simple retroviruses. Though it does not contain a transduced oncogene, it induces tumors very

FIG 3 Provirus insertions in the *AKT1* locus. (A) Exon-intron structure of *AKT1* and position of provirus insertions. The design and symbols are as in Fig. 2. (B) Semiquantitative *AKT1*-specific RT-PCR. A pair of the primers specific for *AKT1* kinase domain exons was used to detect all *AKT1* mRNAs. No increase in *AKT1* expression can be seen in samples carrying provirus insertions in the locus (marked by arrows).

FIG 4 Transcriptional activation of candidate cancer genes. Representative results of semiquantitative CIS-specific RT-PCR are shown. For each candidate cancer gene, two types of RT-PCR were carried out: RT-PCR detecting integration-specific hybrid mRNAs, marked "int." (using a primer specific for the provirus against a primer specific for the gene's exon downstream of the VIS cluster), and RT-PCR revealing all transcripts of the gene, labeled "total" (using primers specific for the downstream exons). For *EGFR* and *RON*, expression of the 3' part of the gene (downstream exons) and 5' part of the gene (upstream exons) are separately displayed, labeled "C-term." and "N-term.," respectively. Strong overexpression of one of the four genes is evident in most of the samples (indicated by boxes). In the case of *EGFR* and *RON* activation, only the 3' part of the genes was overexpressed as a result of intragene integrations. Neither integration-specific products nor the genes' overexpression can be seen in samples with the suffix LiN (for nontumor livers from infected animals; indicated by the large dotted box). The length variation of integration-specific products reflects the variable position of individual integration sites; occasional multiple bands reflect alternative splicing of provirus-adjacent chicken intronic sequences. The primers specific for chicken β -actin (*ACTB*) were used to check the amount and quality of RNA. All RT-PCRs of the same type were carried out in parallel; the products were run on two separate gels, and negative gel pictures were spliced into one image. For the sake of clarity, the samples were rearranged to be in the same logical order in all parts of the image.

efficiently, with a latency of less than 2 months. Moreover, in contrast to most other simple retroviruses, which cause predominantly hematopoietic tumors, MAV-2 induces tumors of the kidneys, lungs, and liver. Throughout its genome MAV-2 is highly homologous to other ALVs (15). Only one short region has a completely dissimilar

FIG 5 Modes of insertional activation of candidate cancer genes. The structure of MAV-2 provirus plus sites of start (bent arrow), splicing (dotted lines), and termination [poly(A)] of its transcript are shown (upper drawing). The activity of the 3' LTR promoter in a nondefective provirus is blocked by transcriptional interference (30). The mechanism of formation of hybrid MAV-2/cancer gene mRNAs is suggested (lower drawings). Wavy and dashed lines represent chicken and rearranged proviral sequences, respectively. Efficient activation of the adjacent gene is achieved either through deletion/rearrangement of the 5' part of the provirus (thus releasing the 3' LTR promoter from transcriptional interference) or by deletion of the 3' LTR [thus eliminating proviral poly(A) signal]. The structures of many rearranged gene-activating proviruses are even more complex; e.g., most of the *EGFR*-activating proviruses carry additional viral sequences upstream of the 5' LTR that contain the 3' LTR oriented in the opposite direction. Detailed data on provirus defects are available (Pecenka et al., unpublished). SD, intronic cryptic splice donor site (or the authentic one in the case of *RON*).

sequence: the U3 part of the LTR, containing a promoter plus enhancer. By testing virus chimeras, this very U3 region was shown to impart distinct oncogenic abilities to MAV-2 (16).

Three types of induced liver tumors and effects of tumor promotion. Though MAV-2-induced liver tumors were macroscopically mostly indistinguishable, they comprised three tumor classes derived from different target cells: hepatic hemangiosarcomas (HHSs), originating from endothelial cells; intrahepatic cholangiocarcinomas (ICCs), arising from bile duct epithelial cells; and hepatocellular carcinomas (HCCs), coming from hepatocytes. Using their histological patterns (V. Karafiat, unpublished data), they could be matched to specific subtypes and/or stages of human or animal tumors described in the literature. HHSs resembled progressed high-grade human and animal hemangiosarcomas (17, 18). The HCC-like lesions were mostly high-grade dysplastic nodules, late preneoplastic stages through which HCC must pass; only ca. one-third fulfilled the criteria of early HCC in humans (19). Among the ICCs there was a continuum of stages from microscopic preneoplastic foci (these were not included in the analysis) to large nodules resembling the human mass-forming ductular type of ICC (20).

