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Characterization of a *myc*-Containing Retrovirus Generated by Propagation of an MH2 Viral Subgenomic RNA

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We have previously isolated, from wild-type MH2 virus that contains the two oncogenes *mil* and *myc*, mutants defective in one or the other oncogene product. We report here the molecular cloning and extensive characterization of MH2 CL25 provirus lacking the v-*mil* oncogene. Our results indicate that this virus corresponds to the propagation of the 2.8-kilobase subgenomic RNA of MH2l.

The avian retrovirus Mill-Hill 2 (MH2) is a replication-defective retrovirus that induces liver and kidney carcinomas in fowl and transforms fibroblasts, macrophages, chondroblasts, and neuroretina cells in culture (1, 2, 7). The genome of MH2 contains two cell-derived oncogenes: v-*myc*, also found in three other retroviruses (MC29, CMII, OK10) (19, 21), and v-*mil* (4, 12), related to the *src* gene of Rous sarcoma virus (RSV) (6, 13). In MH2-transformed cells v-*mil* is expressed as a 100-kilodalton polyprotein resulting from the fusion of *gag* and *mil* sequences (11, 17, 19). v-*myc* is expressed as a 61- to 63-kilodalton nuclear protein encoded by a 2.8-kilobase subgenomic mRNA (9, 17, 22).

We defined distinct effects of the *mil* and *myc* oncogenes by studying the properties of MH2-infected neuroretina cells. Chicken embryo neuroretina cells which normally do not multiply in vitro are induced to proliferate upon infection with MH2 but not with MC29, CMII, and OK10 (2, 3). From two distinct viral stocks of wild-type (wt) MH2 we isolated two mutants, MH2 CL16 and MH2 CL25, lacking the v-*mil* oncogene. These mutants failed to transform neuroretina cells or to induce their proliferation but retained the ability to transform avian embryo cells. To precisely define the genetic organization of these v-*mil*-defective mutants, we molecularly cloned a v-*myc*-containing provirus from MH2 CL25-transformed quail embryo cells. We present here data indicating that this mutant corresponds to the propagation of a retroviral particle containing subgenomic MH2 RNA species.

Molecular cloning of MH2 CL25 provirus. A transformed quail embryo cell clone containing MH2 CL25 provirus was isolated in soft agar and used as a source of DNA to construct a gene library. High-molecular-weight DNA was partially digested with *Eco*RI restriction endonuclease and ligated with purified lambda Charon 4A arms, packaged, and amplified as reported previously (22). Several probes were prepared: a long terminal repeat (LTR) probe was derived from a provirus of RSV, strain Schmidt-Ruppin A (RSV-SRA) (5). Fragments of the retroviral *gag* gene were derived from Prague strain (RSV-PrA) as previously described (22). Two *myc* probes were used: a 5′ v-*myc* probe (*Hpa*I-*Pst*I fragment derived from pMH2Hd [4]) and a 3′ c-*myc* probe representing the 3′ half of chicken exon 3 and described in reference 23. Agarose gel-purified fragments were labeled through nick translation reactions (20) (Amersham nick translation kit) in the presence of [^{32}P]dCTP according to the supplier's instructions. By use of LTR and *myc* probes, two lambda phages were isolated, purified, and amplified. From one of these phages, lambda MH2 CL25, we subcloned a 10-kilobase-pair (kbp) *Eco*RI fragment containing the complete provirus into the *Eco*RI site of pKH47 plasmid DNA (10), yielding plasmid pMH2-CL25. A detailed restriction map of the provirus is shown in Fig. 1.

