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Retention or Loss of v-*mil* Sequences after Propagation of MH2 Virus In Vivo or In Vitro

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During propagation of the defective avian retrovirus MH2 in the presence of replication-competent helper virus, deletion of portions of the viral genome occurred frequently. After transformation of quail cells in vitro, v-*mil* sequences were lost, leading to populations of MH2 viruses which were highly deficient for *mil* gene expression but which could transform macrophage and fibroblast cells in vitro with high efficiency. In contrast, after induction of tumors in quail with *mil*-deficient MH2 viral stocks, a majority of the tumor DNAs contained *mil*$^+$ proviruses, suggesting that there is selection for retention of the v-*mil* gene in vivo and that the *mil* protein may play a role in the oncogenicity of MH2 virus. We also isolated MH2-transformed cell lines which contained deleted proviruses arising from packaging and subsequent integration of the subgenomic v-*myc*-encoding mRNA. Some of these cell lines produced viruses which encoded abnormal v-*myc* proteins and had altered in vitro transforming properties. These altered phenotypes may be caused by mutations within the v-*myc* gene.

Many acute transforming viruses have been isolated and characterized. The biological properties of such viruses result from viral acquisition of unique sequences (oncogenes). The MC29 group of acute transforming avian retroviruses, which includes MC29, MH2, OK10, and CMII, all contain a transduced v-*myc* oncogene. MH2 is unique in the MC29 group of viruses because its genome contains a second cell-derived gene, v-*mil* (6, 23, 25, 26, 41). Although v-*mil* is homologous to v-*raf*, the sole transforming gene of murine sarcoma virus 3611 (23, 25, 26, 41), the v-*mil* gene alone cannot transform avian cells (2, 31). In MH2, the v-*mil* gene is expressed as a *gag-mil* fusion protein of 100 kilodaltons (P100) (5, 6, 19), while the v-*myc* gene is translated from a subgenomic 2.2-kilobase (kb) spliced mRNA to yield p61/63^{myc} (15, 34). MH2 causes endotheliomas and liver and kidney carcinomas and sarcomas in vivo and transforms fibroblasts and macrophages in vitro (4, 11). The other *myc*-containing avian retroviruses, i.e., MC29, CMII, and OK10, have biological properties similar but not identical to those of MH2 (11, 28). This difference in biological properties is presumably due, in part, to the presence of the oncogene v-*mil* (1, 2, 12) as well as to the numerous point mutations identified in the MH2 v-*myc* gene (10, 21, 25, 35).

Several in vitro-constructed MH2 deletion viruses and spontaneously occurring MH2 mutants have been isolated and characterized (2, 22, 28, 30, 34, 35, 48). MH2 viruses that are *mil*− *myc*$^+$ transform chicken and quail embryo cells and macrophages (22, 28, 34, 48), while both *mil* and *myc* sequences are required for transformation of chicken neuroretinal cells and chondroblasts (1, 2). *mil* sequences also play a role in MH2 transformation of chicken macrophages by stimulating production of chicken myelomonocytic growth factor, resulting in autocrine growth (12). Proviruses which appear to arise from reverse transcription and integration of the *myc*-encoding subgenomic RNA have also been isolated. These viruses transform quail embryo cells (32, 35).

We have previously described variants arising after propagation of MH2 in vitro which lack detectable levels of the v-*mil* protein. Such variants are still capable of full transforming activity in Japanese quail cells (28, 34). In particular, we characterized a *mil*-deficient viral isolate, MH2YS3 (28, 34). Analysis of cells transformed by MH2YS3 in vitro reveals no detectable P100$^{gag\text{-}mil}$ polyprotein, which is easily detectable in MH2 quail nonproducer cells (MH2QNP) (28). No P100-encoding viral clone can be isolated from the original MH2(MH2AV-A) population, suggesting that much of the transforming virus present has undergone genomic alterations resulting in *myc* but not *mil* expression. In the present study we characterized the proviruses present in a variety of transformed cell lines and tumor tissues induced by MH2 and found that the genome of MH2 frequently underwent genetic alterations, resulting in proviruses with unique structures, including deletions and point mutations. Although the v-*mil* gene was frequently lost from cells infected and maintained in tissue culture, analysis of primary tumors revealed that there was a strong selection for proviruses containing *mil* sequences in vivo.

MATERIALS AND METHODS

Viruses and tissue culture. The original stock of MH2(MH2AV-A) was obtained from P. Vogt. MH2QNP cells were derived from this viral stock by Hu et al. (19). MH2YS3 was isolated in this laboratory by growing the wild-type stock on quail macrophage cultures. MH2 viral clones were obtained by picking single foci under agar from quail embryo fibroblast (QEF) cultures infected with MH2YS3 (MH2 clones 26 and 28). Cell clones derived from MH2 28 cells were obtained by plating MH2 28 cells in soft agar at limiting dilution and picking individual soft agar colonies. MH2 28-BB and 28-U cell lines were derived by infection of quail peripheral blood macrophage cultures with MH2 virus and growth of individual transformed foci in medium containing quail primary-cell-conditioned medium (CM* [28]). MH2QNP cells (19) were obtained from J. Neil and P. Vogt. QEF were obtained from fertile Japanese quail eggs. Transformed fibroblasts were grown in GM+D (28); transformed macrophage cell lines were grown in CM as described (28).

Tumorigenicity assays. Newborn Japanese quail were injected with 0.1 ml of viral supernatant in the wing web. Tumors were removed aseptically, finely minced, and placed

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in 100-mm tissue culture dishes; tumor cells were grown in CM* (28). DNA was extracted from a portion of each tumor.