The penetrance of liver tumors in MAV-2-infected animals was rather low (ca. 15%). To obtain sufficient number of samples for thorough analysis, we used two types of proven tumor-promoting treatments: injection of nontumor cells into the bloodstream (3) and chronic liver injury by carbon tetrachloride (21). We must stress here that the promoting effect of injected cells was not based on faster or more effective virus dissemination via virus-producing cells. Our experiments (unpublished data) showed, counterintuitively, that the virus spread after the injection of cells was delayed compared to that in infection with virions. Nevertheless, in both cases the virus dissemination was quite aggressive. By 3 days postinfection (dpi) (virions) or ca. 4 dpi (cells), the virus had spread into all organs, reaching a plateau of a few proviruses per cell (depending on the organ). The plateau then did not change noticeably until the animal's death, which suggests the establishment of strong superinfection resistance. Moreover, the same promoting effect as via the injection of virus-producing cells was obtained when chicken embryos were first infected with virions and then after hatching (9 days later) were injected with cells (3). Importantly, the injected cells did not proliferate (or probably even survive) in the tumors, since we could not detect even traces of their progeny using very sensitive PCR assays (3). Thus, they would not interfere with the search for CISs.

We must also stress that using tumor promotion is not just a convenient experimental tool but also a natural component of tumorigenesis, including in humans. In particular, liver oncogenesis is highly dependent on nongenetic factors initiated by chronic liver insult, such as viral or other types of infection, fibrosis, and chronic inflammation, that trigger wound healing processes and disrupt tissue homeostasis (22). Importantly, the tumors examined in our experiments contained the same activated cancer genes and had the same histological appearance whether they were induced with or without promotion, suggesting that they had originated from the same pool of MAV-2-primed transformed cells.

Nevertheless, promotion acted on different tumor types with very different efficacies (Table 2; see also Table S2). Avian species have a strong natural predisposition to ICCs (23), while in mammals, the most frequent liver tumors are HCCs (24, 25). Without the help of promotion, however, MAV-2 seemed to induce mainly HCCs and HHSs (unpublished data). Cell promotion not only increased the number of HCCs and HHSs but also led to the appearance of ICCs. Treatment with CCl₄ very selectively promoted ICCs, which was rather unexpected since in mouse models CCl₄ promotes only HCCs and not ICCs (21).

Four CISs with very high statistical significance. Four major CISs were identified among 325 VISs (Table S1), characterized by a very high statistical significance given by a high number of independent insertions and a narrow cluster of insertions (Fig. 2). Each major CIS could be unequivocally associated with a single gene encoding a

member of classical signaling cascades and known to be causally linked to cancerogenesis: the small signaling GTPase *Hras* and the receptor tyrosine kinases *EGFR*, *MET*, and *RON* (Table 1). All insertions into major CISs were situated either in the gene's promoter region upstream of the coding sequences or in a narrow region inside the gene itself (Fig. 2).

Altogether, 86% of tumors contained provirus insertions into one of the major CISs, while in 14% of samples no CIS could be identified (Table 2; see also Table S2). However, the failure to detect an insertion into any major CIS does not imply the absence of such an insertion and is in fact expected considering the limitations of our screening technique (see Materials and Methods).

Avian retroviruses can integrate into essentially any locus in a host genome in a quasirandom fashion, though their preferences for individual loci vary (26–28). Unlike gammaretroviruses and lentiviruses, however, they do not form clusters of insertions (29). Thus, the remarkable recurrence of insertions into four major CISs must be a consequence of oncogenic selection: insertion into the CIS endowed the cell clone with the ability to outgrow other clones and form a tumor. This conclusion is further supported by the correlation between the gene and tumor histotype: *HRAS* activation was found only in HHSs, *MET* only in ICCs, *EGFR* (with three exceptions) in HCCs, and *RON* (with a single exception) in ICCs or combined HCC/ICCs (Table 2; see also Table S2). Further strong evidence lies in the fact that no tumor carried two or more insertions into major CISs (either the same or different). If the CISs were simply hot spots of integration, many tumors would harbor more than one CIS insertion, given the presence of multiple proviruses in nearly all tumor clones (3.7 on average) and the high proportion of tumors carrying one CIS insertion (86%).

