Molecular cloning of the avian acute transforming retrovirus MH2 reveals a novel cell-derived sequence (*v-mil*) in addition to the *myc* oncogene

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Mill-Hill-2 virus (MH2) proviral DNA was cloned from a transformed non-producer cell culture (MH2QB2) through insertion of randomly cut high mol. wt. cellular DNA in the lambdoid vector L47.1. Restriction analysis of a suitable recombinant phage by Southern DNA blotting and hybridization with different probes allowed us to characterize the genetic organization of the provirus and to identify a novel MH2-specific sequence of at least 1.1 kbp. Such a sequence, for which we propose the name *v-mil*, from *Mill-Hill-2* virus, is not homologous to *v-myc*, the previously described oncogene of MH2, nor to avian leukaemia virus-related sequences. Evidence is presented here that *v-mil* has a cellular counterpart (*c-mil*) phylogenetically conserved in birds and mammals, including man, and expressed as a single RNA species at least in some tissues. MH2 virus might thus be regarded, like avian erythroblastosis virus or E26, as another example of retroviruses having recombined with more than one cellular gene.

Key words: MH2/*v-mil*/*v-myc*/oncogenes/leukemia/carcinoma

Introduction

Mill-Hill-2 virus (MH2) is an avian acute defective leukemia virus (DLV) classified along with MC29 (the prototype virus), OK10, and CMII in the myelocytomatosis-inducing virus group (for review, see Graf and Stehelin, 1982). All four viruses acquired, by an as yet unknown recombination process, a similar transforming sequence, *v-myc*, derived from a conserved cellular eukaryotic gene, *c-myc* (Roussel *et al.*, 1979; Sheiness and Bishop, 1979; Graf and Stehelin, 1982). Transcriptional activation of *c-myc* sequences have been implicated in the onset of spontaneous neoplasms, such as plasmacytomas (Mushinski *et al.*, 1983) and Burkitt lymphomas (Erikson *et al.*, 1983) while *v-myc*-containing viruses have been described to cause fibroblast and macrophage transformation, *in vitro* (Graf and Beug, 1978). *In vivo* different kinds of tumors develop after infection of susceptible animals, mainly liver and kidney carcinomas, in addition to myelocytomatosis (Graf and Beug, 1978; Alexander *et al.*, 1979). Transformation by MC29-related viruses was thought to be mediated by the synthesis of a polyprotein containing both *gag* and putative oncogenic determinants (Bister and Duesberg, 1980). Such proteins are produced by all the members of the family (MC29:P110, MH2:P100, CMII:P90, OK10:P200) (Bister *et al.*, 1977, 1979; Hu *et al.*, 1978; Hayman *et al.*, 1979) but only for MC29 was it possible to confirm directly its supposed function (Ramsay *et al.*, 1980).

Conversely, for MH2, indirect evidence against the presence of *v-myc* determinants in the putative transforming fusion protein P100 has been, since then, provided by several independent studies (Neil *et al.*, 1981; Linial, 1982; Ramsay *et al.*, 1982). In addition to this discrepancy, recent work has indicated that MH2, in comparison with MC29, shows a higher tumorigenicity when injected into young immunocompetent quails (Linial, 1982), and differs substantially in the modality of expression of its viral oncogene (Pachl *et al.*, 1983; Saule *et al.*, 1983). According to Pachl *et al.* (1983) the transformation potential of MH2 is to be attributed to a non-*gag*-linked *myc* protein of ~57 kd encoded by a subgenomic mRNA. Studies carried out in our laboratory on a transformed, non-producer cell clone (MH2QB2) have indeed confirmed the existence of this messenger (Saule *et al.*, 1983). In addition, they have suggested the probable deletion, in this MH2 genome, of most or all of the structural *pol* and *env* genes and the presence, instead, of an unidentified nucleotide sequence inserted between the *gag* and the *myc* domains. In this article we report the molecular cloning of an integrated MH2 provirus from MH2QB2 cells and the identification of a novel MH2-specific, additional sequence. By hybridization studies with ³²P-labelled probes on Southern and Northern blots, we demonstrate that this sequence has a cellular counterpart in normal DNA from avian and mammalian species, found as a conserved locus transcribed at least in some normal tissues as a single polyadenylated RNA species.