DNA analysis. DNA from tissue culture cells was prepared from nuclei by using proteinase K digestion and phenol-chloroform extraction in the presence of a high salt concentration (13). DNA from tumor tissue was extracted by subjecting the tissue to Dounce homogenization in 1% sodium dodecyl sulfate (SDS)–0.15 M NaCl–0.1 M EDTA (pH 8.0), followed by incubation at 60°C. After chloroform extraction, DNA was spooled from the ethanol, suspended in 10 mM Tris (pH 7.4)–1 mM EDTA–0.5% SDS, and subjected to proteinase K digestion. After phenol-chloroform extraction, tumor DNAs were ethanol precipitated. For Southern blot analysis, tumor and tissue culture DNAs were digested with restriction endonucleases (Boehringer Mannheim) in the appropriate buffer (7), size fractionated on 0.8% agarose gels in TBE buffer (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA), and transferred to nitrocellulose paper by using the method described by Southern (40). Nitrocellulose filters were prehybridized for at least 4 h in Stark buffer and hybridized with ^{32}P-labeled nick-translated probes in Stark buffer with 10% dextran sulfate for 16 to 24 h (13). Filters were washed at room temperature for 30 min in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and for 1 h at 65°C in 0.1× SSC–0.1% SDS, air dried, and exposed to X-ray XAR-5 film (Eastman Kodak Co.) at −70°C with intensifying screens.

cDNA analysis. Virion RNA was isolated from viral particles following concentration by ultracentrifugation as previously described (33). A cDNA library was synthesized from poly(A)$^+$ virion RNA isolated from MH2 28-Z virus by using murine leukemia virus reverse transcriptase (Life Sciences) and oligo(dT) (P-L Biochemicals) priming (36). The second strand was synthesized by using RNase H-generated primers and the Klenow fragment of DNA polymerase I (Boehringer Mannheim) (33). *Eco*RI linkers were added, and the cDNA fragments were inserted into the *Eco*RI site of λgt10 (20). Ligated, concatemeric DNA was packaged in vitro by using Gigapack (Vector Cloning Systems). Clones that were *myc* positive were isolated by using standard hybridization and plaque purification techniques (3). After positive clones were identified, bacteriophage particles were concentrated and purified from plate lysates by DEAE-cellulose (Whatman D-52) chromatography (17). Phage DNA was isolated by conventional precipitation methods (17) and subcloned into bacteriophage M13 derivative mp18 by using standard techniques (47). mp18m13 templates were prepared by using polyethylene glycol precipitation followed by phenol-chloroform extraction and ethanol precipitation (45). Nested deletions of DNA fragments cloned into mp18 were made by using the sequential exonuclease III and S1 digestion method of Henikoff (18). Dideoxy-sequencing reactions were carried out by using [^{35}S]dATP (New England Nuclear Corp.) essentially as previously described (45).

DNAs used as nick-translated probes. DNAs were nick translated by using standard techniques (37). The sources and characterization of probes used are as follows: *myc*, *Cla*I-to-*Eco*RI fragment (exon 3) of the chicken cellular *myc* gene cloned in pBR322 (29); 5′ long terminal repeat (LTR), fragment of the viral LTR containing the U5 and R regions and about 50 bases of U3 (L fragment [32]); *mil*, v-*mil*-containing plasmid pMH2-BH obtained from K. Bister (21); *pol*, SR-A RSV *Pst*I *pol* fragment in mp18 obtained from E. Hunter; and *env*, *env* gene-specific plasmid (p-env) obtained from E. Hunter.

Protein analysis. To examine viral proteins present in transformed cells, cells were radiolabeled with [^{35}S]methionine for 30 min at 37°C, immunoprecipitated, and size fractionated on SDS-polyacrylamide gels (39). Anti-*myc* sera were prepared in rabbits against a synthetic carboxy-terminal chicken *myc* polypeptide and were obtained from R. Eisenman (15).

RESULTS

Frequent deletion of *mil* sequences in vitro. In our previous studies with MH2, we found that it is difficult to detect P100$^{gag\text{-}mil}$ protein after infection of tissue culture cells with stocks of MH2 virus containing replication-competent helper virus (28, 34). P100$^{gag\text{-}mil}$ is easily detected in cell line MH2QNP, even after maintenance of these cells (which lack replication-competent virus) in continuous culture for several years. These cells were used in the original experiments of Hu et al. (19) to define the *gag* gene products of MH2. We therefore decided to reclone our MH2 stock in an attempt to isolate nonproducer cells containing proviruses which would remain stable after passage in tissue culture. MH2-transformed clones were derived from our P100-deficient viral stock by picking foci induced by infection of QEF cultures at limiting viral dilutions and growing these foci into cell lines. Southern blot analysis was done on these cell lines to determine the genetic composition of the MH2 proviruses (Fig. 1). We were unable to isolate any transformed lines lacking helper-virus genomes, presumably because of the large excess of helper virus in our stocks (approximately 1,000-fold excess). The cellular DNAs were digested with the restriction endonuclease *Kpn*I and hybridized with 5′ LTR, *myc*, or *mil* probes. *Kpn*I was particularly useful for mapping integrated MH2 proviruses, as the only proviral *Kpn*I sites are in the U3 region of the LTRs, allowing excision of the provirus as a single DNA fragment. We first determined the structure of the quail c-*myc* and c-*mil* genes after *Kpn*I digestion. Uninfected QEF DNA was digested with *Kpn*I and hybridized to *myc*, *mil*, and viral LTR probes (Fig. 1A). Hybridization to a *myc* probe (Fig. 1A, lane a) yielded a 5.4-kb c-*myc* fragment, while hybridization to a *mil* probe (lane b) yielded two c-*mil* fragments of about 3.2 and 10 kb. We occasionally detected other fragments with the *myc* probe; these additional fragments may represent other members of the *myc* gene family. A 5′ LTR probe (lane c) did not hybridize to any fragments in QEF DNA under the stringency conditions used. The structure of the wild-type *myc*$^+$ *mil*$^+$ MH2 provirus was determined by *Kpn*I digestion of MH2QNP cell DNA. MH2QNP cells contained a single 5.4-kb provirus detected with all three probes (Fig. 1A). Because the 5.4-kb proviral band comigrated with the c-*myc* band in these *Kpn*I digestions, double digestions with *Kpn*I and other restriction enzymes were done to demonstrate the existence of v-*myc* sequences in the proviruses (data not shown).

