Correction. In the article “Avian carcinoma virus MH2 contains a transformation-specific sequence, mht, and shares the myc sequence with MC29, CMII, and OK10 viruses” by Nancy C. Kan, Christos S. Flordellis, Claude F. Garon, Peter H. Duesberg, and Takis S. Papas, which appeared in number 21, November 1983, of Proc. Natl. Acad. Sci. USA (80, 6566-6570), the authors wish to clarify the following. The authors referred to the recent finding of a high degree of relationship (identity) between transforming sequences of the avian retrovirus MH2 (the mht gene) and the murine retrovirus 3611-MSV (the raf gene) as “unpublished data.” The mht sequence is unpublished data of the authors, and the raf sequence is unpublished data of G. Mark and U. Rapp (Laboratory of Viral Carcinogenesis, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21701). The sequence comparison was made by J. Lautenberger.

Correction. In the article “Amiloride reduces the taste intensity of Na⁺ and Li⁺ salts and sweeteners” by Susan S. Schiffman, Elaine Lockhead, and Frans W. Maes, which appeared in number 19, October 1983, of Proc. Natl. Acad. Sci. USA (80, 6136-6140), the authors request that the following acknowledgment be added. “F.W.M. was supported by grants from the Netherlands Organization for the Advancement of Pure Research ZWO and from the National Science Foundation to Dr. R. P. Erickson.”
Avian carcinoma virus MH2 contains a transformation-specific sequence, mht, and shares the myc sequence with MC29, CMII, and OK10 viruses

(acute leukemia virus/oncogene/recombinant DNA)

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ABSTRACT Avian carcinoma virus MH2 has been grouped together with MC29, CMII, and OK10, because all of these viruses share a transformation-specific sequence termed myc. A 5.2-kilobase (kb) DNA provirus of MH2 has been molecularly cloned. The complete genetic structure of MH2 is 5'-Δgag(1.9-kb)-mht(1.2-kb)-myc(1.3-kb)-Δenv(?) and noncoding c-region (0.2-kb)-3'. Δgag, Δenv, and c are genetic elements shared with nondefective retroviruses, whereas mht is a unique, possibly MH2 transformation-specific sequence. Hybridizations with normal chicken DNA and cloned chicken c-myc DNA indicate that the mht sequence probably derives from a normal cellular gene that is distinct from the c-myc gene. The genetic structure of MH2 suggests that the Δgag and mht sequences function as a hybrid gene that encodes the p100 putative transforming protein. The myc sequence of MH2 appears to encode a second transforming function. Therefore, it seems that MH2 contains two genes with possible oncogenic function, whereas MC29, CMII, and OK10 each carries a single hybrid Δgag-myc transforming gene. It is remarkable that, despite these fundamental differences in their primary structures and mechanisms of gene expression, MH2 and MC29 have very similar oncogenic properties.

On the basis of a common nucleotide sequence termed myc, the avian carcinoma virus Mill Hill 2 (MH2) has been classified with three other replication-defective, oncogenic avian retroviruses MC29, CMII, and OK10 into the MC29 subgroup (1-4). The oncogenic properties of these viruses, in particular those of MH2 and MC29, are very similar: both MH2 and MC29 induce liver and kidney carcinomas and rarely leukemias in the animal and transform fibroblasts and hematopoietic cells in culture (4-9).

The transforming gene of MC29 has been shown to be a genetic hybrid composed of a partial, retroviral structural gene, Δgag, linked to the myc sequence of MC29 (10, 11). The Δgag-myc gene of MC29 encodes a p110 [110 kilodalton (kDa)] transforming protein. Similar hybrid gag-myc proteins are thought to be encoded by CMII and OK10 (3, 4, 12). Because MH2 also encodes a gag-related protein of similar size, p100 (7), it was assumed that the MH2 p100 protein was a structural homolog of the MC29 transforming protein. Nevertheless, recent analyses have suggested that the p100 protein is not serologically related to the myc portion of the p110 protein of MC29 (unpublished data) and that the myc sequence of MH2 may be expressed via a subgenomic mRNA (13, 14) to encode a 57-kDa protein (13).

