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Activation and Transduction of c-*mil* Sequences in Chicken Neuroretina Cells Induced To Proliferate by Infection with Avian Lymphomatosis Virus

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We report that nondividing neuroretina cells from chicken embryos can be induced to proliferate following infection with Rous-associated virus type 1 (RAV-1), an avian lymphomatosis retrovirus lacking transforming genes. Multiplication of RAV-1-infected neuroretina cells is observed after a long latency period and takes place initially in a small number of cells. We also show that serial virus passaging onto fresh neuroretina cultures leads to the generation of novel mitogenic viruses containing the *mil* oncogene. DNA analysis indicated that RAV-1 is the only provirus detected in cells infected at virus passage 1, whereas neuroretina cells infected at subsequent virus passages harbor *mil*-containing proviruses. Three viruses, designated IC1, IC2, and IC3, were molecularly cloned. Restriction mapping indicated that in each virus, truncated c-*mil* sequences were inserted within different portions of the RAV-1 genome. In addition, IC1 and IC2 viruses have transduced novel sequences that belong to the 3′ noncoding portion of the c-*mil* locus. All three viruses induce neuroretina cell multiplication and direct the synthesis of *mil*-specific proteins. Proliferation of neuroretina cells infected at passage 1 of RAV-1 was not associated with any detectable rearrangement of c-*mil*, when a v-*mil* probe was used. However, these cells expressed high levels of an aberrant 2.8-kilobase mRNA hybridizing to *mil* but not to a long terminal repeat probe. Therefore, transcriptional activation of a portion of c-*mil* could represent the initial events induced by RAV-1 infection and lead to retroviral transduction of activated c-*mil* sequences.

Avian lymphomatosis viruses (ALVs) are replication-competent retroviruses which lack transforming genes but cause tumors in birds after a long latency period. These viruses do not transform any cell type in vitro. The integrated provirus of ALVs consists of three genes essential for replication (*gag*, *pol*, and *env*) flanked by long terminal repeats (LTR) that contain promoter, enhancer, and polyadenylation signals required for transcriptional regulation and recognized by the host cell (42).

ALVs induce mainly B-cell lymphomas, but also erythroblastosis, nephroblastomas, and fibrosarcomas (8, 40). Induction of lymphomas and erythroblastosis is associated with an increased expression of the cellular oncogenes c-*myc* and c-*erbB*, respectively, as a consequence of viral integration within these genes (13, 18, 32). ALVs were also shown to cause tumors by their ability to transduce in vivo cellular oncogenes, including *src* (17, 20), *fps* (29), *erbB* (19, 28, 45), and *fos* (30). There has been one report of in vitro transduction of a cellular oncogene in chicken fibroblasts chronically infected by a transformation-defective mutant of B77 Rous sarcoma virus (39). Because of the low frequency of these events, early steps of interactions between the ALV genome and regulation of cellular gene expression have been difficult to study in vivo and in vitro.

We have previously shown that differentiating neuroretina (NR) cells from avian embryos constitute a useful host system for studying the effects of viral oncogenes on cell growth regulation. As a consequence of oncogene expression, these nondividing cells are rapidly and massively induced to proliferate upon infection with various transforming retroviruses (4, 33). We report that chicken NR cells infected with the ALV Rous-associated virus type 1 (RAV-1) can also acquire sustained growth capacity. However, multiplication of RAV-1-infected NR cells markedly differs from that induced by retroviral oncogenes, since it is initially observed in a small number of infected cells, after a long latency period. We also show that serial passaging of RAV-1 onto fresh NR cells led to the transduction of novel retroviruses containing the *mil* oncogene, previously shown to express mitogenic activity in NR cells (1).

MATERIALS AND METHODS

Cell cultures and viruses. NR cultures were prepared from 8-day-old Brown Leghorn chicken embryos (Gs^+ Chf^+) of the Edinburgh strain, as previously described (33). Cultures were maintained and passaged in Eagle basal medium supplemented with 5 to 8% fetal calf serum.

Chicken embryo fibroblasts (CEF) were prepared from 11-day-old embryos by standard procedures and grown in Dulbecco modified Eagle medium containing 3 to 5% newborn calf serum, 1% heat-inactivated chicken serum, and 10% tryptose phosphate broth.

RAV-1 is a subgroup A lymphomatosis virus routinely grown in CEF. Fibroblasts were infected with serial threefold dilutions of an RAV-1 virus stock, and the titer of the virus was determined by resistance to superinfecting transforming Rous sarcoma virus (RSV) of the same subgroup. Virus cloned by two cycles of endpoint dilution was used as a source of RAV-1 in these studies.

NR cells seeded in 35-mm dishes (2×10^6 cells per dish)

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were infected with 0.1 ml of undiluted virus, as previously described (33).

