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The *mil/raf* and *myc* Oncogenes: Molecular Cloning and In Vitro Mutagenesis *

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A. Introduction

Avian retrovirus MH2 is a member of the MC29 subgroup of avian acute leukemia viruses which includes the four independently isolated viruses MC29, CMII, OK10, and MH2 [4]. The genetic hallmark of these viruses is the presence in their genomes of unique transformation-specific sequences, termed v-*myc* [4, 5], which are of cellular origin [3–5, 13]. In contrast to MC29, CMII, and OK10 which induce predominantly leukemic diseases, MH2 induces predominantly liver and kidney carcinomas [1, 2].

The genetic structure of MH2 was recently analyzed in detail. A molecularly cloned MH2 provirus was shown to contain a novel oncogene, termed v-*mil,* in addition

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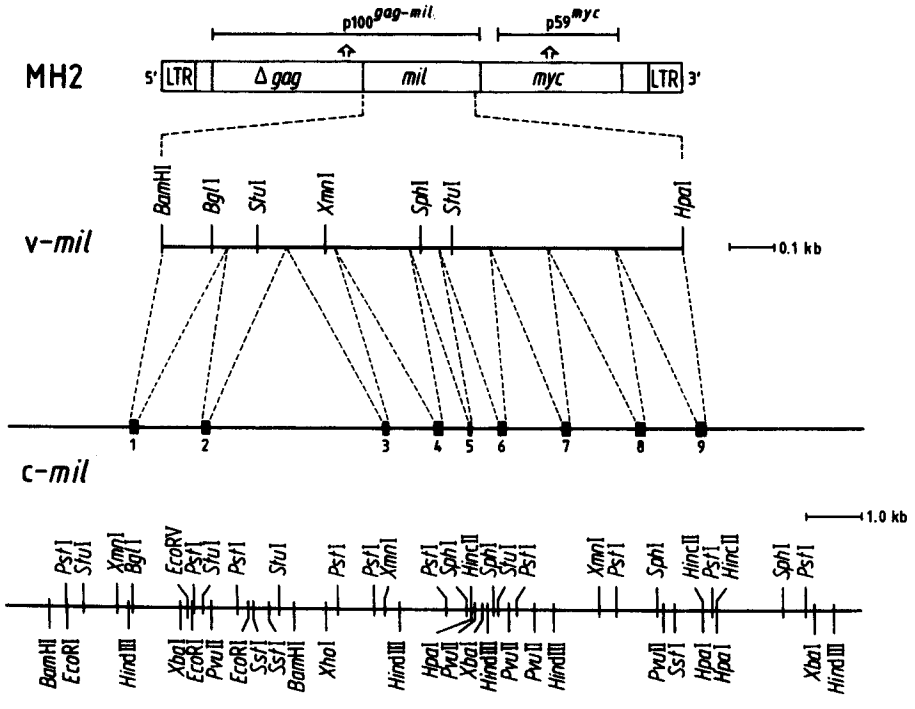

Fig. 1. Genome structure of MH2 and relationship between v-*mil* and c-*mil.* A schematic diagram of cloned proviral MH2 DNA and of its gene products is shown at the top. *Δgag* indicates the presence of partial complements of the *gag* gene. Below this diagram a restriction map of the v-*mil* oncogene is shown. At the bottom of the figure a detailed restriction map of the chicken c-*mil* locus is presented. The exon–intron arrangement as determined from Southern blots and from heteroduplex analysis is shown between the restriction maps of the viral and the cellular oncogene. *Numbered black boxes* represent the regions of c-*mil* homologous to v-*mil* sequences. These presumed exons are numbered in the 5′–3′ direction (see note added in proof)