The hybridization pattern obtained allowed the following observations. (i) The subcloned MH2 CL25 provirus contained a *Kpn*I restriction site within its LTRs as attested by the 2.2-kbp band hybridizing with LTR, *gag*, and *myc* probes. Such a band was also observed with the DNA of quail embryo cells transformed by the original isolate of MH2 CL25 pseudotyped by Rous-associated virus 1 (RAV-1) (Fig. 2). (ii) By *Sac*I digestion of the provirus, an internal 0.30-kbp restriction fragment was found that hybridized with both *gag* and 5′ *myc* probes (Fig. 1). This 0.30-kbp fragment replaced a 3.2-kbp fragment observed previously in the provirus of wt MH2 analyzed similarly, which in addition hybridized to a *mil* probe (4), indicating that in the MH2 CL25 mutant the *gag* and *myc* sequences had become proximal. Such a *gag-myc* junction could be explained by an extensive deletion of the *mil* sequences, or it could have resulted from the integration of a reverse-transcribed spliced subgenomic viral RNA. We thus analyzed the precise boundary between the *gag* and *myc* portions within the 0.30-kbp *Sac*I fragment. The fragment was purified on agarose gel, cleaved by *Hin*fI restriction endonuclease, and subjected to nucleotide sequencing by the Maxam and Gilbert procedure (15). Figure 1 presents our results, compared with the nucleotide sequence of the *gag* gene of RSV-SRA (24) (since the similar region in wt MH2 has not been sequenced before) and with the nucleotide sequence of the *myc* gene of wt MH2 (6, 13).

It appears that the *gag-myc* junction in MH2 CL25 occurred precisely between the splice donor site of the *gag* sequence and the splice acceptor site of the *myc* gene, bringing the open reading frames together in the correct phase. Therefore, the MH2 CL25 *myc* protein may initiate at the AUG of *gag* located 18 nucleotides upstream from the splice donor site (Fig. 1).

The *myc* gene of molecularly cloned MH2 CL25 is biologically active. We next examined whether our pMH2-CL25 was biologically active. Supercoiled pMH2-CL25 was used

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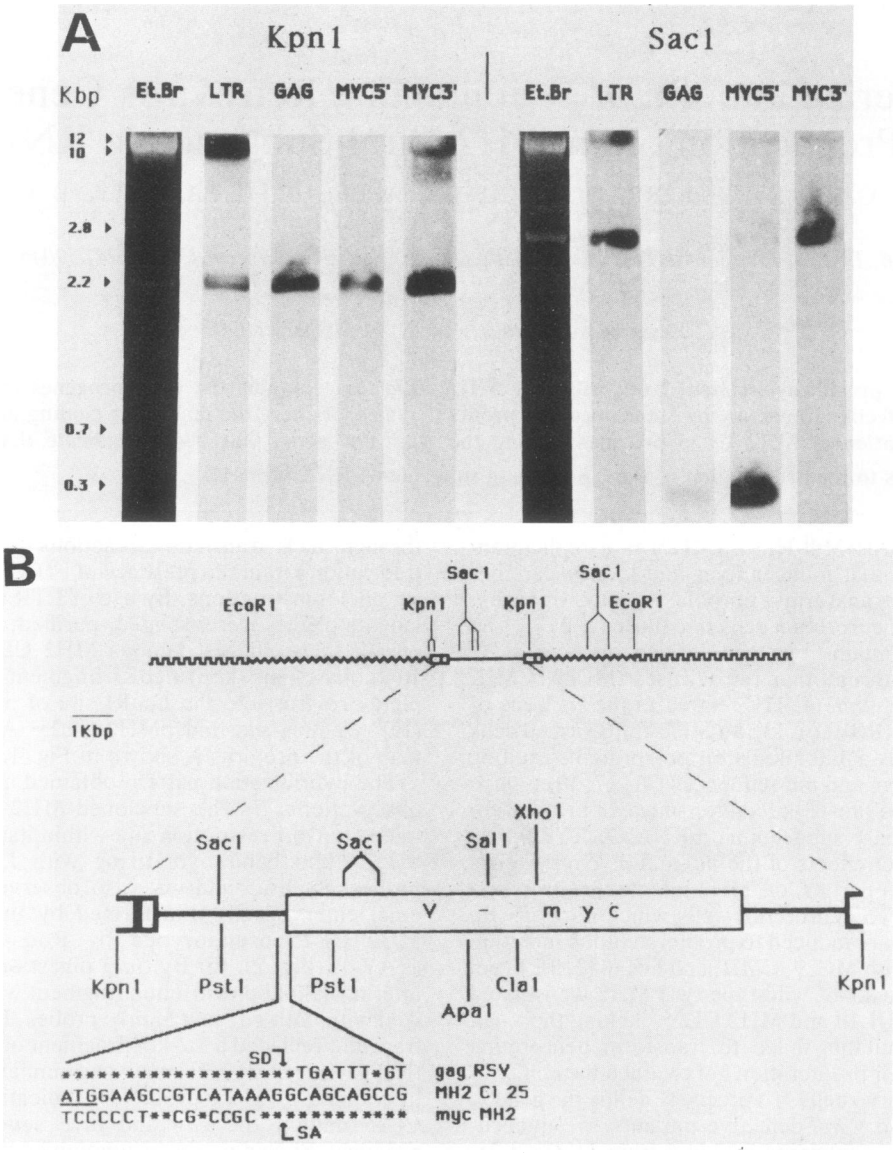