DNA purification and restriction enzyme analysis. High-molecular-weight DNA was purified from cells by standard procedures (16). DNAs were digested to completion with restriction endonucleases under conditions recommended by the suppliers (Bethesda Research Laboratories, Inc.; New England BioLabs, Inc.; and Pharmacia, Inc.), fractionated by electrophoresis in 1% agarose gels, and transferred to nitrocellulose filters in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by the method of Southern (38). Hybridization was performed by the method of Wahl et al. (44) with probes radioactively labeled by nick translation (35).

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated by using the guanidium thiocyanate-cesium chloride method (5). The RNAs were denatured at 60°C in a formamide-formaldehyde mixture (26), fractionated by electrophoresis in 1% agarose–2.2 M formaldehyde gels (25), transferred to nitrocellulose filters in 20× SSC (41), and hybridized to ^{32}P-labeled probes.

Molecular probes. The following probes were used: (i) the 1.1-kilobase-pair (kbp) *Bam*HI-*Hpa*I v-*mil* fragment of the pMH2BS molecular clone (6); (ii) the 350-base-pair (bp) *Eco*RI fragment (*Eco*D) of long terminal repeat (LTR) sequences from the RSV molecular clone pSRA2 (9) (a generous gift of J. M. Bishop); (iii) the *Eco*RI-*Bam*HI fragment of pLTR-L6 containing U5 and leader sequences of RSV (kindly provided by T. Takeya); (iv) the 2.1-kbp *Sac*I-*Eco*RI *gag*-specific fragment of plasmid pB5*gag* (39); (v) the 1.8-kbp *Kpn*I-*Sac*I *env*-specific fragment of plasmid pB5*env* (39) (both pB5*gag* and pB5*env* plasmids were kindly provided by E. Stavnezer); (vi) a plasmid containing *pol* sequences obtained by subcloning the 2.2-kb *Hin*dIII-*Kpn*I *pol*-specific fragment of plasmid pSRA2 into pUC18.

Molecular cloning. High-molecular-weight DNA was partially digested with either *Eco*RI or *Sau*3AI and fractionated by centrifugation through sucrose gradients (26). The 4- to 8-kb *Eco*RI and the 10- to 20-kb *Sau*3AI DNA fragments were ligated to purified arms of λ gt11 (46) or λ EMBL3 (12) DNAs, respectively, and packaged in vitro (26). Recombinant bacteriophages were selected by plaque hybridization (2) with ^{32}P-labeled v-*mil* and LTR probes and further purified and amplified.

DNA sequencing. DNA fragments were subcloned into the *Sma*I site of the M13mp8 bacteriophage vector (27), and nucleotide sequences were determined by the dideoxy-chain termination method (36).

Transfection of NR cells. Eight-day-old chicken embryo NR cells were transfected with cloned DNA by the calcium phosphate method of Graham and Van der Eb (15). Briefly, 20 μg of phage DNA coprecipitated with 3 μg of plasmid pRAV-1 DNA was added in a 0.5-ml volume to 35-mm dishes containing 4×10^6 to 5×10^6 NR cells. After 1 h of incubation at 37°C, 1 ml of Eagle basal medium containing 10% fetal calf serum was added, and incubation was continued for 4 h. This medium was then removed, and the cells were subsequently maintained in Eagle basal medium supplemented with 8% fetal calf serum until proliferation became evident.

Protein labeling and immunoprecipitation. Labeling of NR cultures with [^{35}S]methionine and preparation of cell extracts were done as previously described (34). *mil* proteins were immunoprecipitated with either rabbit anti-*gag* serum or anti-*mil* serum (10). Immunoprecipitates were processed as previously described (34) and analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (24).

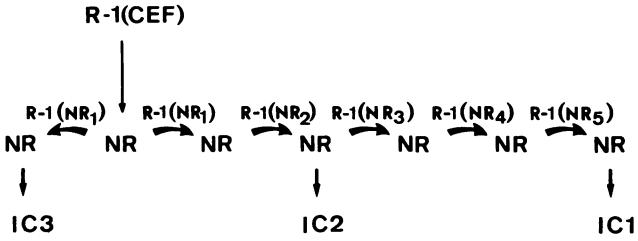

FIG. 1. Experimental protocol for in vitro generation of mitogenic viruses in NR cells infected with RAV-1. Abbreviations and symbols: R-1(CEF), RAV-1 cloned by endpoint dilution and grown in CEF; R-1(NR), RAV-1 harvested from proliferating NR cells; arrows, serial passaging of RAV-1(NR) on NR cells; R-1(NR_1), virus collected from NR cells infected with RAV-1(CEF), i.e., at passage 1 on NR cells; R-1(NR_2), virus collected from NR cells infected with RAV-1(NR_2), i.e., at passage 2 on NR cells and so forth.