Here we have analyzed the genetic structure of a molecularly cloned MH2 DNA provirus. This led to the identification of a MH2-specific sequence, mht. The genetic structure of MH2 suggests that the p100 protein is encoded by a Δgag-mht hybrid gene. Thus, it seems that MH2 contains two possible transforming genes, Δgag-mht and the gene that includes the myc sequence. It follows that, despite their similar oncogenic properties, the onc genes of MH2 and MC29 have fundamentally different structures.

MATERIALS AND METHODS

Source of MH2 Virus and Proviral DNA. High molecular weight genomic DNA was extracted from a MH2-transformed nonproducer quail cell line. MH2 viral RNA was prepared from virus produced by the same cell line superinfected with MC29-associated virus A. These cell lines were generously provided by M. Linal (8, 13).

Nucleic Acid Hybridizations. Southern blot analyses were performed as described (15, 16). Oligonucleotide analyses of the MH2 genome were carried out as described by Duesberg and Vogt (1). Heteroduplex molecules were formed by denaturing DNA fragments with alkali and renaturing in 50% formamide solution at 30°C for 30 min as described (17).

Molecular Cloning of MH2 Proviral DNA. MH2-transformed nonproducer DNA was cleaved with EcoRI and fractionated on a sucrose gradient. The fraction containing the 6.5-kilobase (kb) fragment that hybridized to cloned MC29 proviral DNA was used to ligate to AgWES-AB arms (18). A population of phage produced from the ligation reaction was screened for MH2 proviral sequences as described (16).

RESULTS

Molecular Cloning of the Integrated Proviral Genome. To detect the integrated provirus in the cellular genome, MH2-transformed nonproducer DNA was cleaved with the restriction enzyme EcoRI or BamHI and hybridized to radioactive probes prepared from cloned MC29 proviral DNA (ref. 16; see Fig. 2b). As shown in Fig. 1, lane b, the 2.9-kb probe containing both gag and myc sequences of MC29 hybridized to two EcoRI fragments 15 and 6.5 kb in size. When the probe was dissected into gag- and myc-containing pieces, the 6.5-kb EcoRI fragment was shown to hybridize to both the gag- and myc-containing probes, whereas the 15-kb EcoRI fragment was shown to hybridize only to the myc-containing probe (Fig. 1, lanes d and f). Southern blot analysis of BamHI digests of the non

Abbreviations: kb, kilobase(s); kDa, kilodalton(s); LTR, long terminal repeat.

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These data were shown in Fig. 2b. The Xho I site within the myc sequence of MH2 is due to a single-base mutation at position 2,900 in MC29 DNA (11) (unpublished data). However, between the Pst I sites in MH2 there was an extra sequence of about 1.2 kb, which was not present in the genome of MC29. Furthermore, the restriction pattern of this fragment did not correspond to that of either the gag or pol sequences of Rous sarcoma virus (RSV) (22), indicating that the extra sequence in MH2 was unrelated to these viruses. We refer to this MH2-specific sequence as mht.

**Heteroduplex analysis.** Heteroduplex molecules were formed between the 6.5-kb EcoRI fragment containing the proviral genome of MH2 and the 9.1-kb EcoRI fragment of MC29 proviral DNA (Fig. 2c and d) (16). Two forms of hybrid molecules were observed. The linear form (Fig. 3a) revealed a single-stranded loop about 1.5 kb in length, signaling a MH2-specific sequence, probably mht, that maps between the Δgag and myc sequences shared with MC29 DNA. To the 5' side of the loop, the two molecules formed a perfect hybrid. Based on the known location of the 5' LTR in the MC29 clone (11, 16), the 5' end of the 6.5-kb EcoRI-resistant MH2 DNA clone must be at or within (unpublished data; see Fig. 2d) the 5' LTR of the MH2 provirus. To the 3' side of the loop there was a forked structure at the end, indicating that the env sequence in the 9.1-kb MC29 clone was not present in MH2. The formation of a circular structure between MH2 and MC29 DNA (Fig. 3b) is consistent with the joining of the 5' LTR of MC29 with the 3' LTR of the MH2 clone, as diagramed in Fig. 3b. Thus, the MH2 clone starts probably at an EcoRI site within the 5' LTR and terminates at an EcoRI site of a cellular DNA sequence downstream of the 3' LTR. It would appear then that the 3' LTR lacks the EcoRI site present in the 5' LTR. These results are in good agreement with the restriction enzyme analysis.