Results

Molecular cloning of the MH2 provirus

A transformed quail non-producer fibroblast cell clone containing the MH2 provirus, (MH2QB2, Saule *et al.*, 1983), was used as a source of proviral DNA for the preparation of a gene library. High mol. wt. DNA (HMW DNA), extracted from cell cultures, and partially cut by *Mbo*I restriction enzyme was ligated with gel purified lambda L47.1 arms, packaged and amplified. After isolation and amplification of a suitable recombinant, lambda MH2Q1, phage DNA was isolated and digested with different restriction enzymes. Fragments were run on agarose gels, transferred to nitrocellulose sheets and hybridized with different [³²P]nick-translated probes. These were prepared from cloned DNA fragments corresponding to the long terminal repeat (LTR) of Schmidt-Ruppin avian sarcoma virus or to the three retroviral structural genes *gag*, *pol* and *env*. Other probes were from fragments of cloned *v-myc*^{MC29} DNA, representing essentially the whole *myc* gene or its 5' or 3' regions (see Figure 1 and Materials and methods). From the hybridization pattern obtained (Figure 1), the following conclusions were drawn: (i) the cloned provirus we isolated contained the two LTRs, as attested by the three bands (6.0, 5.5 and 2.5 kbp) seen with an LTR probe on an *Eco*RI digest; (ii) the MH2 partial (Δ) *gag* gene was mainly localized in a 1.3-kbp *Bam*HI fragment at the 5' end of the provirus, whereas a *Sal*I and a *Xho*I site, near the 3' end, mapped within the *v-myc*^{MH2} region; (iii) the proviral 2.0-kbp *Bam*HI-*Sal*I internal fragment, hybridizing

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Fig. 1. Characterization of cloned MH2 proviral DNA. Lambda MH2Q1 phage DNA was digested with *EcoRI* (E), *BamHI* (B), *BamHI-SalI* (B/S) or *XhoI* (X) restriction enzymes. Digests (1 µg/lane) were separated on 1% agarose gels, transferred onto nitrocellulose sheets and hybridized with the nick-translated probes listed across the top. (►) Size of significant hybridization-positive MH2Q1 bands. Doublets at the top of the autoradiograms represent partially reannealed ('cos' ends) phage arms containing some hybridizable proviral material. The faint signal at ~7 kbp in the *XhoI* digest (X), *gag* channel, is artefactual. (—) Molecular size markers. A restriction map of phage MH2Q1 is shown at the top of the figure. Solid lines represent L47.1 arms, the open box the cellular insert and hatched lines map proviral LTRs. Stretches coding for P100, the *gag* fusion polyprotein of MH2, and p57^{myc} are indicated. Bottom line: probes from cloned *v-myc*^{MC29} DNA used in this study: *v-myc* probe (representative): *Pst* fragment (1510 bp); *myc5'* probe: *Sac-Sal* fragment (478 bp); *myc3'* probe: *AvaI-SphI* fragment (315 bp). Restriction endonucleases indicated are: Av, *AvaI*; B, *BamHI*; E, *EcoRI*; H, *HindIII*; P, *PstI*; Sc, *SacI*; SI, *Sall*; Sp, *SphI*; X, *XhoI*.

only with a *v-myc5'* probe, was too large to contain only *v-myc* sequences; (iv) no *env* and a negligible amount of *pol* sequences were detected in the provirus.

