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Molecular and Biological Properties of c-*mil* Transducing Retroviruses Generated during Passage of Rous-Associated Virus Type 1 in Chicken Neuroretina Cells

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IC1, IC2, and IC3 are novel c-*mil* transducing retroviruses generated during serial passaging of Rous-associated virus type 1 (RAV-1) in chicken embryo neuroretina cells. They were isolated by their ability to induce proliferation of these nondividing cells. IC2 and IC3 were generated during early passages of RAV-1 in neuroretina cells, whereas IC1 was isolated after six consecutive passages of virus supernatants. We sequenced the transduced genes and the *mil*–RAV-1 junctions of the three viruses. The 5′ RAV-1–*mil* junction of IC2 and IC3 was formed by a splicing process between the RAV-1 leader sequence and exon 8 of the c-*mil* gene. The 5′ end of IC1 resulted from homologous recombination between *gag* and *mil* sequences. Reconstitution experiments showed that serial passaging of IC2 in neuroretina cells also led to the formation of a *gag-mil*-containing retrovirus. Therefore, constitution of a U5–leader–Δc-*mil*–ΔRAV-1–U3 virus represents early steps in c-*mil* transduction by RAV-1. This virus further recombined with RAV-1 to generate a *gag-mil*-containing virus. The three IC viruses transduced the serine/threonine kinase domain of the cellular gene. Hence, amino-terminal truncation is sufficient to activate the mitogenic property of c-*mil*. Comparison of the transforming properties of IC2 and IC1 showed that the transduced *mil* gene, expressed as a unique protein independent of *gag* sequences, was weakly transforming in avian cells. Acquisition of *gag* sequences by IC1 not only increased the rate of virus replication but also enhanced the transforming capacity of the virus.

Oncogenes of retroviruses arose by transduction of cellular proto-oncogenes (47) by viruses carrying only replicative genes, such as avian leukosis viruses (ALV) (4). Generation of an acutely transforming retrovirus not only requires the transducing event but also involves several changes in both viral and oncogenic sequences. Viral oncogenes differ from their cellular homologs by base changes, loss of part of the cellular gene sequences, or fusion to viral coding sequences to create a hybrid protein (4). Transduction of oncogenes generally deletes large portions of the replicative genes. Therefore, transforming retroviruses are most often replication defective and need association with a helper virus. It is generally assumed that integration of ALV in the vicinity of a proto-oncogene constitutes the first step in retroviral transduction. This and further events are difficult to investigate during in vivo generation of transforming viruses.

We recently described a biological model for reproducible in vitro transduction by ALV Rous-associated virus type 1 (RAV-1). We reported that serial passaging of RAV-1 on chicken embryo neuroretina (NR) cells resulted in retroviral transduction of cellular oncogenes which induce the proliferation of these nondividing cells. We characterized a retrovirus, IC10, which contains a new member of the *mil-raf* gene family, designated R*mil* (11, 30). Three other viruses, IC1, IC2, and IC3, transduced c-*mil* sequences inserted within different portions of the RAV-1 genome (29). IC2 and IC3 were isolated during early passages of RAV-1 on NR cells, whereas IC1 was isolated at later passages of culture supernatants on these cells. Therefore, it appears that this model system yields recombinant viruses containing proto-oncogenes of the same family as that of murine sarcoma virus 3611, isolated by a procedure which included both in vitro and in vivo experiments (40).

In this study, we characterized the content of transduced c-*mil* sequences and the structure of the RAV-1–*mil* junctions of IC1, IC2, and IC3 viruses. We report that early steps in transduction involve a splicing process between the leader sequence of RAV-1 and activated c-*mil* sequences. The transduced sequences contain the serine/threonine kinase domain of the cellular gene, which is sufficient to induce NR cell proliferation. We also show that *gag-mil*-containing retroviruses arise by further recombination between these early viral forms and the RAV-1 genome. Acquisition of *gag* sequences does not influence mitogenic activity but enhances the rate of replication and the transforming capacity of c-*mil* transducing retroviruses.

MATERIALS AND METHODS

Cell cultures and viruses. NR cell cultures were prepared from 7-day-old brown leghorn chick embryos (gs^+ chf^+) of the Edinburgh strain as previously described (37). They were maintained and subcultured in Eagle basal medium supplemented with 5 to 8% fetal calf serum.

Wild-type MH2 (RAV-1) was recovered from quail embryo fibroblasts cotransfected with pMH2-Hd (7) and helper pRAV-1 DNA. PA200-MH2 (RAV-1) is a spontaneous variant of MH2 (RAV-1) carrying a large deletion of the 3′ portion of the v-*myc* oncogene, which was previously characterized (2, 28). We used virus obtained from NR cells cotransfected with a PA200 DNA clone and helper pRAV-1 DNA. IC1, IC2, and IC3 are novel c-*mil* transducing retroviruses which were recently isolated (29). Virus produced by NR cells transfected with IC1 or IC2 molecular clones and RAV-1 DNA was used in this study.