We looked at seven lines derived from MH2 infection and found two different types of proviruses. Most of the lines examined (six of seven) had the structure seen in MH2 26 cells (Fig. 1B), in which a 5.4-kb provirus was detected with the 5′ LTR probe (lane c). This probe also detected higher-molecular weight *Kpn*I DNA fragments from the helper-virus genomes present, which tended to obscure the MH2 provirus. Double digestions also revealed that the 5.4-kb *myc*$^+$ band contained v-*myc* sequences (data not shown). However, using the *mil* probe (lane b), we were unable to detect any *mil* sequences in the MH2 26 5.4-kb provirus. We

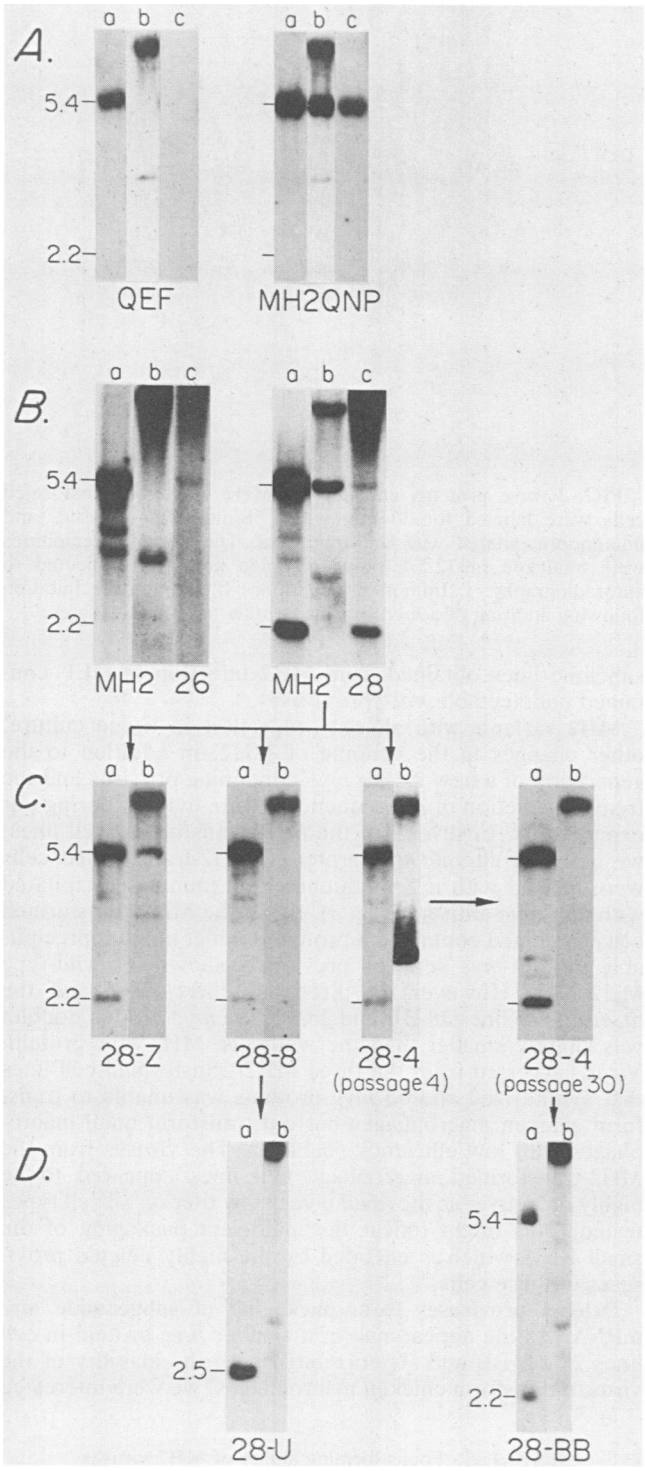


FIG. 1. Southern blot analysis of MH2-transformed cell lines. All DNAs were digested with *Kpn*I except for MH2 28-U (lane a in D), which was digested with *Eco*RI. DNAs were size fractionated on 0.8% agarose gels, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled probes. Lanes: a, Exon 3 chicken c-*myc*; b, v-*mil*; c, 5′ LTR. (A) QEF and MH2QNP. (B) MH2-transformed cells; viral clones MH2 26 and 28. (C) Cell clones of MH2 28-transformed cells. (D) Macrophage clones transformed by 28-8 virus (28-U) or 28-4 virus (28-BB).

estimated that less than 10% of the proviruses contained *mil* sequences. In one cell line, MH2 28, a 5.4-kb provirus could be detected with the *mil* probe as well as with the 5′ LTR probe (Fig. 1B, lanes b and c). Double digestion demonstrated that the 5.4-kb MH2 28 provirus also contained *myc* sequences (data not shown). Because the sizes of the proviruses were identical in the MH2 26 and 28 cell lines, the provirus in MH2 26 cells must have acquired sequences to replace the *mil* sequences. Since the 5.4-kb fragment did not hybridize to viral *env* or *pol* probes (data not shown), the origin of these replacement sequences appears to be cellular.