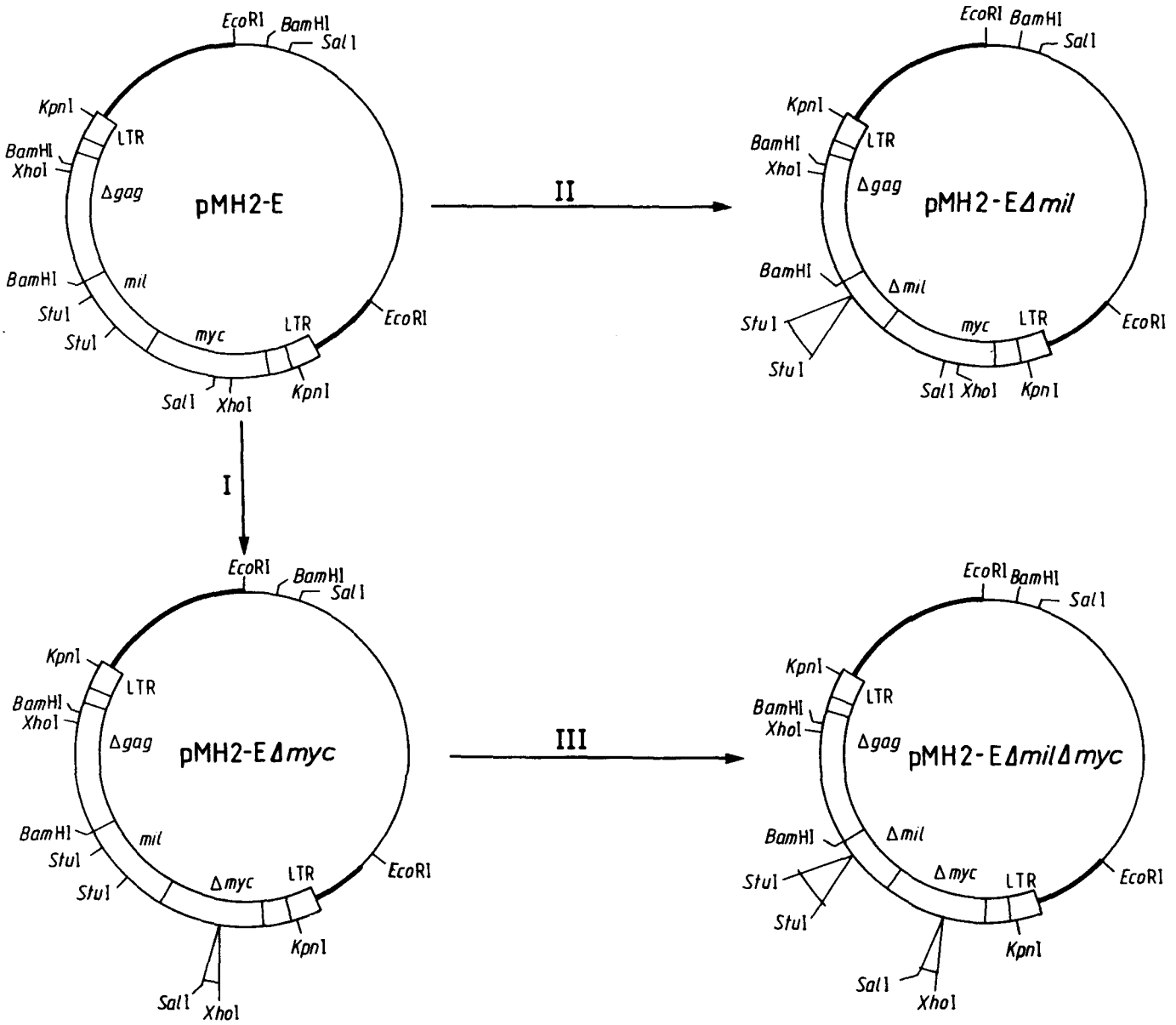

Fig. 2. In vitro mutagenesis of the cloned MH2 provirus. I: construction of an MH2 *Δmyc* provirus. The cloned MH2 provirus was digested with *Sal*I. The ~ 6.5 kilobases fragment containing 5′-LTR-*Δgag-mil*-5′-*myc* of the provirus was isolated. In parallel, the MH2 provirus was digested with *Sal*I and *Xho*I. The ~ 5.6 kilobases fragment containing all plasmid sequences essential for ampicillin resistance and all MH2 proviral sequences 3′ of the *Xho*I site in v-*myc* was isolated. After ligation of these two fragments and transformation of competent bacterial cells, the desired plasmids were selected by growth on tetracycline plates. The structure of four recombinant plasmids was verified and all four turned out to contain the desired MH2 *Δmyc* provirus. The *Δmyc* preserves the reading frame for the v-*myc* protein 3′ of the *Xho*I site. II: construction of an MH2 *Δmil* provirus. The cloned MH2 provirus was digested with *Stu*I. The ~ 11.9 kilobases fragment containing all of the provirus with the exception of a ~ 440 base pairs v-*mil* fragment was isolated and religated. III: construction of an MH2 *ΔmilΔmyc* provirus. To obtain the double deletion mutant the MH2 *Δmyc* provirus was cleaved with *Stu*I, the ~ 11.7 kilobases fragment was isolated and religated. *Boxes* proviral DNA; *thick lines* cellular DNA; *thin lines* plasmid DNA (pBR322)