**Oligonucleotide analysis of the Δgag, mht, and myc regions of MH2.** Δgag region: Because the gag genes of avian tumor viruses are highly conserved (4) and the numbers and sequence locations of gag oligonucleotides in RSV are known (22), RNA from any nondefective avian tumor virus hybridized by cloned MH2 DNA should give an estimate of the complexities of the MH2-specific gag region. Fig. 4A shows the RNase T1-resistant (T1) gag oligonucleotides of avian sarcoma virus PRCI and PRCII-associated virus hybridized by the 3.3-kb Xho I-Xho I DNA fragment of MH2 that contains the Δgag, mht, and myc regions (Fig. 2c and d). The partial sequence of these T1 oligonucleotides was determined as described (1, 23), and the positions of these T1 oligonucleotides in the known gag sequence of RSV (22) were identified. The numbers in Fig. 4A are the initial sequence positions in the gag gene of RSV (22). The 3'-most oligonucleotide identified—i.e., at position 1,748—locates the 3' border of the Δgag sequence in MH2 at about 1.75 kb. This is a minimal estimate, because other gag oligonucleotides closer to the 3' end may be present as unresolved species in the map shown in Fig. 4A. It follows that the defective gag gene of MH2 terminates within the p12-coding region of the gag gene.

**mht region:** To identify T1 oligonucleotides of the MH2-specific mht region, a MH2 RNA-DNA hybrid was formed under conditions that blocked hybridization with gag and myc sequences by competitive hybridization with MC29 RNA (Fig. 4B). The composition of the oligonucleotides in the hybrid is reported in Table 1. Several of these (numbers 1-4, 6a,b, and 7a,b) have been diagnosed as MH2-specific (ref. 1). Competition experiments with OK10 RNA (Fig. 4C) and CMII RNA

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**Fig. 1.** Southern blot analysis of integrated MH2 proviral genome in quail DNA. MH2-transformed quail nonproducer DNA was cleaved with BamHI (lanes a, c, and e) and EcoRI (lanes b, d, and f), size-fractionated on a 1% agarose gel, and transferred onto a nitrocellulose filter by the method of Southern (19). The filter was hybridized with nick-translation DNA fragments in 35% formamide solution at 37°C for 36 hr and then washed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7, at 65°C for 1 hr. In lanes a and b, the 2.9-kb BamHI-BamHI fragment of MC29 (ref. 16; Fig. 2b) was used as the probe. The 2.9-kb fragment was cleaved with Pst I, and the 3' Pst I-BamHI fragment (1.5 kb) containing only the myc sequence was used in lanes c and d, whereas the 5' BamHI-Pst I fragment (1.2 kb) containing only the gag sequence was used in lanes e and f. Values are given in kb.
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**FIG. 2.** Comparison of the major structural features between MC29 and MH2 proviral genomes. Restriction enzyme maps of MC29 and MH2 proviral genomes are shown in b and c, respectively. The restriction sites shared by both viruses are listed below the maps, whereas the sites unique to each virus are listed above (b) and below (c) the maps. MH2 DNA contains an extra sequence of 1.4 kb between the two PsiI sites. Genetic maps of MC29 and MH2 are shown in a and d, respectively; 1.2 kb of the extra sequence in MH2 is designated mht. The reading frame of myc gene in MH2 is assumed to be the same as that in MC29. The dotted lines in MH2 5' long terminal repeat (LTR) represent sequences not present in the cloned 6.5-kb EcoRI fragment.