Yet another conspicuous phenomenon corresponds well with the notion of oncogenic selection of cellular clones with a specific type of provirus integration. Nearly all proviruses that had integrated into the major CISs were highly defective, while in the great majority of cases the “non-CIS” proviruses were nondefective, suggesting that only defective proviruses were able to drive the efficient overexpression of affected cancer genes. Details of this phenomenon are available (Pecenka et al., unpublished).

Considering provirus positions and orientations, all insertions into CISs corresponded to the promoter insertion mechanism where the transcription of a resident cellular gene is driven by the inserted proviral promoter (30). No case of enhancer insertion (where provirus regulatory sequences boost, at a distance, the gene's transcription from its own promoter) or the formation of a recombinant oncogene-transducing retrovirus was observed.

Limited relevance of minor common sites of integration. Besides the major CISs, there were also seven minor ones: loci hit independently in two or three tumors (Table 1). Based on *P* values, even when assuming a fully random choice of integration sites, the probability that genes residing in minor CISs are involved in oncogenesis is not significantly higher than the probability for any other cellular gene. For the most promising minor CIS, the *AKT1* locus, the proviruses were scattered throughout a very wide region (86 kb, upstream, inside, and downstream of *AKT1* [Fig. 3]), which would imply different mechanisms of activation in each case (promoter insertion, gene truncation, and enhancer insertion). Most importantly, the integrations had no effect on *AKT1* expression (Fig. 3), nor did they lead to the formation of MAV-*AKT1* fusion mRNAs (data not shown). Insertions in *AKT1* occurred in different tumor histotypes together with insertions in *HRAS*, *MET*, and *RON* (Table S1 and Table S2). This could implicate *AKT1*, a frequent cancer gene in human tumors (31), as driving chicken liver tumor progression. However, all three respective lesions were in the smallest size category.

Mechanisms of cancer gene activation: a strong resemblance to human tumors. Two modes of cancer gene activation operated in chicken liver tumors. Insertions in the *HRAS* and *MET* loci did not affect the genes' coding regions but rather caused the overexpression of mRNAs encoding normal proteins (Fig. 4). In contrast, proviruses in the *EGFR* and *RON* loci were positioned in the middle of the genes, thus separating 5' exons from the rest of the genes. Inserted proviruses drove the high expression of

truncated receptors lacking all or most of their ligand-binding extracellular domains. Such truncated forms of receptors display defective downregulation and constitutive kinase activity even in the absence of a ligand (32–34).

These mechanisms of cancer gene activation mimic the mechanisms found in other animal models as well as in humans. The typical mechanism of *RAS* genes oncogenic activation in tumors consists of point mutations. However, the overexpression of wild-type (WT) Ras proteins has also been shown to have a transforming effect (35) and was found to be one of the oncogenesis-related alterations both in model organisms (insertional activation in mice [36]) and in human tumors (WT *RAS* gene amplification [35] or protein stabilization [37]). Similarly, the overexpression of WT *MET* was shown to be oncogenic (38), and gene amplification or overexpression in the absence of amplification was observed in several human tumor types (39). Recently, *MET* was found as a recurrent target of insertional mutagenesis in ALV-J-induced chicken hemangiomas (40).

Insertional activation of *EGFR* has already been described for ALV-induced chicken erythroblastosis where the receptor's extracellular domain was found to be truncated exactly the same way as in MAV-2-induced hepatocarcinomas (41). An analogically truncated *EGFR*, so-called *EGFRvIII*, in which exons 2 to 7 are deleted, is the most common oncogenic form of *EGFR* in several types of human tumors (34).