Our cloned provirus lambda MH2Q1 (Figure 1) apparently corresponded to the provirus in the starting MH2QB2 cell DNA (Figure 5A), as attested with a *gag* probe by the 3.4-kbp internal band seen in a *XhoI* digest as well as by the 5.5-kbp band seen with *EcoRI*, that cuts within LTRs (compare Figures 1 and 5A). An additional band at 11.5 kbp was present in the *EcoRI* digest or at 12.0 kbp in a *HindIII* digest of MH2QB2 DNA (Figure 5A). These bands represented a second but incomplete MH2 provirus, integrated in MH2QB2 cells at a different locus, and containing only one LTR (data not shown). This additional deleted provirus was judged irrelevant to the aim of this paper, since, as reported later, our cloned provirus was proven to be biologically active.

Characterization of the MH2 specific sequence

To analyze further the organization of MH2 proviral DNA, a 7.8-kbp *HindIII* fragment, containing the provirus and some flanking cellular sequences (Figure 1), was inserted into a plasmid vector pKH47 yielding the recombinant plasmid pMH2-Hd, a detailed restriction map of which is presented in Figure 2. By comparison with MC29 provirus, it can be seen that five restriction sites of the *v-myc*^{MH2} domain map at apparently identical positions in *v-myc*^{MC29}. These data and the hybridization pattern obtained with the *myc* probes indicated that *myc5'* and *myc3'* sequences in MH2 were adjacent and located immediately upstream of the 3' LTR, thus confirming the localization previously assigned on the basis of RNA studies (Saule *et al.*, 1983). To assess the *v-myc*^{MH2} upstream limit, the proviral *BamHI-SalI* 2.0-kbp fragment (lacking *gag*-related sequences, see Figure 1) was subcloned in the corresponding sites of plasmid pKH47 to yield plasmid pMH2-BS. When pMH2-BS was digested with *HincII*, a 1.85-kbp fragment including the proviral *BamHI-HincII* 1.1-kbp region failed to hybridize with a representative *v-myc*^{MC29} probe (Figure 3). We thus considered this 1.1-kbp region as the major part of an as yet uncharacterized sequence, specific to the MH2 provirus, for which we propose the name *v-mil* from *Mil*-Hill-2 (MH2) virus. We utilized the

Fig. 2. Restriction analyses of the MH2 provirus. The 7.8-kbp *HindIII* fragment containing the whole provirus and some cell sequences was inserted in plasmid pKH47. A restriction map of the recombinant plasmid, pMH2-Hd, (second line) can be compared with that of the cloned *v-myc*^{MC29} oncogene (first line). The third line presents a finer restriction map of the *v-mil*-containing 2.0-kbp *BamHI-SalI* fragment, subcloned in plasmid pMH2-BS. Solid lines represent plasmid DNA, wavy lines flanking cellular DNA and open boxes proviral sequences. The localization of the 1.1-kbp *v-mil*-specific probe is also indicated. L: LTR. Restriction endonucleases used were: B, *BamHI*; C, *Clai*; E, *EcoRI*; H, *HindIII*; Hc, *HincII*; K, *KpnI*; P, *PstI*; Pv, *PvuII*; Sc, *SacI*; Sm, *SmaI*; SI, *Sall*; Sp, *SphI*; Xh, *XhoI*; Xb, *XbaI*.

Fig. 3. Characterization of the *v-mil* sequence. DNA from plasmid pMH2-BS was digested with *HincII* restriction enzyme, electrophoresed on agarose gel and stained with ethidium bromide (EtBr). Size-separated bands were transferred onto nitrocellulose and hybridized with the representative *v-myc* probe (*v-myc*). Two bands, out of four, were not detected: the plasmid-derived 3.5-kbp band, and a 1.85-kbp fragment. This fragment contains 0.75 kbp of plasmid-derived DNA and 1.1 kbp of proviral sequences representing the major part of the specific domain (*v-mil*) present in the MH2 virus. (M) Molecular size markers: *EcoRI* digest of phage 13i DNA (Sergeant *et al.*, 1982) run on the same gel.