RESULTS

Isolation of acutely mitogenic viruses from NR cells infected with RAV-1. Cultures of NR dissected from 8-day-old chicken embryos contain exclusively differentiating neurons and glial cells that rapidly cease to divide under our culture conditions (7). These resting cells can be maintained but not propagated in culture for about 2 months.

Five NR cultures, each containing 2×10^6 cells seeded in 35-mm dishes, were infected at a multiplicity of infection of 0.1 with RAV-1(CEF), a stock of RAV-1 purified by endpoint dilution and grown in CEF. As previously reported, RAV-1(CEF)-infected NR cells initially resembled uninfected cells in their normal morphology and limited growth capacity (3). However, after 4 to 6 weeks, one or two areas of multiplying cells were observed in three of five infected dishes. These foci were composed of a small number of flat cells with an epithelial-type morphology. In two dishes, NR cells ceased to divide after one passage, whereas cells of the third culture could be subcultured four times at a split ratio of 1:3. These cells will be referred to as the RAV-1(CEF)-infected NR proliferating cells. Given the small number of cells initially induced to proliferate, we estimated that cell proliferation continued through 10 to 15 generations. Multiplying cells remained flat and adherent to the substrate throughout their lifetime.

Medium harvested from the RAV-1(CEF)-infected NR proliferating cells was used to infect several dishes of fresh NR cells. Virus present in these supernatants will be designated RAV-1(NR_1). This time, foci of dividing cells were observed in all RAV-1(NR_1)-infected NR cultures after 3 to 4 weeks. Infected cultures were pooled, and multiplying cells were selected by further subcultivation. Supernatants of these cultures were harvested and used to infect fresh NR cells. This procedure, which was repeated three more times, resulted in accelerated cell proliferation upon each viral infection. Viral stocks collected after passage 5 on NR cells were able to induce cell multiplication within 7 days, suggesting that they contained an acutely mitogenic virus(es). This experimental protocol is summarized in Fig. 1.

Proliferating NR cells harbor *mil*-containing proviruses. To identify the mitogenic virus(es), DNA was extracted from infected NR cells at each passage, digested with *Eco*RI, and

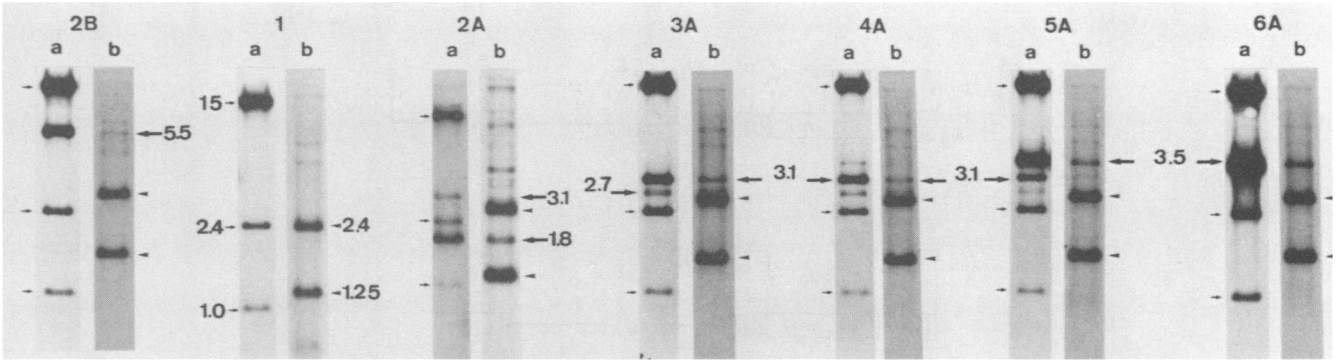

FIG. 2. Southern blot analysis of DNAs from RAV-1-infected NR cells. DNA (10 μg per lane) was digested with *Eco*RI, electrophoresed on 1% agarose gels, blotted on nitrocellulose filters, and hybridized to radioactive v-*mil* (lanes a) and LTR *Eco*D (lanes b) probes. Fragments indicated by → correspond to endogenous c-*mil* *Eco*RI fragments, whereas bands designated by ◄ correspond to 5′ and 3′ fragments of RAV-1 provirus. The sizes of DNA fragments are expressed in kilobase pairs. (A) DNA was extracted from multiplying NR cells infected with RAV-1(CEF) (panel 1) and from NR cells infected at passages 2 (2A), 3 (3A), 4 (4A), 5 (5A), and 6 (6A) of RAV-1(NR) on fresh NR cultures. (B) Virus supernatant collected from NR cells infected at passage 1 of RAV-1(NR) (panel 1) was used in a parallel experiment to infect NR cells, and DNA was extracted from those proliferating cells (panel 2B).

analyzed by Southern blot hybridization with v-*mil* and LTR probes (Fig. 2).