FIG. 1. Restriction map of pMH2-CL25 DNA. The restriction map of MH2 CL25 phages and plasmids was established with several endonucleases. The viral domains were located by Southern blot analyses with specific ^{32}P-labeled probes corresponding to LTR sequences from RSV-SRA, the *gag* gene from Pr-RSV-A, a 5′ v-*myc* probe derived from wt MH2, and a 3′ c-*myc* probe. (A) *Kpn*I or *Sac*I digestions were performed on pMH2-CL25 DNA. Fragments were separated on 1% agarose gels, visualized by ethidium bromide (EtBr) staining, and then transferred to nitrocellulose and hybridized with the probes quoted above. (B) Organization deduced from Southern blot analysis of MH2 CL25 DNA and nucleotide sequence of the *gag-myc* junction. A 162-nucleotide *Hin*fI fragment was sequenced by the Maxam and Gilbert procedure (15). The determined sequence was compared with the *gag* sequence of RSV (24) and with the v-*myc* sequence of wt MH2 (6, 13). Asterisks denote common nucleotides; SD, splice donor site of the *gag* gene; SA, splice acceptor site of the *myc* gene. Symbols: ——, MH2 CL25 proviral DNA, ∿∿∿, plasmid DNA; ∿∿∿, cellular DNA; ▭, LTR.

to transfect quail embryo cells. DNA (30 μg) was precipitated by the calcium phosphate method on 10^6 quail embryo cells (8). After overnight exposure, cultures were reseeded in 100-mm dishes and maintained in low-serum medium (Dulbecco modified Eagle medium supplemented with 5% fetal calf serum). Ten days later distinct foci had appeared (about 1 focus per μg of DNA). Groups of morphologically transformed cells were picked, pooled, and grown up in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. These cells were checked for the presence of MH2 CL25 proviral DNA and v-*myc* protein. Figure 2 shows the Southern blot analyses of DNA from quail embryo cells transformed by wt MH2 (RAV-1), MH2 CL16 (RAV-1), and MH2 CL25 (RAV-1) and cells transfected with pMH2-CL25. After *Kpn*I restriction endonuclease digestion, known to cut within the proviral LTRs, a 2.2-kbp *myc*-hybridizing band was detected in MH2 CL16- and MH2 CL25-transformed cell DNAs (in addition to the 5.5-kbp band corresponding to the endogenous c-*myc* gene seen in normal quail embryo cells [data not shown]). After *Eco*RI digestion of the same DNAs, a similar 2.2-kbp *myc*-hybridizing band appeared in MH2 CL16 DNA but not in MH2 CL25 DNA (in addition to the 16-kbp endogenous c-*myc* gene band [23]). This was expected for MH2 CL16 since this clone was derived from wt MH2 QB2 cells in which both restriction sites were found previously in the proviral LTRs (4). The