*Bam*HI-*Hinc*II fragment as a *v-mil*-specific probe. This probe did not hybridize to the other *v-onc* genes found in avian acute defective leukemia viruses, namely, *v-erb-A*, *v-erb-B*, *v-myb* or the recently described second specific sequence *v-ets* of E26 myeloblastosis retrovirus (Leprince *et al.*, 1983) nor to the *src* gene of Rous sarcoma virus (data not shown).

The cloned MH2 is biologically active

We examined whether our MH2 clone was biologically active and could lead to recovered infectious virus. pMH2-Hd DNA restricted by *Hind*III cleavage was utilized, without further purification, to perform co-transfection assays together with helper virus (RAV-1) DNA at a ratio of 5 to 1. Quail embryo fibroblasts transfected using the CaPO_4 method were amplified, and examined 10 days later. Developing foci were observed in each Petri dish. Production of infectious, transforming rescuable MH2 pseudotype virus was shown by the infection of QEF or CEF monolayers as well as chicken macrophages, with supernatants from transfected cultures. As an example, Figure 4 shows QEF monolayers transformed by MH2 virus from transfected cultures (Figure 4C) or from MH2QB2 control cells superinfected with RAV-1 (Figure 4D).

Transfection experiments were then repeated without the

Fig. 4. Transforming ability of cloned pMH2-Hd. Quail embryo fibroblast cultures were transformed by the CaPO_4 method as described in Materials and methods. (A) Primary focus 10 days after transfection with *Hind*III-cleaved plasmid pMH2-Hd (x 100). (B) MH2Q3 transformed culture obtained by pooling four primary foci (x 100). (C) QEF culture infected with MH2 virus from pMH2-Hd/RAV-1 co-transfected cultures (x 200). (D) - QEF culture infected with MH2 virus from superinfected (RAV-1) MH2-QB2 cultures (x 200). Scale bars: 50 μm .

addition of helper virus DNA in order to obtain transformed non-producer cell cultures. We obtained ~ 0.2 foci/ μg of DNA on 100 mm Petri dishes. Pools, each containing four single foci (Figure 4A) were amplified as mixed cultures (Figure 4B represents one such culture, MH2Q3, used in this study). At present, after >20 passages MH2Q3 cultures have not lost their growth potential. MH2Q3 cells were checked for the presence of integrated MH2 provirus as well as for the absence of integrated helper virus sequences, by analysis of cellular DNA and mRNA. As shown in Figure 5A, digestion of HMW DNA by *Eco*RI or *Xho*I restriction enzymes revealed the expected internal proviral bands. Similarly, data presented in Figure 5B showed that MH2Q3 cells accumulated the viral RNA species found in MH2QB2 cultures.

Finally, to determine if the transformed cells were producing the expected P100 protein containing *gag* determinants, ^{35}S -labelled total cellular extracts from MH2Q3, MH2QB2 and QEF control cultures were challenged with rat anti-*gag* immune serum. Immunoprecipitated proteins, characterized in relation to their apparent mol. wt. by polyacrylamide gel electrophoresis, are shown in Figure 5C. As can be seen, a single band migrated with the same mobility as P100^{MH2} for MH2QB2 and MH2Q3 cells. This finding indicated that a *gag-mil* fusion polypeptide, indistinguishable in size from P100^{MH2}, was synthesized in MH2Q3 cells.

We concluded from these experiments that our cloned MH2 provirus was biologically active and indistinguishable from the input virus by all the criteria tested.

v-mil sequences have a cellular counterpart

Retroviral-specific nucleotide sequences described so far have been demonstrated to be of eukaryotic origin and to be conserved in the genome of various species. This remarkable sequence stability throughout speciation has been regarded as evidence for their importance in the economy of the cell, although the biological role of these sequences is far from being fully understood. For these reasons, we investigated the presence of a possible *c-mil* locus in DNA from the chicken as