We also noted the appearance of a new proviral fragment of 2.2 kb, which contained 5′ LTR and *myc* sequences in several of the cell lines, including MH2 28 (Fig. 1B). This *Kpn*I fragment could be detected with both the *myc* and 5′ LTR probes (Fig. 1B) but not with the *mil* or *gag* probes (data not shown for the *gag* probe). Since *Kpn*I cuts only within the MH2 LTRs, this fragment most likely represents acquisition of a deleted MH2 provirus, which contains only LTR and *myc* sequences.

We were interested in whether cells containing only the 2.2-kb provirus could be isolated. To this end, cell clones of MH2 28 (which contained both the 2.2- and 5.4-kb proviruses) (Fig. 1B) were picked after plating MH2 28 cells in soft agar; 14 cell clones were analyzed for proviral content. Southern blot analysis of several of the clones is shown in Fig. 1C. Clone 28-Z was unique among the 14 clones examined. It resembled the original population of MH2 28-transformed cells and contained a 5.4-kb mil^+ myc^+ provirus and a 2.2-kb myc^+ provirus, which was also detected with an LTR probe (data not shown for LTR probe). Cell clone 28-8 resembled the majority of the 14 cell clones analyzed (11 of 14). These clones harbored both a 5.4-kb provirus that hybridized with the 5′ LTR (data not shown) and *myc* probes but not with the *mil* probe and a 2.2-kb proviral fragment. A 5.4-kb mil^- myc^+ provirus as well as the 2.2-kb myc^+ provirus was also detected in cell clone 28-4 (Fig. 1C). Interestingly, in clone 28-4 and in one other cell clone, we detected a 3.0-kb proviral fragment which hybridized to *mil* and *env* sequences (data not shown for *env* hybridization). This 3.0-kb mil^+ band was seen only in low-passage (passage 4) cells. After 26 additional passages in tissue culture (passage 30), the 3.0-kb *mil-env* proviral fragment disappeared from MH2 28-4 cells (Fig. 1C). These results indicate that the MH2 *mil* gene was frequently lost in vitro. None of the clones examined contained only the 2.2-kb *Kpn*I myc^+ fragment.

We also used MH2 28 viral clones to generate transformed macrophage lines. Two stably transformed quail macrophage cell lines were obtained. These lines, 28-BB and 28-U, were analyzed for MH2 proviruses (Fig. 1D). Unlike the fibroblast-derived cell lines, the macrophage cell lines lacked full-length 5.4-kb provirus detectable after *Kpn*I digestion with a 5′ LTR probe (data not shown). Clone 28-BB contained a 2.2-kb myc^+ provirus. No *mil* sequences other than endogenous c-*mil* could be detected. Macrophage clone 28-U contained no detectable provirus after digestion with *Kpn*I. After digestion with *Eco*RI, a 2.5-kb myc^+ provirus was detected (Fig. 1D). Since the MH2AV helper virus in our MH2 stocks contains *Eco*RI sites within its LTRs (unpublished data), a likely origin of this provirus was through recombination with helper virus. No *mil*-containing fragments other than endogenous c-*mil* could be detected with either *Kpn*I (Fig. 1D) or *Eco*RI (data not shown). Thus, in tissue culture, transformed macrophage cell lines could be

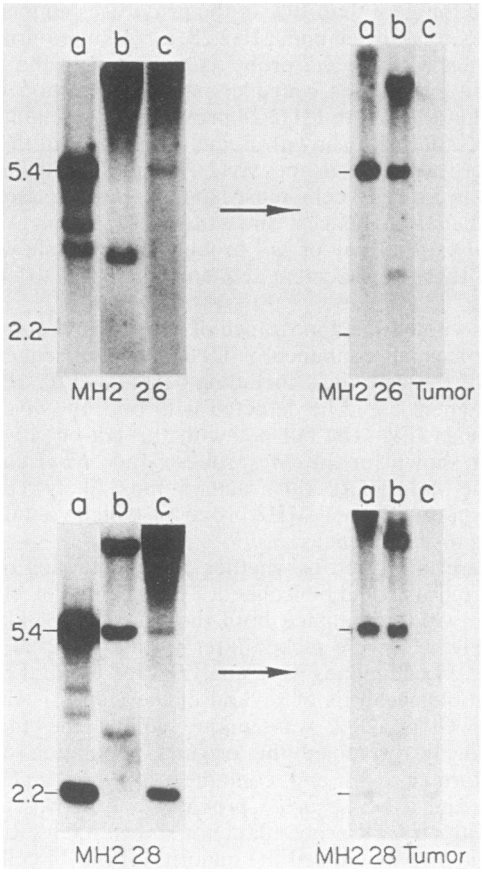


FIG. 2. Southern blot analysis of MH2-transformed tissue culture cells and primary tumor tissues. DNA was extracted from original tumor tissues or from tissue culture cells, and digested with *Kpn*I and subjected to Southern blot analysis as described in the legend to Fig. 1. Virus from the tissue culture cells was used for in vivo injections. Filters were hybridized to exon 3 chicken c-*myc* (a), v-*mil* (b), or 5′ LTR (c).

maintained indefinitely which contain only highly deleted MH2 proviruses.