to v-*myc* [8, 10]. This novel oncogene, like all other viral oncogenes, is of cellular origin [9, 10], and it was shown to be the avian counterpart of the murine *raf* gene [11, 14], the oncogene of the murine retrovirus 3611-MSV. In this communication, we report on two different strategies for the construction of proviral DNA species which will be useful to resolve the individual contributions of the *mil* and *myc* oncogenes in the induction of the specific tumors observed in chickens infected by MH2.

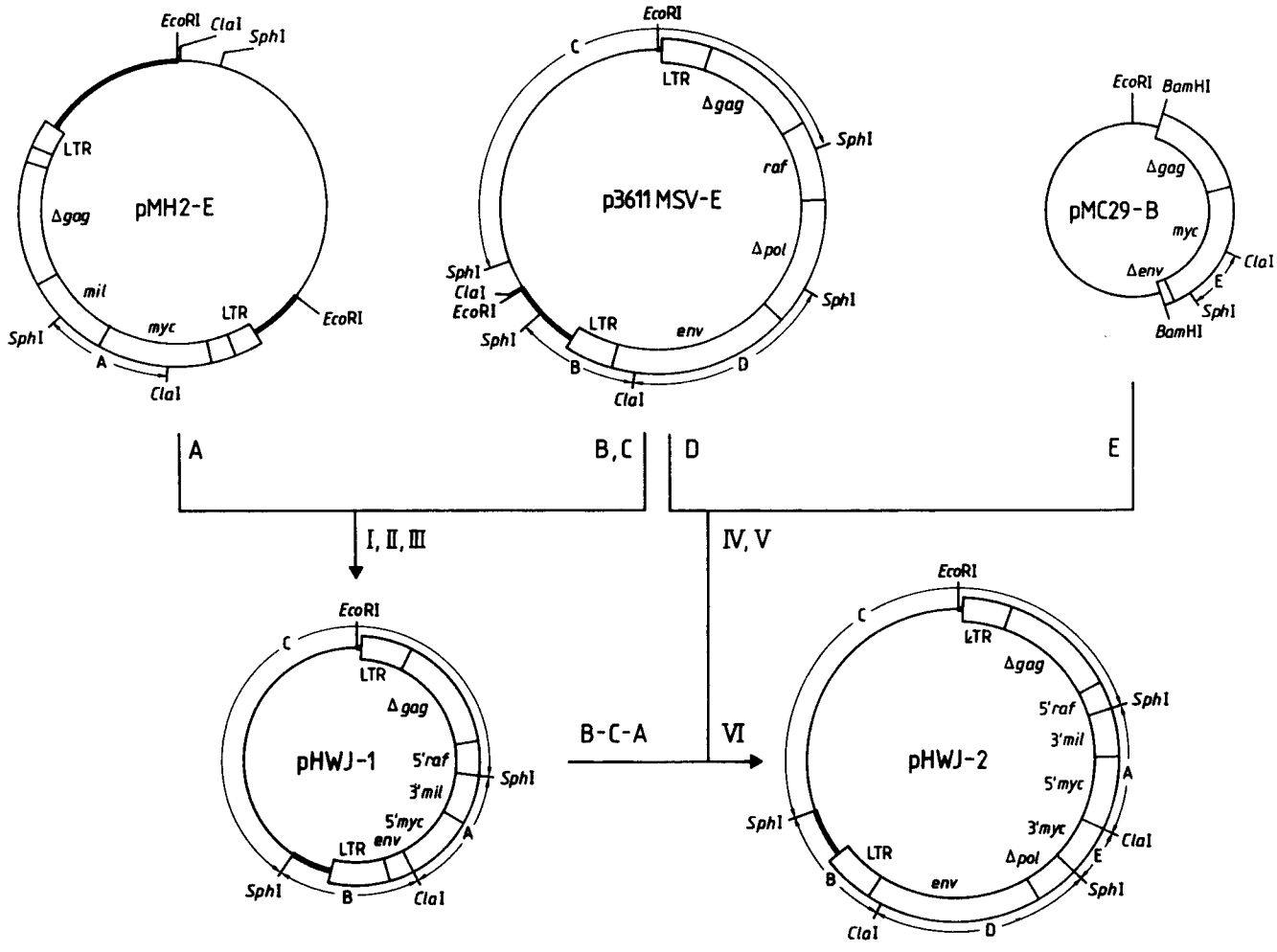

Fig. 3. Construction of a murine provirus containing the *raf/mil* and the *myc* oncogene. For the assembly of pHWJ-1 which contains a v-*raf/mil* hybrid oncogene and the 5′ half of MH2 v-*myc*, plasmids containing the cloned proviruses of MH2 or of 3611-MSV were cleaved with the restriction endonucleases *Cla*I and *Sph*I. The fragments denoted *A–C* were electroeluted from an agarose gel (I). Fragments *A* and *B* were ligated and subsequently cleaved with *Sph*I (II). The products of these reactions were ligated to fragment *C* which had been dephosphorylated with bacterial alkaline phosphatase in advance. The plasmids obtained after transformation of competent *Escherichia coli* cells with this reaction mixture were selected for size and orientation (III). For the construction of the plasmid pHWJ-2 which contains the v-*raf/mil* hybrid oncogene and a complete v-*myc* gene, plasmids containing the cloned provirus of 3611-MSV or the 3.0 kilobases *Bam*HI fragment of the MC29 provirus were cleaved with *Cla*I and *Sph*I. The fragments denoted *D* and *E* were isolated (IV), ligated, and subsequently cleaved with *Cla*I. The desired *D–E* fragment was gel-purified (V) and ligated with the *B–C–A* DNA of pHWJ-1 which had been dephosphorylated after cleavage with *Cla*I. The plasmids obtained after transformation were selected for size and orientation (VI)

B. Results and Discussion