Similarly, a truncated oncogenic form of *RON* is the major gene product in some types of human tumors: so-called *sRON* and its protein product *RONΔ55* (33, 42, 43). *sRON* formation results from an epigenetic switch of a transcription start to an alternative promoter in exon 10 (32). Its translation starts at an internal Met codon in exon 11 at the position equivalent to the start of translation of insertionally activated *RON* in chicken cholangiocarcinomas. The truncated chicken *EGFR* and *RON* genes also closely imitate the structure of the transduced acute viral oncogenes *v-erbB* and *v-sea*, respectively (*erbB* and *sea* are the historical names of the chicken orthologs of *EGFR* and *RON*, respectively) (41, 44).

Conclusion. MAV-2-induced liver tumors likely originate from cells in which the provirus-driven constitutive overexpression of either normal (*HRAS* and *MET*) or truncated (*EGFR* and *RON*) forms of cancer genes propels oncogenic transformation. Tumor formation is strongly reliant on tumor-promoting conditions. This model might be a useful tool for investigating the interplay between genetic and nongenetic factors during tumor progression. Several features make the model very convenient: short tumor latency, easy searching for the involved insertionally activated genes, the simple and uniform genetic landscapes of tumors, the possibility of analyzing several different tumor types, and high sensitivity to (and in fact dependence on) nongenetic factors.

MATERIALS AND METHODS

Animals, cells, and viruses. Outbred Brown Leghorns and inbred congenic CB and CC White Leghorns (45) were used. MAV-2 was the MAV-2(N)-type virus isolated from the AMV-BAI-A complex stock by plaque purification (46). Chicken embryo fibroblasts (CEFs) were prepared from outbred Brown Leghorn or inbred White Leghorn (line CB) embryos and infected with MAV-2 in culture. Semistable MAV-2-producing clonal fibroblastoid cell lines originated from the kidneys of MAV-2-infected CB embryos (A210 and B211) or from the lung of an adult MAV-2-infected CB chicken (P4) (3).

Tumor induction. Two hundred 12-day-old chicken embryos were injected with cell-free MAV-2 stock (4×10^6 PFU) or with 1×10^5 MAV-2-producing cells (CEFs or A210, B211, or P4 cells) into the chorioallantoic vein. Injection of virus-producing cells coupled virus infection and injection of tumor-promoting cells and had previously been shown to have the same tumor-promoting effect as stepwise injection of virions and cells (3). One-third of the cells-injected animals were treated with repeated intraperitoneal administrations of carbon tetrachloride (20% solution in olive oil, 2 μ l/g of animal weight, twice a week for the first 3 weeks posthatching), a procedure known to strongly promote liver cancerogenesis in mice (21). The animals were sacrificed and examined at 15 weeks posthatching, or earlier when they showed signs of wasting. Macroscopically discernible liver nodules with sizes between 1 and 30 mm were resected for histological analysis and isolation of DNA and RNA.

All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (47) and approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

Isolation of DNA and RNA was done as described previously (3).

TABLE 3 Primers used in this study^a

Primer type and target	Sequence or position	
	Forward primer	Reverse primer
iPCR primers		
Target sequence		
Right flank	CCTGGGTTGATGGCCGGACCG (R)	TAAGACTACATTTCCCCCTCCCTATGCAAAAGC (3')
Left flank	AAGGCTTCATTTGGTGACCCGACG (5')	GGTGCATCAGGCGATTCCCTTATTTGG (L)
Both flanks	R	L
RT-PCR primers		
Target mRNA		
Total <i>HRAS</i>	GCGTCTCCATGACCGAGTACAAGCTGGTG (HRAS E3+)	CGTCGACACCTCTCCAAGTCAGG (HRAS E6–)
Fusion <i>HRAS</i>	R	HRAS E6–
Total <i>MET</i>	CCTGGACAGGCTGGTGAGTGCAAG (MET E14+)	CCACTGGACAGAATTGGGGAGAGGTC (MET E15–)
Fusion <i>MET</i>	R	MET E15–
Total 5' <i>RON</i>	CCACCCTCAGTGGCTTCGTCTTCG (RON E4+)	CAGGCTGGTGGCAGCAGGAGCTG (RON E6–)
Total 3' <i>RON</i>	TACTACAGCATCCGGCAGACCG (RON E16+)	GCAGGTAGCGGGCCATGTCGTAG (RON E17–)
Fusion <i>RON</i>	R	RON E17–
Total 5' <i>EGFR</i>	TGTGCAATGGCATTGGAATAGGTGAAC (EGFR E8+)	GCTGCTTGGTTTCGGCTCGGATA (EGFR E10–)
Total 3' <i>EGFR</i>	TATCGTGCGAAGCGCACCCCTG (EGFR E16+)	CCAGCAAGCGGCACACATGAGGA (EGFR E19–)
Fusion <i>EGFR</i>	R	GCCTGGTTTGGTGCCTCCCCACTG (EGFR E17–)
Total <i>AKT1</i>	ACTATGGTCGTGCAGTGGACTGGTG (AKT E11+)	CTTGGCATCATCAGGACCGCCTC (AKT E12–)
Fusion <i>AKT1</i>	R	AKT E12–
Control <i>ACTB</i>	CCGTAAGGATCTGTATGCCAAC (ACTB+)	GTGTGGGTGTTGGTAACAGTCC (ACTB–)