DNA from the RAV-1(CEF)-infected NR proliferating cells generated *Eco*RI fragments of 15, 2.4, and 1.0 kbp, representing the normal c-*mil* gene (22) (Fig. 2, lane 1a). Hybridization of this DNA to an LTR probe revealed two major fragments of 2.4 and 1.25 kbp, corresponding to the 5′ and 3′ portions of the RAV-1 genome, respectively (Fig. 2, lane 1b). From passage 2 of the virus onward, DNAs from RAV-1(NR)-infected cells contained additional fragments of various lengths hybridizing to both v-*mil* and LTR probes. Thus, NR cells infected with virus that had been passaged two to four times exhibited a major 3.1-kbp *Eco*RI fragment detected by v-*mil* and LTR probes (Fig. 2, panels 2A to 4A); at passages 5 and 6 of RAV-1(NR), DNA from infected cells contained a predominant fragment of 3.5 kbp detected by both v-*mil* and LTR probes (Fig. 2, panels 5A and 6A). The 3.5-kbp *Eco*RI fragment also hybridized to a *gag*-specific probe (data not shown) and represented a unique *mil*-containing provirus (Fig. 2, panel 6A). In parallel studies, RAV-1(NR_1) was used to infect another NR culture. DNA extracted from these proliferating cells contained a different provirus, generating a 5.5-kbp *Eco*RI fragment that hybridized to both v-*mil* and LTR probes (Fig. 1 and 2, panel 2B) but not to a *gag*-specific probe (data not shown).

Molecular cloning of *mil*-containing proviruses. We molecularly cloned *mil*-containing proviruses from NR cells infected with RAV-1(NR_2) and RAV-1(NR_5) and from cells which contained the 5.5-kbp *mil*-specific *Eco*RI fragment.

High-molecular-weight DNAs were partially digested with *Eco*RI or *Sau*3AI, ligated to purified arms of either λ gt11 or λ EMBL3 DNAs, and packaged in vitro. Recombinant clones hybridizing to both v-*mil* and LTR probes were selected, further purified, and amplified. Two *mil*-containing proviruses designated Institut Curie no. 1 and 2 (IC1 and IC2) were thus isolated: IC1 was isolated from NR cells infected with RAV-1(NR_5) and containing the 3.5-kbp *mil*-specific *Eco*RI fragment, and IC2 was isolated from NR cells infected with RAV-1(NR_2) and harboring the 3.1-kbp *Eco*RI fragment. A third provirus, designated IC3, was isolated from cells infected with RAV-1(NR_1), which contained the 5.5-kbp *Eco*RI fragment hybridizing to v-*mil*.

A restriction map of the three proviruses was obtained by digestion of phage DNAs with several restriction endonucleases, Southern blotting, and hybridization to probes representing different portions of the RAV-1 genome (Fig. 3). Analysis of the hybridization patterns showed that in all three proviruses, c-*mil*-derived sequences recombined with different portions of the RAV-1 genome. IC1 and IC2 contain the entire *env* gene but differ by the amount of their *gag* sequences. IC3 contains the entire *pol* and *env* genes but lacks most of the *gag* gene.

IC1 and IC2 proviruses contain novel c-*mil* sequences. Restriction mapping of IC1 and IC2 proviruses showed the presence of three *Hpa*I sites and one *Pst*I site located within the *mil-env* junction (Fig. 2). To characterize the structure of this region, *Hpa*I fragments of about 250 bp (fragment A) and 300 bp (fragment B) were purified from the molecular clone of IC2 and subcloned into the *Sma*I site of M13mp8 vector. Clones containing inserts in both orientations were obtained, and their nucleotide sequence was compared with that of the v-*mil* gene of MH_2 (14, 23), the chicken c-*mil* gene (21), and the *env* gene of Pr-RSV-C (37) (Fig. 4).

Nucleotides 1 to 43 were 100% homologous to both v-*mil* and chicken c-*mil* sequences. These sequences include the termination codon of both v-*mil* and c-*mil* (nucleotides 29 to 31). Beyond nucleotide 43, homology with published c-*mil* sequences extended up to the *Pst*I site corresponding to nucleotide 195 of the IC2 provirus. Between nucleotides 423 and 568 there was a 96% homology with the beginning of *env* sequences of Pr-RSV. The presence of unknown sequences between nucleotides 196 and 422 suggested that the c-*mil* sequences acquired by the IC2 virus included a thus far uncharacterized 3′ noncoding region of the cellular gene.

To determine the origin of these nucleotides, we subcloned the 318-bp *Hpa*I fragment B of IC2 DNA into the *Sma*I site of pUC8 (43) (Fig. 5C). A 220-bp *Ban*II-*Hin*dIII fragment of the recombinant plasmid was purified and used as a radioactively labeled probe (Fig. 5C).