(data not shown) gave essentially the same results. It is concluded that most or all of these oligonucleotides represent the mht sequence and that the mht sequence is not present in three other viruses of the MC29 subgroup.

**myc region:** To define the myc region of MH2, a hybrid of MH2 RNA with a 10-kb BamHI-resistant region of the cellular proto-myc DNA, mcv3 (20, 21), was analyzed. After mapping, the sequence of each MH2 myc oligonucleotide was superimposed on the known sequence of MC29 (11) and then labeled by the sequence position of its MC29 equivalent. It can be seen in Fig. 4D that MH2 RNA contains identical or closely related oligonucleotide sequence equivalents of most MC29 and cellular myc oligonucleotides up to the position defined by oligonucleotide 3,353 that extends to position 3,367 in MC29 DNA (11, 20, 21).

Because the 3' junction of myc and proto-myc is at position 3,629 in MC29 DNA (11, 21), the following competition-hybridization experiment was carried out to determine whether the 3'-terminal 250–300 myc nucleotides of MC29 were missing in MH2. A molar equivalent of proto-myc DNA was first hybridized with 5 molar equivalents of MH2 RNA and then with a 0.3 molar equivalent of MC29 (32P labeled) RNA. The T1-resistant hybrid was then mapped. The experiment directly confirmed that the MC29 myc oligonucleotides 3,350–3,386 in the 3' region are not subject to competition by MH2 RNA and hence have no sequence equivalents in MH2 RNA (Fig. 4E). We conclude that MH2 shares most of all of the 5' 1.3 kb but lacks the 3' 0.3 kb of the myc sequence of MC29. Because the region that includes the 5' translation-termination codon (TAG) of the MC29 myc region (11) is still included in the region shared with MH2 (Fig. 2), it would appear that both viruses contain similar myc coding domains.

There is one MH2-specific oligonucleotide, number 4, that was hybridized by proto-myc clone mcv3 (Fig. 4D) but was not present in MC29, OK10, or CMII virus, based on competition hybridizations (Fig. 4B and C) and previous analyses (24). This oligonucleotide could be at the 5' or 3' end of the mht sequence, because the BamHI-resistant mcv3 region extends about 3 kb over the 5' and 3 kb over the 3' boundary of the proto-myc gene and thus includes non-myc, perhaps proto-mht, information.

**DISCUSSION**

We have cloned the integrated proviral genome of MH2. The size of the DNA provirus is 5.2 kb and its complete genetic structure is 5'-Δgag(1.9-kb)-mht(1.2-kb)-myc(1.3-kb)-Δenv(?)-noncoding c-region (0.2-kb)-3'. Thus, MH2 differs from MC29, CMII, and OK10 in its unique mht sequence. Hybridization with MC29, PRCII, and RSV (not shown) indicated that mht is not related to these onc genes. However, preliminary data suggest that mht is closely related to the specific sequence of an oncogenic murine retrovirus (v-raf) (25) (unpublished data). Among the sequences shared with MC29, the 5' noncoding and Δgag region of MH2 (1.9 kb) is larger than that of MC29 (1.7 kb) (11), and the myc region of MH2 lacks about 250 nucleotides at its 3' end compared to MC29.

A recent study (14) has indicated that the genetic structure of the MH2 genome is 5'-Δgag-Δenv-myc-c-3', whereas the structure of a MH2 variant was thought to be similar to that
hybridized with hybridized 

of MH2 equivalents sequence first 

myc 

diagnosed by their digestion of and 

and 

4.27 ± 0.22 kb; b, 1.88 ± 0.09 kb; c, 1.63 ± 0.11 kb; d, 1.34 ± 0.09 kb; e, 2.24 ± 0.14 kb; f, 1.03 ± 0.08 kb. (B) (n = 10 molecules) a, 4.12 ± 0.35 kb; b, 1.88 ± 0.06 kb; c, 1.71 ± 0.07 kb; d, 1.22 ± 0.03 kb; e, 0.31 ± 0.01 kb; f, 1.0 ± 0.10 kb; g, 1.5 ± 0.14 kb. 

described in this report. Because the MH2 viral RNA hybridized by our molecularly cloned MH2 proviral DNA (Fig. 4B) shares several mht-specific oligonucleotides with the standard MH2 strain analyzed previously (Fig. 4 B and C, Table 1) (1), we feel that the genetic structure described here is that of standard MH2.