Selection for the *mil* gene in vivo. To examine the effect of the MH2 *mil* gene in vivo, virus harvested from lines MH2 26 (*mil*$^-$) and MH2 28 (*mil*$^+$) was used to induce tumors in Japanese quail. Both virus stocks, as well as virus from other mutant *mil*$^-$ cell lines (MH2 24 and MH2 45), induced tumors efficiently with short latency periods (data not shown). These results corroborate earlier results (28, 34), which suggest that the *mil* gene is not required for efficient tumor induction by MH2. However, when we examined the tumors induced by both the MH2 *mil*$^+$ and *mil*$^-$ viral stocks, we detected a 5.4-kb *mil*$^+$ *myc*$^+$ provirus in each case. Four *mil*$^+$-induced tumors were analyzed and shown to contain a *mil*$^+$ provirus. One example is shown in Fig. 2. Thus, although we could not detect *mil*$^+$ proviruses in our original MH2 viral stock (data not shown) or in many of the viral subclones, such as MH2 26 (Fig. 1B), a subpopulation of proviruses containing the *mil* gene must persist in the MH2 26-transformed cells, as well as in cells transformed by other mutant *mil* subclones. Viral genomes containing the *mil* gene appear to be more highly oncogenic than mutant *mil*$^-$ genomes, since tumors preferentially arose from cells infected with *mil*$^+$ viruses. This is in contrast to the case of tissue culture infection, in which six of seven of the viral

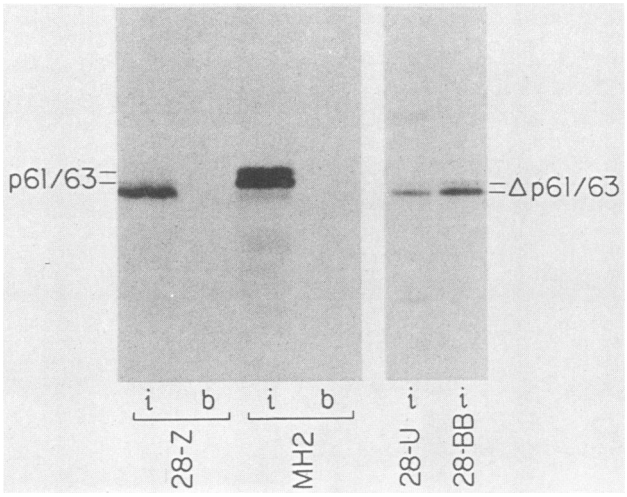


FIG. 3. *myc* proteins encoded by MH2 viruses. Transformed cells were labeled for 30 min with [^{35}S]methionine, lysed, and immunoprecipitated with anti-*myc* sera. The immunoprecipitates were analyzed in 12.5% polyacrylamide gels and subjected to autoradiography. i, Immunoprecipitation; b, immunoprecipitation following addition of a *myc*-specific peptide to the cell lysates.

subclone lines obtained from MH2 infection of QEF contained undetectable *mil*$^+$ proviruses.

MH2 variants with altered properties. In tissue culture, other changes in the genome of MH2, in addition to the generation of a new 2.2-kb *myc*-containing provirus and the frequent deletion of *mil* sequences, were evident during our experiments. In several of the MH2-transformed cell lines, we detected altered *myc* proteins. MH2-transformed cells were labeled with [^{35}S]methionine and immunoprecipitated with anti-*myc* antisera (Fig. 3). All of the MH2-transformed cells examined contained a protein doublet immunoprecipitable by anti-*myc* sera, as previously shown for wild-type MH2 (15). However, in three cell lines (28-Z and the macrophage lines 28-BB and 28-U), the *myc* protein doublet was slightly smaller than the wild-type MH2 *myc* protein. Virus harvested from the three MH2-transformed cell lines that synthesized altered *myc* proteins was unable to transform chicken macrophages but did transform quail macrophages with low efficiency (Table 1). The viruses from the MH2-transformed macrophage cell lines appeared to be highly defective, as they had a very low titer on all cell types tested. This might reflect the inefficient packaging of the small RNA which is encoded by the highly deleted proviruses in these cells.

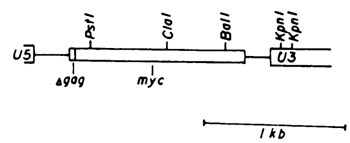
Deleted proviruses from packaging of subgenomic *myc* mRNA. As the appearance of a smaller *myc* protein in cell lines 28-Z, -BB and -U correlated with the inability of the virus to transform chicken macrophages, we were interested

TABLE 1. Focus-forming ability of MH2 viruses

MH2 virus	Focus-forming units/ml[a]			
	QEF	CEF	Qmϕ	Cmϕ
28-4	6,000	700	840	110
28-Z	9,000	900	75	<2
28-BB	42	<5	~10	<2
28-U	24	<5	~2	<2

[a] CEF, Chicken embryo fibroblasts; Qmϕ, quail macrophages; Cmϕ, chicken macrophages.

A



B

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TCCGCCGACCACTATTCCCTAACGGATCACGTCGGGGTCACCAAATGAAGCCTTCTGCTT      60

                            leader
CATTCGAGGTGTTCGCAATCGTTAGGGACTCAACGGTCGGCCACCAGGACGGCGGTGGCG     120

ATCCTGTCCTCATCCGTCTCGGCTTATTCGGGGAGCAGACGATGACCCTAGTAGAGGGGG     180

 packaging signal
CTGCGGCTTAGGAGGGCAGAACGCTGAGTGCCGTCGGAGGGAGCTCTACTGCAGGGAGCC     240

AACATACCCTACCGAGCACTCAGAGAGTCTTGGAAGACGGGAAGGAAGCCCGACGACTGA     300

                                                  gag
GCGGTCCACCCCAGACGCGGTTCTGGTCGCCCTGTGGATCAAGCCATGGAAGCCGTCATA     360
                                             MetGluAlaValIle
 myc
AAGGCAGCAGCCGCCGCGATGCCGCTCACCGTCAGCCTCCCCAGCAAGAACTACGATTAC     420
LysAlaAlaAlaAlaAlaMetProLeuThrValSerLeuProSerLysAsnTyrAspTyr
                           Ser                                   MH2