DNA from chicken embryos was digested with *Eco*RI, *Hin*dIII, and *Sph*I, electrophoresed on agarose gels, and analyzed by Southern blotting with both v-*mil* and the 220-bp *Ban*II-*Hin*dIII fragment as probes. The results showed that sequences contained in the *Ban*II-*Hin*dIII probe hybridize to fragments of the c-*mil* locus (Fig. 5A).

Total cellular RNAs extracted from chicken and quail NR cells were electrophoresed, transferred to nitrocellulose filters, and hybridized to both v-*mil* and the *Ban*II-*Hin*dIII

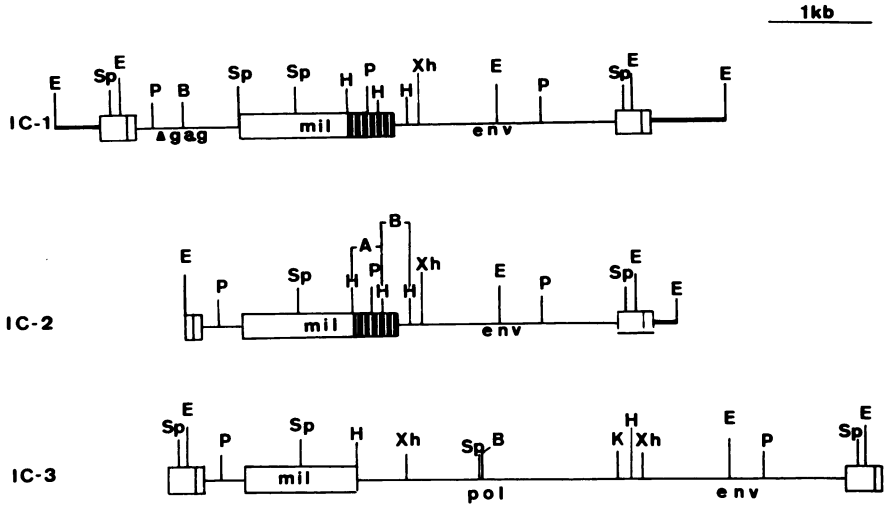

FIG. 3. Restriction maps of molecular clones of IC proviruses. DNAs were extracted from NR cells infected at passages 6 (IC1), 3 (IC2), and 2 (IC3) of RAV-1(NR) on NR cells. DNAs were partially digested with *Eco*RI (IC1 and IC2) or *Sau*3AI (IC3) and ligated to purified arms of either λ gt11 (IC1 and IC2) or λ EMBL3 (IC3) DNAs and packaged in vitro. Recombinant phages hybridizing to both v-*mil* and LTR probes were purified as described in Materials and Methods. DNAs of IC1, IC2, and IC3 proviruses were digested with restriction enzymes, blotted, and hybridized to v-*mil*, LTR *Eco*D, *env*, *gag*, and *pol* radioactive probes. Restriction maps of IC1 and IC2 proviruses were established by analysis of full-length biologically active molecular clones. The restriction map of IC3 provirus was reconstructed from two partial clones with large overlaps within the *mil-pol* region. Flanking cellular sequences of IC3 are not presented. Restriction enzymes: E, *Eco*RI; Sp, *Sph*I; P, *Pst*I; B, *Bam*HI; H, *Hpa*I; Xh, *Xho*I; K, *Kpn*I. Symbol: ▭, c-*mil* sequences of IC1 and IC2 proviruses that are not present in the v-*mil* gene of MH2.

probes. In cells from both species the 3.4-kb c-*mil*-specific mRNA also hybridized to the *Ban*II-*Hin*dIII probe (Fig. 5B). Finally, hybridization to DNA clones IC1 and IC3 indicated that the c-*mil* sequences contained in the *Ban*II-*Hin*dIII probe were present in IC1 but not in IC3 provirus (data not shown).

Taken together, these results confirmed that sequences corresponding to nucleotides 196 to 422, present in IC2 and