Two lines of evidence indicate that the MH2-specific sequence, mht, is derived from a normal cellular gene. First, hybridization of EcoRI-digested normal chicken DNA with the mht sequence revealed two bands <6 kb and one band >10 kb (unpublished data). Because the chicken c-myc gene is contained in a single 15-kb EcoRI fragment (20), it is clear that the two small EcoRI fragments that hybridized to the mht sequence are different from the c-myc locus. Second, the 10-kb BamH1 clone that contains the chicken c-myc gene as well as about 3 kb of its 5' and 3 kb of its 3'-flanking cellular DNA (20, 21) did not hybridize to the mht sequence, except possibly one MH2-specific oligonucleotide—i.e., number 4 (Fig. 4D). However, further work characterizing the cellular mht prototype is necessary to delineate cellular mht and myc sequences.

Based on the genetic structure of the cloned MH2 provirus, we propose that the 5' 1.7-kb Agag region, together with about 1 kb of the adjacent mht region, function as a hybrid gene encoding the p100 protein of MH2. p100 is thought to be the transforming protein of MH2 (4, 7), and the finding of an mht-related sequence in an oncogenic murine retrovirus is consistent with this view. The myc region of MH2 appears to be part of a second viral gene. Although our data do not rule out the possibility that Agag and myc function together to encode p100 via a spliced mRNA, the following arguments favor the first proposal. (i) In all retroviral genes encoding gag-related transforming proteins of the Agag–x design, the Δagag and x sequences map adjacently on the viral genome (4). (ii) We have detected many translation termination codons present in all three reading frames in a region 100 nucleotides 5' to the mht–myc junction (un-

FIG. 3. Electron micrographs of typical heteroduplex molecules formed between MH2 0.5-kb and MC29 9.1-kb EcoRI DNA fragments. Contour length measurements of various segments are expressed as mean kilobases ± SD. Internal calibration standards were derived independently by using single- and double-stranded pBR322 molecules present in the same field as heteroduplex molecules. (A) (n = 35 molecules) a, 4.27 ± 0.22 kb; b, 1.88 ± 0.09 kb; c, 1.63 ± 0.11 kb; d, 1.34 ± 0.09 kb; e, 2.24 ± 0.14 kb; f, 1.03 ± 0.08 kb. (B) (n = 10 molecules) a, 4.12 ± 0.35 kb; b, 1.88 ± 0.06 kb; c, 1.71 ± 0.07 kb; d, 1.22 ± 0.03 kb; e, 0.31 ± 0.01 kb; f, 1.0 ± 0.10 kb; g, 1.5 ± 0.14 kb. 