GACTACGACTCGGTGCAGCCCTACTTCTACTTCGAGGAGGAGCAGGAGAACTTCTACCTG     480
AspTyrAspSerValGlnProTyrPheTyrPheGluGluGluGlnGluAsnPheTyrLeu
                                             Glu          Ser    MH2

GCGGCGCAGCAGCGGAGCAGCGAGCTGCAGCCTCCAGCCCCGTCCGAGGACATCTGGAAG     540
AlaAlaGlnGlnArgSerSerGluLeuGlnProProAlaProSerGluAspIleTrpLys
                                                                 MH2

AAGTTTGAGCTCCTGCCCGCGCCGCCCCTCTCGCCCAGCTGCCGCTCCAACCTGGCCGCC     600
LysPheGluLeuLeuProAlaProProLeuSerProSerCysArgSerAsnLeuAlaAla
                                                                 MH2

GCCTCCTGCTTCCCTTCCACCGCCGACCAGCTGGAGATGGTGACGGAGCTGCTCGGGGGG     660
AlaSerCysPheProSerThrAlaAspGlnLeuGluMetValThrGluLeuLeuGlyGly
                                                                 MH2

GACATGGTCAACCAGAGCTCCATCTGCGACCCGGACGACGAATCCTTCGTCAAATCCATC     720
AspMetValAsnGlnSerSerIleCysAspProAspAspGluSerPheValLysSerIle
                                                                 MH2

ATCATCCGGGACTGCATGTGGAGCGGCTTCTCCGCCGCCGCCAAGCTGGAGAAGGTGGTG     780
IleIleArgAspCysMetTrpSerGlyPheSerAlaAlaAlaLysLeuGluLysValVal
                                                                 MH2

TCGGAGAAGCTCGCCACCTACAAAGCCTCCCGCCGGGAGGGGGACCCCGCCGCCGCCTCC     840
SerGluLysLeuAlaThrTyrLysAlaSerArgArgGluGlyAspProAlaAlaAlaSer
                                             Gly                 MH2

CGACCCGGCCCGCCGCCCTCGGGGCCGCCGCCTCCTCCCGCCGGCCCCGCCGCCTCGGCC     900
ArgProGlyProProProSerGlyProProProProProAlaGlyProAlaAlaSerAla
                                                                 MH2

GGCCTCTACCTGTACGACCTGGGAGCCGCGGCCGCCGGCTGCATCGGCTCCTCGGTGGTC     960
GlyLeuTyrLeuTyrAspLeuGlyAlaAlaAlaAlaGlyCysIleGlySerSerValVal
            His                                                  MH2

TTCCCCTGCCCGCTCGGCAGGCGCGGCCCGCCCGGCGCCGCGCTCGCGGCTCTGCTGGGG    1020
PheProCysProLeuGlyArgArgGlyProProGlyAlaAlaLeuAlaAlaLeuLeuGly
                              .           GlyPro                 MH2

GTCGACGCGCCGCCCACGGCCGGCGGCGGCTCGGAGGAAGAACAAGAAGAAGATGAGGAA    1080
ValAspAlaProProThrAlaGlyGlyGlySerGluGluGluGlnGluGluAspGluGlu
                     Val                                         MH2
 ClaI
ATCGATGTCGTTACATTAGCTGAAGCGAACGAGTCTGAATCCAGCACAGAGTCCAGCACA    1140
IleAspValValThrLeuAlaGluAlaAsnGluSerGluSerSerThrGluSerSerThr
                                                      Gly        MH2

GAAGCATCAGAGGAGCACTGTAAGCCCCACCACAGTCCGCTGGTCCTCCGAGCGGTGTCAC   1200
GluAlaSerGluGluHisCysLysProHisHisSerProLeuValLeuGluArgCysHis
                                                                 MH2

GTCAACATCCACCAACACAACTACGCTGCTCCTCCCTCCACCAAGGTGGAATACCCAGCC    1260
ValAsnIleHisGlnHisAsnTyrAlaAlaProProSerThrLysValGluTyrProAla
                                                                 MH2

GCCAAGAGGCTAAAGTTGGACAGTGGCAGGGTCCTCAAACAGGTCAGCAACAACCGAAAA    1320
AlaLysArgLeuLysLeuAspSerGlyArgValLeuLysGlnValSerAsnAsnArgLys
                                                                 MH2

TGCTCCAGTCCCCGCACGTCAGACTCAGAGGTGAACGACAAGAGGCGAACGCACAACGTC    1380
CysSerSerProArgThrSerAspSerGluValAsnAspLysArgArgThrHisAsnVal
                                                                 MH2

TTGGAGCGCCAGCGAAGGAATGAGCTGAAGCTGAGCTTCTTTGCCCTGCGGGACCAGATA    1440
LeuGluArgGlnArgArgAsnGluLeuLysLeuSerPhePheAlaLeuArgAspGlnIle
                                                                 MH2

CCCGAGGTGGCCAACAACGAGAAGGCGCCCAAGGTTGTCATCCTGAAAAGAGCCACGGAG    1500
ProGluValAlaAsnAsnGluLysAlaProLysValValIleLeuLysArgAlaThrGlu
                                                                 MH2

TACGTTCTGTCTATCCAATCGGACGAGCACAGACTGATCGCAGAGAAAGAGCAGTTGAGG    1560
TyrValLeuSerIleGlnSerAspGluHisArgLeuIleAlaGluLysGluGlnLeuArg
                                                                 MH2

CGGAGGAGAGAACAGTTGAAACACAAACTTGAGCAGCTAAGGAACTCTCGTGCATAGGAA    1620
ArgArgArgGluGlnLeuLysHisLysLeuGluGlnLeuArgAsnSerArgAla
                                                                 MH2