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v-mil    :  GTTAACATCCACAAGACTGCCTGTTTTTTAGGATTGTGCTCCC
c-mil    :  GTTAACATCCACAAGACTGCCTGTTTTTTAGGATTGTGCTCCCCTTCCCTTAATCTCTCC
IC-2     :  GTTAACATCCACAAGACTGCCTGTTTTTTAGGATTGTGCTCCCCTTCCCTTAATCTCTCC
             HpaI                                                    60

c-mil    :  AGTGATGGGAAAGGAACAGAAGTAAGAGAAGTTGTGCTTTTAATGCCTCAGTGTACAGGA
IC-2     :  AGTGATGGGAAAGGAACAGAAGTAAGAGAAGTTGTGCTTTTAATGCCTCAGTGTACAGGA
                                                                   120

c-mil    :  TCAGTGCCAGCAGGATCATCCGCATCCCCGTTTAAGAACAAGCTGCTAAGGATGTTTGCA
IC-2     :  TCAGTGCCAGCAGGATCATCCGCATCCCCGTTTAAGAACAAGCTGCTAAGGATGTTTGCA
                                                                   180

c-mil    :  GTTCTTACCCTGCAG
IC-2     :  GTTCTTACCCTGCAGGGACACAGCTCCACATTGCCTTTTTCTACAGTTTCTTCTACTGGG
                 PstI                                              240

IC-2     :  ATAGTTAACAGACTACAGATCAAGAGATCTATTACTATTTTTTTAATAGATGCACTTGAG
              HpaI                                                 300

IC-2     :  CCTTATTTTTCCCATACACAGAAGAGATGGATGTTTCTTTGGCAGTGTTTCTGGGTTGAT
                                                                   360

IC-2     :  TTGAATCTGCTGATAAACTGGTATTTTTTACAGCAGGGATGACCTGCCTTGGCATTTGAG
Env RSV  :                                                  GAGGTGA
                                                                   420

                          BanII
IC-2     :  GAAAGAAAGATGAGGCGAGCCCTCTTTTTTGCAGGCATTTCTGACTGGATACCCTGGGGAG
Env RSV  :  CTAAGAAAGATGAGGCGAGCCCTCTTTTTTGCAGGCATTTCTGACTGGATACCCTGGGAAG
                                                              G    480

IC-2     :  ACAAGCAAGAAGGACTCCAAGAAGAAGCCGCCAGCAACAAGCAAGAAGGACCCGGAGAAG
Env RSV  :  ACGAGCAAGAAGGACTCCAAGGAGAAACCGCTAGCAACAAGCAAGAAAGACCCGGAGAAG
              A                                                    540
                                    HpaI
IC-2     :  ACACCCTTGCTGCCAACGAGAGTTAAC
Env RSV  :  ACACCCTTGCTGCCAACGAGAGTTAAT
                                    568
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FIG. 4. Nucleotide sequence of the *mil-env* junction of IC2 provirus and comparison with v-*mil* sequences of MH2, chicken c-*mil*, and *env* gene of Pr-RSV-C. Sequence numbering begins at the first nucleotide of the *Hpa*I subcloning site of IC2. Novel c-*mil* sequences extend from the end of the *Pst*I site at nucleotides 195 to 423.

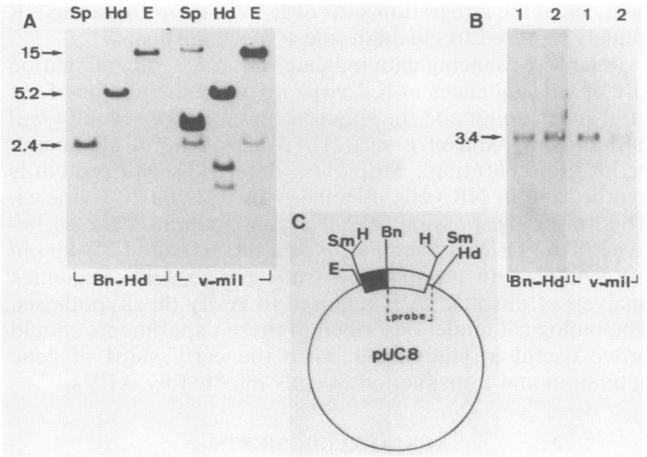

FIG. 5. Novel sequences in the *mil-env* junction of IC2 derive from the chicken c-*mil* locus. (A) Southern blot analysis of normal chicken DNA. Chicken fibroblast DNA (10 μg per lane) was digested with *Sph*I (Sp), *Hin*dIII (Hd), and *Eco*RI (E) and electrophoresed on 1% agarose gels. Southern blots of the gels were hybridized, under stringent conditions, with ^{32}P-labeled *Ban*II-*Hin*dIII and v-*mil* probes. (B) Northern blot analysis of total RNA of chicken (lanes 1) and quail (lanes 2) NR cells. Total RNAs (15 μg) were electrophoresed on 1% agarose–2.2 M formaldehyde gels, blotted, and hybridized with ^{32}P-labeled *Ban*II-*Hin*dIII and v-*mil* probes. An RNA ladder (Bethesda Research Laboratories) was run as a size marker. The size of RNA is expressed in kilobases. (C) Plasmid vector containing the *Ban*II-*Hin*dIII probe. The 318-bp blunt-ended *Hpa*I B fragment (see Fig. 3) of IC2 provirus was inserted into the blunt-ended *Sma*I (Sm) site of pUC8 vector. The probe was obtained by digestion with *Ban*II (Bn) and *Hin*dIII (Hd), electrophoresis on a 0.7% agarose gel, electroelution of the 220-bp fragment, and further purification with phenol-chloroform-isoamyl alcohol extractions.