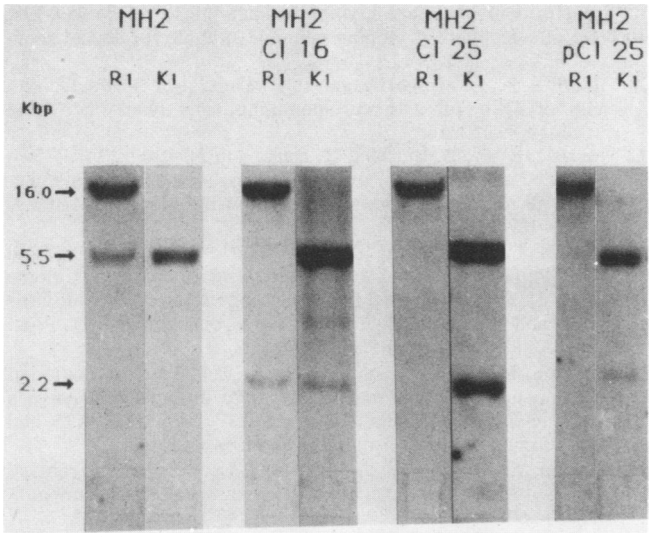

FIG. 2. Southern blot analysis of DNA from quail embryo cells transformed by wt MH2 and mutants. High-molecular-weight DNA from infected cells was digested with *Eco*RI (RI) and *Kpn*I (K). Size-separated cellular DNA fragments were transferred to nitrocellulose and hybridized with the 3′ c-*myc* probe. The transforming viruses used (RAV-1 helper pseudotypes) are indicated on top of the figure. pMH2-CL25 corresponds to quail cells transformed by the plasmid containing the molecularly cloned MH2 CL25.

progenitor of MH2 CL25 would be in this respect more related to the one described by Jansen et al. (12) that similarly lacks the LTR *Eco*RI site.

Finally, to determine whether the transformed cells produced the expected p61-63 *myc* doublet protein (9), [^{35}S]methionine-labeled total cellular extracts were challenged with rabbit anti-*myc* serum prepared by immunization with a bacterially expressed polypeptide corresponding to the product of the 5′ part of exon 3 from the human *myc* gene (F. Ferre, manuscript in preparation). Immunoprecipitation was performed as previously described (2). Immunoprecipitated proteins, characterized by their apparent molecular weight in polyacrylamide gel, are shown in Fig. 3. As can be seen, the p61-63 doublet was detected in cells transformed by wt MH2 or the mutants studied (lanes 1); such bands were not seen with preabsorbed serum (lanes 2).

We concluded from these experiments that the molecularly cloned provirus pMH2 CL25 was biologically active and apparently indistinguishable from the starting mutant isolate.

We then addressed whether pMH2-CL25 could produce recovered infectious virus. Transformed quail embryo cells obtained upon transfection with pMH2-CL25 DNA were superinfected with RAV-1 helper virus. Supernatant medium collected 2 weeks later was titrated for transforming activity by a focus assay on quail embryo cells (29) and contained 0.6 $\times$ 10^3 focus-forming units per ml. This titer was roughly similar to those obtained in similar conditions with cultures infected with wt MH2 (1.5 $\times$ 10^3 focus-forming units per ml), MH2 CL16 (0.7 $\times$ 10^3 focus-forming units per ml), and MH2 CL25 (0.9 $\times$ 10^3 focus-forming units per ml), all pseudotyped with RAV-1 helper virus. We concluded from these results that the virus rescued from pMH2-CL25 provirus was not defective in packaging.

Our results show that the MH2 CL25 and MH2 CL16 mutants derive from wt MH2 and appear to correspond to the encapsidation of subgenomic RNA in viral particles that can propagate as RAV pseudotypes.