CTCTTGGACATCACTTAGAATACCCCAAACTAGACTCCGTGTGATAGCTGGTTGGATCGT    1680

TAATCGGACGGCTGGCACACGGAATGTAGGAGGTCGCTGAGTAAGTACGAACAAAATTTA    1740

CGTTGTAATAAGGTGAGACTTGACCTACAATTGTTCAAATAATGCTTCCTGTAGAAATGTT   1800

TAGCATTAGGCATCTTGCGCTGCTCCGCGATGTACGGGTCAGGTATAATGTGCAGTTTGA    1860

CTGAGGGGACCATGATATGTATAGGCGAAAAGGCGGGGCTTCGGTTGTACGCGGTTAAGG    1920

                                                     U3
GGTCCCCTCAGGATATAGTAGATACGCTTTTGCATAGGGAGGGGGAAATGTAGCCTTACA    1980

CAATAGCCTTACACAGTTATGTTATGTAACGATGAAACAGCAATACGCCTTATAAGGAGA    2040

GAAAGGGTACCGTGCATGATGATTGGTGGAAGTAAGGTGGTACCATCGCGCCTTATTAGG    2100

AATATAACAGACGGGTCTCACCGGGATTGGATAGACCGCTTAGTTCCGCATAGTAGAGAT    2160

GTTGTATTTAAGTAGCTAGCTTGATACAATAAACGCCATTTGACCA                  2205
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FIG. 4. Restriction map and sequence analysis of MH2 28-Z cDNA clones. (A) Restriction map of MH2 28-Z. (B) Sequence analysis of MH2 28-Z. Dideoxy sequencing reactions were performed by using [^{35}S]dATP on mp18 templates containing nested deletions. The sequence and amino acids are given for MH2 28-Z; MH2 amino acids are listed below the amino acid sequence of 28-Z.

in characterizing the molecular structure of virus produced by these cell lines. To do this, a cDNA library was synthesized from 28-Z virion RNA and screened for *myc*-containing clones. Three *myc*$^{+}$ cDNA clones were analyzed; these clones contained overlapping portions of the genome of MH2 28-Z. A composite sequence and restriction map are presented in Fig. 4. The genome of the 28-Z virus consisted of a v-*myc* gene preceded by six amino acids of the *gag* gene sequence, corresponding to the splice site in the *gag* gene (13, 38). The cDNA clone contained the leader sequences (14) encoding a packaging region required for packaging of avian retroviral RNA (27). The absence of the 5′-most portion of the U5 region from the clone was presumably due to incomplete cDNA synthesis. The 3′ end of the MH2 28-Z genome contained the untranslated sequences homologous to c-*myc* which are found in wild-type MH2 (9, 22, 24, 44) and U3 sequences (Fig. 4). The viral cDNAs we analyzed

have a structure very similar to the integrated 2.2-kb provirus characterized by Patschinsky et al. (35), with the exception of the U5 and U3 sequences. The cDNA clones of MH2 28-Z contained MH2-like U3 sequences, while the clone described by Patschinsky et al. (35) had LTR sequences more similar to those of Rous sarcoma virus Prague C strain. Our sequence analysis of the 28-Z cDNA clone derived from encapsidated MH2 RNA firmly establishes the role of subgenomic mRNA in the genesis of the deleted 2.2-kb provirus, as has been previously suggested (32, 35).

Although the *myc* proteins found in MH2 28-Z-transformed cells were smaller than those of the wild type, the sequence of the MH2 28-Z cDNA clone is nearly identical to the sequence of wild-type MH2 (25). The 28-Z v-*myc* gene contained the four amino acid deletions described for MH2 (25); however, no additional deletion was detected that could account for the lower molecular weight of the 28-Z protein. MH2 contains 27 amino acid substitutions and a 4-amino-acid deletion (25), compared with the c-*myc* gene (44). These amino acid substitutions and deletions are clearly not essential for the transforming activity of MH2, as another *myc*-containing avian retrovirus, CMII, contains only a single amino acid substitution (43). When compared with the MH2 sequence reported by Kan et al. (25), 28-Z has the following nine amino acid substitutions: Ser → Thr (position 9), Glu → Gln (position 29), Ser → Leu (position 34), Gly → Asp (position 149), His → Tyr (position 179), Gly → Ala (position 212), Pro → Leu (position 213), Val → Ala (position 225), and Gly → Ser (position 257) (amino acid numbers correspond to the c-*myc* sequence [44]). The amino acid substitutions at positions 34, 225, and 257 are the same as the amino acids in the MH2 provirus described by Patschinsky et al. (35). Several of the amino acid changes are conservative (positions 9, 29, 149, 212, and 257); positions 179 and 225 are neutral changes, while the other amino acid changes (positions 34 and 213) are nonconservative according to Dayhoff et al. (8). These amino acid changes could conceivably result in the molecular-weight shift of the v-*myc* protein as well as in the ability of the virus to transform chicken macrophages.

DISCUSSION

Our results demonstrate that the genome of MH2 was very unstable when propagated in the presence of a replication-competent helper virus. As can be seen in our analysis of MH2-transformed cells, the *mil* gene was frequently lost from the MH2 provirus in cells propagated in vitro in the presence of replication-competent helper virus, suggesting that there is no selective advantage for retention of *mil* sequences in tissue culture. In one cell line, MH2 28-4, a new *mil*-containing provirus was detected. However, this proviral fragment was rapidly lost from the genome of the transformed cells, again suggesting that there is no in vitro growth advantage for cells harboring a *mil* gene under LTR control (Fig. 1C). In many cell lines such as MH2 26, in which *mil* sequences could not be detected in the MH2 genome, full-length provirus was still present, as demonstrated by the hybridization of the LTR probe to a 5.4-kb fragment (Fig. 1B). To maintain the full length of the provirus after deletion of *mil* sequences, recombination must have occurred with additional cellular sequences, as no hybridization to the 5.4-kb provirus was seen with *env* and *pol* probes.