Fig. 5. Mapping and expression of the integrated MH2 proviruses before and after molecular cloning. **Panel A:** HMW DNA from either MH2QB2 cells, used for cloning or MH2Q3 cells, transformed by the cloned provirus, were digested with the endonucleases listed across the top of the figure, run on agarose gels (20 μ g/lane), transferred onto nitrocellulose and hybridized with a *gag* probe. **Panel B:** polyadenylated RNA from infected MH2QB2 cells or from MH2Q3-transfected cells were denatured with glyoxal, separated by agarose gel electrophoresis, Northern-blotted and hybridized with either *gag* or *myc* probes. Arrow heads indicate the 5.0- and 2.2-kb eukaryotic rRNA markers. **Panel C:** MH2QB2, MH2Q3 and QEF (control) cell cultures were labelled with [35 S]methionine, lysed and incubated with a rat anti-*gag* serum. Immunoprecipitated proteins were separated by polyacrylamide gel electrophoresis and submitted to fluorography.

Fig. 6. *c-mil* is conserved in evolution and expressed. **Panel A:** *Eco*RI-digested HMW DNA from different species were size separated by gel electrophoresis (20 μ g/lane), blotted according to Southern (1975) and hybridized under non-stringent conditions (1.2 M NaCl) with the *v-mil* probe (see text and Figure 3). Chicken: embryo fibroblasts; quail: embryo fibroblasts; rat: AT1A cell line; human: placental tissues. (—) Molecular size markers. Arrow heads indicate the bands detected by the *v-mil* probe. (—) marks the position of the distinct *c-myc* locus in *Eco*RI-digested chicken DNA. **Panel B:** polyadenylated RNA from whole chicken embryos, quail embryo fibroblasts and human erythroid cell line K562, were denatured with glyoxal, separated by agarose gel electrophoresis, and hybridized under non-stringent conditions with the probes listed across the top of the figure. Arrow heads indicate the 5.0- and 2.2-kb eukaryotic rRNA markers.

well as from phylogenetically distant animals. Results of Southern hybridizations with the *v-mil*-specific probe, on blots of *Eco*RI-digested HMW DNA from chicken, quail, rat and human cells are presented in Figure 6A. Several positive bands were observed in each channel, indicating the existence of a cellular counterpart (*c-mil*) to *v-mil* sequences in all four species tested.

To examine if these *c-mil* sequences corresponded to a defined DNA locus, we searched for *v-mil*-related mRNA in chicken and quail embryos as well as in a human cell line. Figure 6B presents the autoradiograms of Northern blots, hybridized with *v-mil* and carrying size-separated, intracellular polyadenylated RNA from the three species. For each channel a single band was observed, of 4.0 kb (chicken), 3.4 kb (quail) and 3.6 kb (man). The 4.0-kb band was also seen in chicken muscle and bone marrow (data not shown).

We conclude that the *v-mil* sequence appears to have been transduced from similar cellular sequences, *c-mil*, in the chicken or a closely related species. The *c-mil* sequences are conserved phylogenetically in higher vertebrates, including man, and belong to a locus distinct from *c-myc* since the respective DNA restriction patterns appear unrelated and the mRNA transcripts differ in size in the same tissue (3.4–4.0 kb for *c-mil*, 2.7 kb for *c-myc*, see Figure 6B).

Discussion

The molecular cloning in a biologically active form of the MH2 provirus confirms the distinct genetic make up of MH2 as compared with the prototype *myc*-containing retrovirus MC29 (Saule *et al.*, 1983). The *myc* gene in MH2 is located at the 3' end of the provirus, a position compatible with the *myc*-containing spliced subgenomic RNA we previously described for this virus (Saule *et al.*, 1983) which yields the *myc* protein of 57 kd (p57^{myc}) described by Pachl *et al.*