IC1 proviruses, originated from the chicken c-*mil* locus and were contained in the c-*mil* transcript.

Mitogenic activity and expression of *mil*-containing proviruses in NR cells. Transfection of NR cells with IC1 and IC2 DNAs, in association with helper RAV-1 DNA, resulted in efficient cell proliferation. Similarly, supernatants collected from cells harboring the IC3 provirus induced NR cell division, indicating that these *mil*-containing proviruses are biologically active (data not shown).

Lysates of [^{35}S]methionine-labeled NR cells containing the IC1, IC2, or IC3 proviruses were immunoprecipitated with either *gag*- or *mil*-specific antiserum, and immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6). NR cells transfected with IC1 DNA synthesize a 68-kilodalton (kDa) polyprotein resulting from the fusion of *gag* and *mil* sequences. In contrast, NR cells transfected with IC2 DNA or infected with IC3 virus contain a 41-kDa protein immunoprecipitated only by anti-*mil*-specific antibodies.

c-*mil* expression of NR cells induced to divide by RAV-1 infection. As shown above (Fig. 2, panel 1A), NR cells induced to proliferate with RAV-1(CEF) do not harbor any *mil*-containing provirus. Moreover, digestion of DNA from these cells with various restriction enzymes did not reveal rearrangement of the c-*mil* locus when a v-*mil*-specific probe was used (data not shown). To understand the molecular events leading to the appearance, during subsequent virus passages, of *mil*-containing viruses, we investigated the pattern of c-*mil* transcription in NR cells infected with RAV-1(CEF).

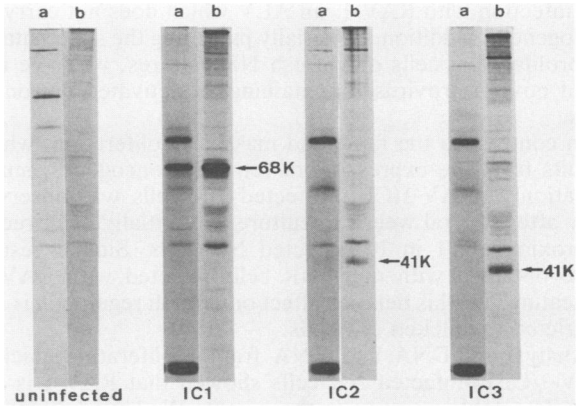

FIG. 6. Synthesis of *mil*-specific proteins in NR cells containing the IC proviruses. Uninfected NR cells and NR cells transfected with molecular clones of IC1 or IC2, or infected with IC3 virus, were metabolically labeled with [^{35}S]methionine. Cell extracts were immunoprecipitated with either rabbit anti-*gag* antiserum (lanes a) or rabbit anti-*mil* antiserum (lanes b). The products of the reaction were run on an 8.5% polyacrylamide gel.

Northern blot analysis of RNA extracted from these infected NR cells showed the presence, in addition to the 3.4-kb endogenous c-*mil* mRNA, of an abundant 2.8-kb transcript hybridizing to v-*mil*, but not to LTR, which was not detected in uninfected NR cells (Fig. 7A). Moreover, lysates of [^{35}S]methionine-labeled infected cells contained a 41-kDa protein immunoprecipitated only by anti-*mil*-specific antibodies (Fig. 7B), whereas the product of the normal c-*mil* gene is the p71-p73 c-*mil* protein (31).

DISCUSSION

We have shown that nondividing NR cells from 8-day-old chicken embryos acquire sustained growth capacity follow-

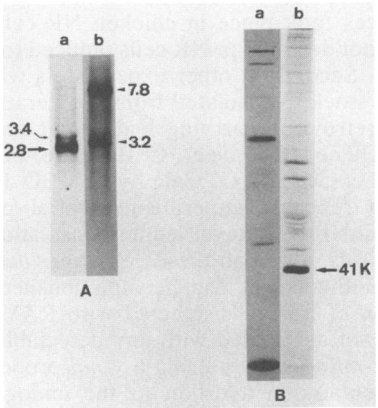

FIG. 7. Activation of c-*mil* in RAV-1-infected NR cells. (A) Northern blot analysis of total RNA of RAV-1-infected cells. Total RNA, extracted from multiplying NR cells infected with RAV-1(CEF), was denatured by formamide-formaldehyde treatment, fractionated on 1% agarose–2.2 M formaldehyde gels, and transferred onto nitrocellulose filters. Blots were hybridized with v-*mil* (lane a) and LTR leader probe (lane b). An RNA ladder (Bethesda Research Laboratories) was run as a size marker. The sizes of RNAs are expressed in kilobases. (B) Immunoprecipitation of *gag* and *mil* proteins of RAV-1-infected cells. NR cells induced to proliferate at passage 1 of RAV-1(CEF) were labeled with [^{35}S]methionine. Cell extracts were immunoprecipitated with anti-*gag* (lane a) and anti-*mil* (lane b) antisera and treated as described in the legend to Fig. 5. The 41-kDa protein is marked (41K).