FIG. 4. RNase T1-resistant oligonucleotides of the gag, mht, and myc sequences of carcinoma virus MH2. (A) A 3.3-kb Xho I-resistant fragment of cloned MH2 DNA, which spans Δgag–mht–myc (see Fig. 2 c and d), was hybridized for 12 hr at 50°C in 70% formamide containing 0.35 M NaCl and 20 mM Na phosphate with 32P labeled RNA of avian sarcoma virus PRCII and PRCH-associated virus containing a complete gag gene. After digestion of unhybridized RNA with RNase T1, the T1-resistant hybrid was isolated and mapped as described (20). gag oligonucleotides were first diagnosed by their RNase A-resistant sequence and then labeled by their sequence positions in the complete gag sequence of RSV (22). (B) MH2-specific mht oligonucleotides that are unrelated to MC29 and OK10 viral RNAs. About 1 µg of the 3.3-kb Xho I–Xho I MH2 DNA fragment was first hybridized with 10 molar equivalents of MC29 and ring-necked pheasant virus (RPPV) RNAs and then challenged with a 0.3 molar equivalent of MH2 32P labeled RNA (106 cpm/µg). The composition of T1 oligonucleotides is listed in Table 1. (C) Same as B except that MH2 DNA was hybridized with 10 molar equivalents of OK10 RNA. (D) The myc oligonucleotides of MH2. MH2 32P labeled RNA (4 × 106 cpm; 106 cpm/µg) was hybridized with 3 molar equivalents of a 10-kb BamHI-resistant region of cellular myc DNA, mc3 (20), and mapped as for A–C. The myc oligonucleotides were first diagnosed by their RNase A-resistant sequences and then labeled by their sequence positions in the complete nucleotide sequence of MC29 (11). (E) The myc oligonucleotides of MC29 that are not present in MH2. About 1 µg of proto-myc DNA was hybridized with 5 molar equivalents of MH2 RNA and then with a 0.3 molar equivalent of MC29 32P labeled RNA and the hybrid was mapped as described (B–D). The myc oligonucleotides of the map were diagnosed and labeled by their sequence positions in the complete sequence of MC29 virus (11).
Table 1. MH2-specific (mht) oligonucleotides

<table>
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<th>Number</th>
<th>RNase A-resistant fragments</th>
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<tr>
<td>1*</td>
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</tr>
<tr>
<td>2*</td>
<td>4U, 6C, G, AC, AU, 2AaC, AaU</td>
</tr>
<tr>
<td>3*</td>
<td>4U, 4C, G, 3AU, 2AU, AaU</td>
</tr>
<tr>
<td>4*</td>
<td>2U, 2C, G, 7AU, AaU, AaU</td>
</tr>
<tr>
<td>5</td>
<td>U, AG, AaU, AaU</td>
</tr>
<tr>
<td>6a</td>
<td>2U, 3C, AC, AU, AaC, 2aG</td>
</tr>
<tr>
<td>6b</td>
<td>3C, AC, 2AU, AG, AaU</td>
</tr>
<tr>
<td>7a</td>
<td>U, 4C, G, AC, AU, AaU</td>
</tr>
<tr>
<td>7b</td>
<td>2U, 2C, G, AU, 2AaC</td>
</tr>
<tr>
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</tr>
<tr>
<td>21</td>
<td>4C, AaG</td>
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*Oligonucleotide numbers 1, 2, 3, 4, 6a, b, and 7a, b were referred to as 4a, 6a, 3, 1, and 11a, b,c in a previous analysis (1).

Indicates the presence of two or more oligonucleotides.

published data). (iii) In preliminary experiments, sera containing antibodies against mcyt determinants failed to precipitate p100 of MH2 but precipitated p110 of MC29. (iv) It has been suggested that the mcyt sequence of MH2 is expressed as a 57-kDa protein via a spliced subgenomic mRNA (13) rather than as a Δagg-mhc hybrid protein as in MC29. Thus, it appears that MH2 contains two genes with possible oncogenic function, the Δagg-mht gene and the gene that contains the mcyt sequence. However, whether these two genes must cooperate or whether only one of them is responsible for the oncogenic properties of MH2 requires further investigation. Preliminary evidence indicates that our MH2 proviral clone transforms chicken embryo fibroblasts upon transfection together with myeloblastosis-associated virus DNA. Future analysis of mutated MH2 DNA would indicate whether both the mht and mcyt genes of MH2 are necessary for its transforming function. Avian erythroblastosis virus is also thought to contain two genes with oncogenic function, Δagg-erb and erb. Recent studies have suggested that the erb gene is sufficient for neoplastic transformation and that the Δagg-erb gene may have only an ancillary function in oncogenicity (26).

Considering the fundamental differences in their primary structures and mechanisms of gene expression, it is remarkable that MH2 and the other viruses of the MC29 subgroup nevertheless have very similar oncogenic properties.

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