(1983). This *myc* oncogene, composed from two cell-derived exons, was shown to share only 60–70% homology with *v-myc*^{MC29} (Roussel *et al.*, 1979; Sheiness *et al.*, 1980). Comparison of the restriction maps of our MH2 proviral clone and that obtained from nucleotide sequencing of the MC29 genome (Reddy *et al.*, 1983) indicates that the whole first exon of *v-myc* is conserved in the two viruses without major variations. Conversely, when *v-myc* sequences mapping downstream of the unique *Clal* site in the two viruses were compared, we observed differences in the presence of at least two restriction sites, a *XhoI* site present only in MH2 and a *SphI* site present only in MC29. This region, nevertheless, retained sufficient homology with *myc* sequences in MH2, as demonstrated by hybridization with a probe representative of the 3' end of *v-myc*^{MC29}. This preliminary analysis of differences between the *v-myc* oncogene in the two viruses awaits confirmation by nucleotide sequencing of the *v-myc*^{MH2} domain, and their possible importance with respect to transformation remains to be assessed.

In addition to the *myc* oncogene, we have demonstrated that the MH2 provirus contains a second specific nucleotide sequence, that we named *v-mil* (for *Mill-Hill-2* virus), not found in the other avian retroviruses tested and thus distinct from the oncogenes *v-src*, *v-myc*, *v-myb*, *v-erb-A*, *v-erb-B* and also distinct from the *v-ets* specific sequence we recently described for the E26 myeloblastosis virus (Leprince *et al.*, 1983). This novel *v-mil* sequence is of cellular origin, it is located between the partial *gag* gene and the *myc* gene in the MH2 provirus, and is translated in infected cells as a *gag-mil* fusion protein of 100 kd (P100^{gag-mil}), found in the cytoplasm of the cells and able to bind to ssRNA *in vitro* (Bunte *et al.*, 1983).

The genetic organization of MH2 closely resembles that of avian erythroblastosis virus (AEV) (Roussel *et al.*, 1979), which also carries two distinct cell-derived specific sequences: *v-erb-A* and *v-erb-B* (Vennström and Bishop, 1982). Polypeptides encoded by these two sequences are translated from two distinct mRNAs, one of genomic size coding for P75^{gag-erb-A} (Hayman *et al.*, 1978; Anderson *et al.*, 1980) and the other, spliced and devoid of *v-erb-A* sequences (Anderson *et al.*, 1980; Sheiness *et al.*, 1981) that codes for a p65^{erb-B} glycoprotein (Hayman *et al.*, 1983). The same strategy of expression can also be observed in MH2, where a genome-sized viral mRNA encodes a P100^{gag-mil} polypeptide, while a subgenomic mRNA (Saule *et al.*, 1983) directs the synthesis of the p57^{myc} protein (Pachl *et al.*, 1983).

Both P75^{gag-erb-A} and P100^{gag-mil} proteins may modulate the transforming properties of the corresponding viruses. Transformation experiments performed with cloned AEV provirus, mutagenized *in vitro* in the *v-erb-A* or *v-erb-B* domains, recently attributed the major oncogenic activity to the *erb-B* product (Frykberg *et al.*, 1983). The *erb-A* product was not transforming as such in those tests, but had an enhancing effect on *v-erb-B* in erythroid transformation. With regard to MH2, Linial (1982) recently reported the characterization of a non-conditional MH2 mutant (MH2YS3), spontaneously arising in a viral stock, that still transforms but that lacks the ability to produce P100^{gag-mil}. This result suggests that *v-mil* sequences are not essential for MH2-mediated transformation, but does not provide clues about a possible biological function of *v-mil*, nor does it exclude the fact that *v-mil* could be by itself a transforming gene. When compared with MC29, MH2 exhibits a markedly higher tumorigenicity that

was attributed by Pachl *et al.* (1983) to the different strategy for the *v-myc* oncogene expression (p57^{myc} for MH2 versus P110^{gag-myc} for MC29). Our results open the alternative possibility that the *mil* product could be implicated in the enhanced transforming potential of MH2. We are currently constructing mutants in the *mil* or *myc* genes of MH2 to resolve this issue.