^aThe positions of iPCR primers are shown in Fig. 1A. The positions of RT-PCR primers are indicated by the exon numbers according to Fig. 2 and 3, with their orientations indicated by the plus and the – signs. All sequences are in the 5'-to-3' orientation.

Inverse PCR and isolation of junction fragments. One microgram of genomic DNA was digested with the BstYI and BclI restriction enzymes, circularized with T4 DNA ligase in a volume of 500 μ l, and linearized with enzymes cutting in the middle of the LTR (ApaLI, Eco81I, or SphI; three different enzymes were used alternatively to avoid systematically missing CISs that contain an array of target sites for individual enzymes). Each reaction mixture was purified by phenol-chloroform extraction and isopropanol precipitation between individual steps. One hundred nanograms of the final product was used as the template for PCRs under the following conditions: 1 U of *Taq* polymerase plus 0.005 U of DeepVent polymerase (New England BioLabs, Beverly, MA) per 20- μ l reaction mixture, LongAmp buffer supplemented with 500 μ M deoxynucleoside triphosphates (dNTPs), and 500 nM primers. The total Mg^{2+} concentration was adjusted to 2.5 mM with $MgSO_4$. PCR cycles were as follows: 95°C for 20 s, 22 cycles of 95°C for 20 s and 65°C for 12 min. There were then 4 additional steps: 95°C for 20 s, 65°C for 18 min, 95°C for 20 s, and 65°C for 30 min. Three separate PCRs with different pairs of primers were run for each tumor DNA providing 5' LTR junction fragments (primers L and 5'), 3' LTR junction fragments (primers R and 3'), and both (primers L and R) (Fig. 1 and Table 3). The PCR products were resolved overnight in 3% NuSieve GTG agarose (Lonza, Basel, Switzerland) in Tris-borate-EDTA (TBE) supplemented with 50 mM sodium acetate. Individual bands were excised, placed into 100 μ l of 100 mM KCl–0.1 mM EDTA, and melted for 5 min at 70°C. The mixed melted samples (0.5 μ l) were reamplified in 10- μ l reaction mixtures under the same conditions as described above except using 8 plus 2 cycles instead of 22 plus 2. The reaction mixture was purified with ExoSAP IT (USB, Cleveland, OH) before sequencing.

RT-PCR. cDNA was synthesized as described previously (3). CIS-specific RT-PCR was performed using GoTaq polymerase (Promega, Madison, WI) according to the manufacturer's instructions. For fusion *MET* mRNA detection, AccuTaq polymerase (Sigma, St. Louis, MO) was used. The standard cycling protocol was 25 cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, or for 2 min in the case of fusion *MET* mRNA detection). The PCR products were resolved in 2% NuSieve GTG agarose in TBE; for sequencing, individual bands were excised, reamplified, and purified as described above.

For the primers, see Table 1.

DNA sequencing and homology searches. Sequencing reactions were performed using the BigDye terminator cycle sequencing kit (PE Biosystems, Warrington, England) and resolved in an ABI Prism 310 sequencer. Primers L and R were used for sequencing 5' and 3' junction fragments, respectively. Sequence homology searches were conducted using the BLAT algorithm in the chicken genome assembly database (Ensembl, Gallus_gallus-4.0).