The conserved infectious titers show that encapsidation signals are present in these types of molecules. Three kinds of encapsidation signals have been characterized in avian retroviruses. (i) Nishizawa et al. (16) reported packaging sequences (Ni-PS) in the 5′ leader of a temperature-sensitive mutant of RSV. These sequences reside between nucleotides 109 and 356, i.e., between the primer-binding site of the tRNA and the initiation codon of the *gag* gene. A mutant virus, TK15, deleted in this region is defective in packaging. Previous work by Shank and Linial (25) reported sequences that may serve similar functions in the first 600 nucleotides at the 5′ end of the RSV-Pr genome. (ii) Pugatsch and Stacey (18) mapped packaging sequences (Pu-PS) at the *Sst*II restriction site (545 nucleotides from the 5′ end) in the *gag* gene of RSV-SRA. These sequences map 3′ of the splice donor signal and are lost in *env* (and *src*) subgenomic mRNAs that splice out the intronic *gag-pol* (*env*) sequences from the viral genome. Sequences serving similar functions have also been reported for spleen necrosis virus (30). (iii) Sorge et al. (26) reported packaging sequences (So-PS) in the direct repeat unit 3′ to the *src* gene of RSV, slightly upstream from the U3 sequences.

Although no direct evidence is yet available, wt MH2 is likely to contain all three packaging signals described, since the leader and *gag* sequences are well conserved among most avian retroviruses and since the direct repeat unit that may encompass the So-PS signal was found in wt MH2 by Kan et al. (13), although the U3 sequences appeared quite distantly related to RSV. In contrast, the mutant MH2 CL25 (and MH2 CL16) corresponds to subgenomic RNA that should lack the putative Pu-PS signal lost during the splicing process. This is also true for the *env* subgenomic mRNA described by Stacey (27). Since the titers of our mutants MH2 CL25 and MH2 CL16 are still about half of the wt MH2 titer, we conclude that the Pu-PS signal, if present, does not

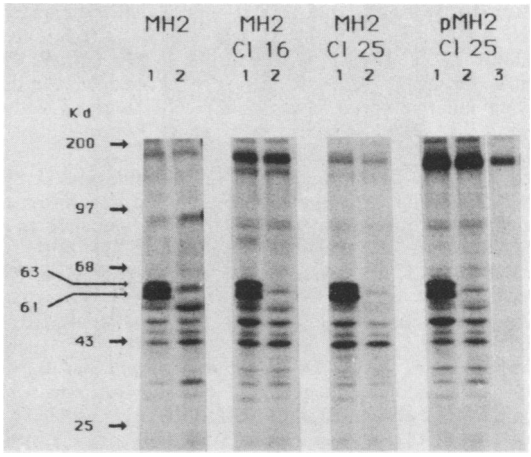

FIG. 3. Immunoprecipitation of v-*myc* proteins in quail embryo cells transformed by wt MH2 and mutants. Cells were labeled for 45 min with [^{35}S]methionine, lysed, and incubated with rabbit anti-human c-*myc* serum prepared with a bacterially expressed polypeptide (lane 1) or the same antiserum preincubated with the corresponding polypeptide (lane 2). Lane 3 corresponds to rabbit preimmune serum. Standard molecular size markers from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) are listed on the left of the figure (Kd, kilodaltons).

play a decisive role in the packaging process of wt MH2 or the mutants.

The titers measured separately for wt MH2 and the mutants in similar conditions would predict a ratio of ca. 60 to 40% of the corresponding particles present in routinely passaged stocks of MH2 virus. Instead, we never detected more than 10% mutant particles in such stocks. Clearly, when promiscuously mixed in such stocks, wt MH2 appears to exhibit a selective advantage over the mutant particles, an observation for which we have as yet no clear explanation.

The isolation and characterization of the MH2 CL25 mutant represents the first demonstration of transmissible pseudotype particles containing an oncogene embedded in a subgenomic mRNA. An analogous situation may have been encountered with *src*-containing deleted proviruses in Syrian hamster tumor cell lines such as H-19 (28) or deletion mutants described by Koyama et al. (14). Such experiments might help in finding the minimum structural genetic elements required to allow the propagation of a retrovirus, and our results may be used for the construction of vectors for the introduction of foreign genes into host cells. Finally, the tumorigenic potential of our mutants is currently being investigated.

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