Three different viruses have now been found in the avian system that contain two distinct cell-derived specific sequences (*erb-A* and *erb-B* for AEV, *mil* and *myc* for MH2, and *myb* and *ets* for E26; Leprince *et al.*, 1983). Such viruses may be unique tools for approaching synergistic effects of oncogenes, a matter now becoming of prime interest with respect to the roles of different oncogenes in distinct steps of the processes leading to carcinogenesis (Land *et al.*, 1983; Ruley, 1983).

The cellular counterpart (*c-mil*) of the *v-mil* sequence appears to constitute at least one DNA locus (pseudogenes have not been ruled out), that has been conserved in the vertebrates tested: chicken, quail, rat and human DNA. Where tested (chicken and man) this locus is transcribed as a polyadenylated mRNA of 4.0 kb in the chicken (3.6 kb in man) at least in some normal tissues (chicken muscle or total embryo) as well as in hematopoietic cells (chicken fresh bone-marrow; human erythroid line K562). The level of expression in different tissues is under investigation. The *c-mil* locus is distinct from the *c-myc* locus since the mRNAs produced differ in size (*c-myc* produces a 2.7-kb mRNA in chicken bone marrow) and, since these loci do not show homology by hybridization experiments. Thus the *c-mil* locus, as other viral oncogene-related cellular genes, probably serves important functions yet to be discovered.

Materials and methods

Cells and nucleic acids

The MH2QB2 cell clone has been previously characterized (Saule *et al.*, 1983). Other cultures utilized as sources of nucleic acids were as follows: chicken embryo fibroblasts (CEF) from 11-day-old eggs, Japanese quail embryo fibroblasts (QEF) from 10-day-old eggs, K 562, a human transformed erythroid cell line (Lozzio and Lozzio, 1975), and AT1A, an AEV-transformed, rat cell fibroblastic culture (Quade, 1979). Human HMW DNA was also obtained from healthy placental tissues following a classical procedure (Saule *et al.*, 1982). The same protocol was utilized for avian embryos, while cell cultures were lysed in Tris-HCl 10 mM pH 7.4 containing EDTA 100 mM, SDS 0.5%, proteinase K 100 µg/ml and incubated overnight at 37°C. Subsequent banding of HMW DNA in CsCl gradients followed by extensive dialysis against Tris-HCl 10 mM pH 7.4, EDTA 1 mM produced DNA sensitive to restriction enzymes. RNA extractions were performed in the presence of guanidinium thiocyanate as described by Glisin *et al.* (1974).

Cloned DNA probes

DNA fragments for the preparation of ³²P probes were obtained from suitable recombinant plasmids by enzyme digestions and agarose gel electrophoresis. Fragments representative of the retroviral genes *gag*, *pol*, and *env* were derived from the Pr-RSV A genome as illustrated by Saule *et al.* (1983). Conversely, the total LTR-representative sequence, obtained through the kindness of J.M. Bishop, was from the RSV-SRA genome (de Lorbe *et al.*, 1980). Three different *myc* probes were utilized throughout the study (see bottom of Figure 1): the *PstI-PstI* fragment of cloned MC29 virus (*v-myc*) was representative of essentially the whole oncogene (Vennström *et al.*, 1981); the probe (*myc* 5') represented the 5' part of *v-myc*^{MC29} as defined by the *SacI-SalI* fragment containing nucleotides 205–683 (Alitalo *et al.*, 1983); the probe (*myc* 3') corresponded to the *AvaI-SphI* fragment containing nucleotides 1097–1412 (Alitalo *et al.*, 1983). The *v-mil* specific probe, described in the text, was obtained from the 1.1-kb *BamHI-HincII* fragment of plasmid pMH2-BS. The fragments described were purified from contaminating sequences by electrophoresis in 0.8% agarose gels and labelled through nick-translation reactions (Rigby *et al.*, 1977) (Amersham nick-translation kit) in the presence of [³²P]dCTP according to the supplier's instruction.