In contrast to the results obtained in vitro, examination of primary tumors induced in Japanese quail or examination of tissue culture cells shortly after explantation revealed v-*mil* sequences in a majority of the cells. This was seen even after induction of tumors by a virus such as MH2 26, which was harvested from tissue culture cells in which v-*mil* sequences could not be detected. Our estimate was that fewer than 10% of the viruses would derive from *mil*$^+$ genomes, yet most of the tumors examined contained full-length v-*mil*$^+$ proviruses. This is presumably a reflection of the persistence of a small number of *mil*$^+$ viruses in our virus stock and of their selection in vivo. This suggests that the *mil* gene product does play a role in rapid induction of MH2 tumors in Japanese quail. This result is somewhat surprising since we have shown that MC29, which contains v-*myc* as its sole oncogene, induces tumors in Japanese quail with a latency period identical to that of MH2 (B. Biegalke, M. Heaney, J. T. Parsons, and M. Linial, submitted for publication), although we cannot rule out the effect of the different LTRs in MC29 and MH2. An artificially constructed virus (JDmyc) containing the MC29 v-*myc* gene encoded by a spliced mRNA lacking the bulk of the *gag* determinants also is highly oncogenic (16; Biegalke et al., submitted). Thus, there was no reason to expect that retention of the v-*mil* gene should have occurred at such high frequencies when we started with viral stocks which contained so few *mil*$^+$ particles. The v-*myc* gene of MH2 contains 27 amino acid substitutions when compared with the chicken c-*myc* sequence, the most changes of any of the *myc*-containing avian retroviruses (25, 43). Thus, an intriguing possibility for the retention of the v-*mil* gene in in vivo transformation is that the numerous mutations have rendered MH2 more defective for transformation than the other v-*myc*-containing viruses, such as MC29. The work of Jansen et al. (22), who found MH2D12 virus, a *mil*$^-$ mutant of MH2, to be much less oncogenic than MH2 is, supports this possibility.

In addition to frequent loss of *mil* sequences, a new 2.2-kb provirus arose rapidly in tissue culture. This provirus, which contained LTR and *myc* sequences, could also be detected directly in some MH2-induced tumor tissues (Fig. 2). Our results strongly suggest that the 2.2-kb provirus arose from reverse transcription and subsequent integration of the subgenomic *myc* mRNA which was encapsidated in virions. In several of our transformed cell lines, the spliced *myc*-encoding mRNA was present in much greater quantity than the 5.4-kb genomic RNA was (34; unpublished data), and in these lines we could detect packaged subgenomic RNA in virions (unpublished data), although little or no subgenomic RNA could be detected in wild-type virions (34). Analysis of the MH2 28-Z cDNA clones directly revealed a structure compatible with integration of a reverse-transcribed mRNA. Similar conclusions have been reached by Patschinsky et al. (35) and Martin et al. (31) for spontaneous variants of MH2 which lack *mil* sequences. It has been recently shown that the genome of human T cell lymphotropic virus type III contains a sequence which appears to regulate splicing of the viral RNA (9). It is possible that other retroviral genomes contain similar elements and that derivatives of MH2 are defective in the putative splicing control element, thereby allowing extensive splicing and subsequent packaging of the spliced v-*myc*-encoding mRNA.

Graf et al. (12) have reported that MH2 requires both the v-*mil* and v-*myc* genes for stable transformation of chicken macrophages, presumably because the v-*mil* gene product is required for autocrine-stimulated growth of transformed chicken macrophages in vitro. This is in contrast to the situation we found for MH2 transformation of quail macrophages. We were able to establish stably transformed quail

macrophage cell lines by using MH2 *mil*$^-$ *myc*$^+$ viruses (cell lines MH2 28-U and 28-BB). These quail macrophage cell lines contained only the 2.2-kb *myc* provirus (Fig. 1D) and grew at low densities in the absence of growth factors (unpublished data). These results suggest that quail macrophages produce factors which act in *myc*-transformed quail cells to yield autocrine growth in a manner analogous to MH2 *mil*$^+$ *myc*$^+$-transformed chicken macrophages. Thus, MH2 v-*myc* alone appears capable of transforming quail macrophages.

MH2 28-Z, 28-BB, and 28-U cells synthesized a *myc* protein doublet which appeared smaller than the wild-type MH2 p61/63^{myc} on polyacrylamide gels (Fig. 3). It has been shown that high levels of v-*myc* expression may be deleterious to the growth of some cell types (42, 46). During the generation of MH2 cell lines, there might be selection for point mutations in v-*myc*, resulting in altered proteins such as those we have detected, which might be less toxic. Sequence analysis of MH2 28-Z cDNA clones revealed nine amino acid changes in the v-*myc* gene as compared with the amino acid sequence of the parental MH2 v-*myc* gene. Five of the amino acid changes were conservative, two were neutral, and two resulted in reversion to the amino acid sequence of c-*myc*. However, two nonconservative amino acid changes were observed, i.e., a proline to a leucine at position 213 and a serine to a leucine at position 34. Any or all of these amino acid changes could affect the protein structure of *myc*, resulting in a faster rate of migration on SDS-polyacrylamide gels. MH2 28-Z virus was unable to transform chicken macrophages, which could result from synthesis of the altered *myc* proteins. This is consistent with the observation that 28-U and 28-BB viruses, which also encoded smaller *myc* proteins, were unable to transform chicken macrophages, although the viruses were derived from quail macrophage cell lines. MH2 28-Z recombinant viruses are currently being generated to determine which amino acid changes are responsible for the altered mobility of the 28-Z v-*myc* protein and for the inability of the 28-Z virus to transform chicken macrophages.

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