The basic structures of the cloned MH2 provirus, of its oncogene v-*mil*, and of the chicken c-*mil* locus are shown in Fig. 1. In order to get information on the role of the *mil* and *myc* oncogenes in tumor induction by MH2 virus, mutants of MH2 were constructed which are deleted in their oncogenes (Fig. 2). A *Δmil* mutant was constructed by deletion of a 443 base pairs *Stu*I fragment of v-*mil* resulting in an out-of-frame deletion. A *Δmyc* mutant was constructed by deletion of an 171 base pairs *Sal*I-*Xho*I fragment of MH2 v-*myc* resulting in an in-frame deletion. This constructed *Δmyc* deletion is similar to deletions observed in natural mutants of MC29 [6] which are still able to transform fibroblasts, but not macrophages efficiently [12] and which are unable to induce tumors in chickens [7]. A mutant with deletions in both oncogenes (*ΔmilΔmyc*) was constructed by a combination of the strategies described. Biologic studies of the *Δmil* and *Δmyc* mutants are under way. First results

suggest that *mil* enhances the induction of cell proliferation and transformation by the *myc* oncogene both in vivo and in vitro (T. Graf, H. W. Jansen, T. Patschinsky, K. Bister, in preparation).

The observation that the avian oncogene v-*mil* and the murine oncogene v-*raf* were derived from cognate cellular genes [11, 14] led to studies of the transforming capacities of the *mil* and *myc* oncogenes in mice. Upon transfection of NIH/3T3 cells with cloned MH2 proviral DNA, no focus formation was observed. Therefore, we decided to construct murine retroviruses containing the avian *mil* and *myc* oncogenes. The detailed strategy for the construction of pHWJ-1, a plasmid containing a complete *raf/mil* oncogene and the 5′ part of v-*myc*, and of pHWJ-2, a plasmid containing both a complete *raf/mil* and a complete *myc* oncogene, is shown in Fig. 3. Upon transfection, both plasmids gave rise to foci on NIH/3T3 cells with those induced by pHWJ-2 being much more prominent. A first striking result from in vivo experiments with these viruses is the observation that the oncogenicity of HWJ-2 virus is markedly higher than that of 3611-MSV (U. R. Rapp, H. W. Jansen, K. Bister, in preparation).

Note added in proof

A recent nucleotide sequence analysis [15] of the c-*mil* locus revealed that there are two additional small regions of homology to v-*mil* 5′ from exon 1 (Fig. 1).

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