ing infection with RAV-1, an ALV which does not carry an oncogene. In addition, by serially passaging the supernatants of proliferating cells onto fresh NR cultures, we have isolated novel retroviruses containing an activated *mil* oncogene.

In contrast to the rapid and massive proliferation, which results from the expression of retroviral oncogenes, multiplication of RAV-1(CEF)-infected NR cells was observed only after several weeks in culture and initially occurred in approximately 1 in 10^6 infected NR cells. Similar results were obtained with quail NR cells infected with RAV-1, indicating that this delayed effect on growth regulation is not restricted to chicken NR cells.

Analysis of DNA and RNA from proliferating chicken RAV-1(CEF)-infected NR cells showed that RAV-1 is the only detectable provirus in these cells. We have previously reported that RAV-1 efficiently replicates in NR cells and that infected NR cells and CEF release comparable amounts of virus (3). Therefore, RAV-1 replication, which occurs in a majority of cells, may be necessary but not sufficient for induction of proliferation, which occurs in an extreme minority of infected cells. Proliferation must result from thus far undefined interactions of the RAV-1 genome and/or RAV-1 gene products with growth-regulatory elements in a small number of NR cells. Alternatively, RAV-1 integration itself may disrupt or modify growth regulation.

In contrast, NR cells infected with RAV-1(NR) contained c-*mil*-derived sequences associated with LTRs and released viruses able to induce proliferation of newly infected cells. Analysis of molecular clones of IC1, IC2, and IC3 proviruses showed that they represent novel replication-defective retroviruses containing the c-*mil* oncogene; the novelty lies in their genetic organization and the presence, in two of them (IC2 and IC1), of additional sequences derived from the 3′ noncoding region of the c-*mil* locus. Multiplication of NR cells induced by these viruses is correlated with the synthesis of *mil*-specific proteins.

We have three reasons to believe that transduction of c-*mil* sequences took place in chicken NR cells. First, IC viruses were not detected in NR cells induced to multiply by RAV-1(CEF). Second, in other experiments with the same RAV-1(CEF) stock, we isolated from proliferating NR cells a mitogenic retrovirus carrying a different oncogene (M. Marx, A. Eychène, D. Laugier, C. Béchade, P. Crisanti, P. Dezélée, B. Pessac, and G. Calothy, EMBO J., in press). Moreover, in repeated experiments, serial passaging of RAV-1 in quail NR cells never led to the isolation of acutely mitogenic viruses, although these cells were actively multiplying (G. Calothy and D. Laugier, unpublished results).

Proliferation of NR cells infected with RAV-1 collected from CEF is not associated with any detectable rearrangement of the c-*mil* locus, by using a v-*mil* probe. However, these cells contain, in addition to the endogenous c-*mil* mRNA of 3.4 kb, an abundant transcript, 2.8 kb in length, hybridizing to a *mil* but not to an LTR probe. Analysis of other NR cultures induced to proliferate by RAV-1(CEF) showed the presence of this transcript and of the 41-kDa *mil* protein in about one-third of the multiplying cultures (data not shown). Therefore, transcriptional activation of a portion of c-*mil* and synthesis of a truncated *mil* protein could represent the initial events induced by RAV-1 infection in a subpopulation of NR cells. These cells would multiply because this protein directly expresses mitogenic capacity (11). It is not known whether the components of the RAV-1 genome, responsible for transcriptional activation, act in the vicinity of or at distance from the c-*mil* locus. Extensive analysis of the integration sites of RAV-1 in proliferating NR cells is required to elucidate these mechanisms.

Recent sequencing data indicate that the 5′ recombination site of *mil* sequences in IC2 virus involved the joining of the viral splice donor and the splice acceptor of the second v-*mil* homologous exon of c-*mil* (21) (A. Eychène et al., manuscript in preparation). Moreover, the 41-kDa *mil* protein is synthesized in NR cells infected with IC2 and IC3 viruses. This raises the possibility that recombination occurred between RAV-1 leader sequences and the activated 2.8-kb *mil* mRNA, already producing a truncated protein. Sequence analysis of this mRNA is required to verify this hypothesis. The biological model described in these experiments should prove useful in studying in vitro the early steps of gene activation and transduction in cells infected by ALVs.

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