Cloning procedures

A MH2QB2 partial *Mbo*I gene library was prepared in L47.1 vector following standard procedures reported elsewhere (Saulle *et al.*, 1983). Recombinant phages were grown to high titer stocks on *Escherichia coli* strain DP50 and their DNA extracted with phenol after phage purification on CsCl gradients (Saulle *et al.*, 1982). Subcloning of the MH2 provirus was performed in plasmid pKH47 (Hayashi, 1980). Fragments and vector DNAs were ligated by T4 DNA ligase (BRL Inc., USA) and used to transform calcium-treated cells of *E. coli* strain HB101. Suitable transformants were detected by antibiotic-resistance tests and colony hybridization with appropriate ³²P-labelled probes (Grunstein and Hogness, 1975). After isolation on agar plates, single colonies were grown to mass cultures in LB (Luria-Bertani) medium. Plasmid DNA was recovered after bacterial lysis induced by a lysozyme-Triton treatment (Perbal and Baluda, 1982).

Nucleic acid analysis

Cellular or cloned DNAs were digested with restriction endonucleases from Boehringer (Mannheim, FRG) and BRL Inc. (USA). Fragments were size-separated by agarose-gel electrophoresis and transferred to nitrocellulose according to Southern (1975). Total cellular RNA was fractionated on oligo(dT)-cellulose (T3, Collaborative Research, Waltham, MA). Polyadenylated fractions were denatured, size-separated on agarose gels and transferred to diazobenzyloxymethyl paper according to Alwine *et al.* (1977). Hybridization of blots to ³²P-labelled DNA, washing and autoradiography at -70°C with Kodak X-ray films and Dupont Lightning Plus X-ray intensifying screens, were as in Saulle *et al.* (1982).

Transfection procedures

Transfection experiments were performed on QEF cultures with *Hind*III-restricted pMH2-Hd DNA with or without the addition of RAV-1 DNA, according to the CaPO₄ method developed by Graham and Van der Eb (1973). Precipitated cellular DNA (25–50 µg) was added in 0.5 ml volumes to 60 mm culture dishes containing 1.5 × 10⁶–2 × 10⁶ cells. After a 1 h incubation at 38.5°C, cells were fed with 10 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% chicken serum (CS). After overnight resting, cultures were reseeded in larger (100 mm) plates and maintained in DMEM 10% FCS, 1% CS. Ten days later groups of four developing foci were picked, pooled in small microculture plates, and grown up in the same medium.

Protein labelling and immunoprecipitation

Labelling was performed on semiconfluent cultures seeded in 35 mm Petri dishes by incubation for 4 h with 5 ml DMEM, 10% FCS containing 0.2 mCi of [³⁵S]methionine. Cells were scraped in Tris-acetate 10 mM pH 7.4 buffer containing 0.4 M NaCl, 1% Nonidet P40, EDTA 1 mM, Apotinin 100 U/ml (TST buffer). After clarification for 10 min at 10 000 g, supernatants were diluted with TST buffer and adsorbed for 3 h at 4°C against non-immune rat serum and Protein A-Sepharose beads (PA). Supernatants were then incubated with anti-gag rat sera (kindly provided by G. Calothy) at 4°C overnight, 5 mg of PA were then added to each sample and absorption allowed for 3 h in the cold. Beads were then washed three times in TST buffer containing 0.1 M NaCl and three times in 5 mM Tris-acetate pH 7.4. Recovered radioactivity was eluted from beads by 5 min boiling in electrophoresis loading buffer (3% SDS, 10% β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8) and loaded onto 10% polyacrylamide gels according to Laemmli (1970). After the run, gels were stained with Coomassie Brilliant blue and prepared for fluorography according to Laskey and Mills (1975). Fluorography was carried out at -70°C with Dupont X-ray intensifying screens and Kodak X-ray films.

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