Efficiency of VIS retrieval. In ca. 14% of the analyzed tumors, no insertion into any of the four major CISs was detected by iPCR. We suggest that in a majority of these cases the CIS insertions were present but were missed for several possible reasons. We have tested and confirmed that both highly GC-rich sequences and long stem-loop structures are no obstacle for PCR under our conditions (very long initiation/elongation steps at decreased temperature). Some junction fragments, however, were certainly lost due to the presence of recognition sites for the linearization enzymes ApaLI, Eco81I, and SphI in the region to be amplified. Since the average frequency of their target sites in the chicken genome is ca. one per 6 kb while the average size of BstYI+BclI-generated fragments is ca. 800 bp, about 13% (800/6,000) of junction fragments will be lost in this way. In the case of nondefective proviruses where both the 5'

and 3' junction fragments could be detected, only ca. 1.7% (0.13%) of VISs would escape detection. However, as a result of extensive rearrangement, the great majority of cancer gene-activating proviruses had only one LTR-containing junction fragment, while the other LTR was missing or unconnected to a genomic sequence (buried inside the rearranged provirus structure). Furthermore, ca. 3% of iPCR fragments are expected to be longer than 5 kb, the size limit of iPCR under our conditions. Finally, 21 out of 234 unique VISs (ca. 9%) could not be unambiguously aligned since the host part of the iPCR product was too short or the VIS was located in a gap of the genomic assembly or in highly repetitive sequence.

The CIS-specific RT-PCR detected fusion mRNA in all samples where insertion into a given CIS was demonstrated by iPCR. However, ca. 10% of samples analyzed only with CIS-specific RT-PCR failed to detect the activation of any of the four major cancer genes. We presume this was mostly due to the nontumor nature of the relevant samples since the same proportion (ca. 10%) of samples repeatedly produced no distinct iPCR fragments, indicating the absence of any clonal cell population. These samples came from the smallest liver nodules; consequently, they were considered to be fibrotic or regenerative lesions (48) and excluded from further analysis.

Our iPCR procedure could detect only VISs contained in major clones and subclones; insertion sites present in less than ca. 10% of tumor cells could not be retrieved. We do not consider this a big drawback, since incorporating very small clones into the analysis would go against the logic of an insertional screen for cancer genes (to find genes that when activated lead to cell clone overgrowth and tumor formation). This is especially true when taking into account that between the day of infection and the day of dissection, each liver cell formed a clone of many cells that could form a cluster. A simplified calculation based on the increase in the liver size suggests ca. 100 cells per clone on average; if the clone size distribution is uneven (e.g., due to the existence of a small number of founder cells in the embryonic liver), some clones could be much bigger.

Statistical analysis. The statistical significance of encountering proviruses integrated into the same genomic locus in independent tumors was calculated based on an assumption of fully random integration. Since an exact calculation would be extremely complicated even with this simplification, we instead formulated equations for the upper and lower bounds of probability for each CIS cluster. When calculating P_{lower} , we divided the chicken genome of length L into nonoverlapping windows of length l (the size of the cluster in base pairs) and calculated the probability with which at least n (number of hits in the cluster) out of N (total number of proviruses) hit the same window. This value obviously underestimates the real probability of random genesis of the cluster since it disregards clusters formed over the boundary between adjacent windows. P_{upper} was constructed similarly except that we counted all possible windows of length l (consequently, each random cluster was counted many times).

The resulting equations were as follows:

$$P_{\text{lower}} = 1 - (1 - P_1)^{L/l}; \text{ for } P_1 \text{ close to zero, } P_{\text{lower}} \sim -L/l \times \ln(1 - P_1)$$

$$P_{\text{upper}} = 1 - (1 - P_1)^L; \text{ for } P_1 \text{ close to zero, } P_{\text{upper}} \sim -L \times \ln(1 - P_1)$$

where P_1 is the probability that at least n proviruses out of N land in one particular window of length l and

$$P_1 = \sum_{k=n}^N \binom{N}{k} \left(\frac{l}{L}\right)^k \left(1 - \frac{l}{L}\right)^{N-k}$$

where $N = 325$ and $L = 1.3 \times 10^9$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00467-17>.

SUPPLEMENTAL FILE 1, XLS file, 0.6 MB.

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We declare that we have no competing interests.

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