NR cultures were transfected with cloned DNAs by the

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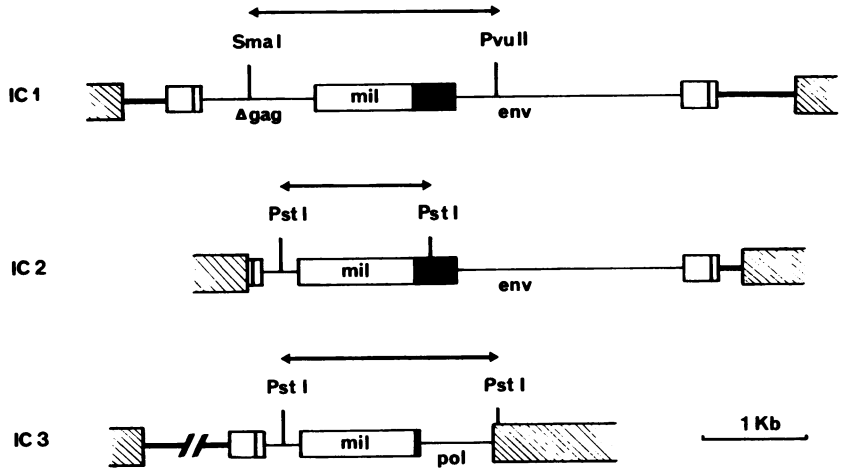

FIG. 1. Sequenced regions of IC1, IC2, and IC3 proviruses. The genetic organization of molecular clones of IC1, IC2, and IC3 proviruses is presented. Restriction sites used to subclone fragments containing c-*mil* and adjacent RAV-1 sequences into the Bluescript vector are indicated. Arrows indicate the sequenced portions of proviruses. Solid boxes represent 3′ noncoding sequences of the c-*mil* gene. Hatched boxes represent lambda vector arms.

calcium phosphate method of Graham and Van Der Eb (15) as previously described (29). Virus was collected when cell proliferation became evident.

Assay of viral infectivity. NR cells were infected as previously described (37). The mitogenic activity of IC viruses was quantitated by infecting NR cells with serial 10-fold dilutions of virus. The mitogenic titer was defined as the reciprocal of the highest dilution inducing cell proliferation after one subcultivation and is expressed as mitogenic units per milliliter.

Measurement of NR cell growth and anchorage independence. NR cells were infected with undiluted virus. When proliferation became evident in infected cultures, cells were passaged twice and seeded at a low density in 60-mm dishes. Cells were counted at various intervals.

For the anchorage-independent growth assay, infected NR cells were subcultured twice and suspended in agar-containing medium at concentrations ranging from 1×10^5 to 3×10^5 cells per 60-mm dish. Solid medium was minimal essential medium containing 0.70% agar, 10% tryptose phosphate broth, 2% heat-inactivated chicken serum, and 8% fetal calf serum. Single-cell suspensions were prepared in the same medium containing 0.35% agar.

Protein labeling and immunoprecipitation. Labeling of NR cells with [^{35}S]methionine and preparation of cell lysates were done as previously described (38). *mil* proteins were immunoprecipitated with either anti-*gag* or anti-*mil* (9) rabbit antiserum. Immunoprecipitates were treated as previously described (37) and analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (27).

DNA purification and restriction enzyme analysis. High-molecular-weight DNA was purified from NR cells by standard procedures (16). DNAs were digested to completion with *Eco*RI restriction endonuclease under the conditions recommended by the supplier (New England BioLabs, 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 (45). Hybridization was performed by the method of Wahl et al. (51) with probes radioactively labeled by nick translation (41). The v-*mil*-specific probe was the 1.1-kilobase-pair (kbp) *Bam*HI-*Hpa*I v-*mil* fragment of the pMH2BS molecular clone (7).

DNA sequencing. The sequenced portions of IC viruses are described in Fig. 1. The sequence was determined as follows. The *Sma*I-*Pvu*II blunt-ended restriction fragment of IC1 virus and the *Pst*I restriction fragments of IC2 and IC3 viruses (Fig. 1) were subcloned into the *Sma*I and *Pst*I sites of the Bluescript vector (Stratagene), respectively. Appropriate blunt-ended restriction fragments of these clones were subcloned into the *Sma*I site of the M13mp8 bacteriophage vector (31), and the nucleotide sequence was determined by the dideoxy chain termination method (42).

RESULTS AND DISCUSSION

Characterization of IC viruses. IC1, IC2, and IC3 are replication-defective retroviruses generated during serial passaging of RAV-1 in chicken NR cells. They were isolated by their ability to induce sustained proliferation of these cells (29). Restriction analysis of molecular clones showed that these viruses transduced c-*mil* sequences recombined within different portions of the helper 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, like IC2, most of the *gag* gene. IC2 was isolated from NR cells infected at the second passage of RAV-1, whereas IC1 was isolated at the sixth passage of culture supernatants in the same experiment. IC3 was isolated at the second passage of RAV-1 on NR cells in an independent experiment. IC2 and IC3 encode a 41-kilodalton *mil*-specific protein ($p41^{mil}$), whereas the *mil* gene of IC1 is expressed through a $P68^{gag\text{-}mil}$ fusion protein.

Sequence analysis of transduced c-*mil* genes in IC viruses. To determine the content of transduced sequences in IC viruses, we subcloned provirus portions containing the *mil* gene (Fig. 1) into the M13mp8 vector and sequenced them by the dideoxy chain termination method of Sanger (42). Sequences were compared with those of a chicken c-*mil* cDNA (26) and the v-*mil* gene of MH2 (48). Reconstitution of the c-*mil* gene is presented in Fig. 2. The organization and numbering of c-*mil* exons is based on (i) the complete coding sequence of a chicken c-*mil* cDNA (26), (ii) the structure of the carboxyl part of the chicken cellular gene (24), and (iii) the numbering of exons proposed for the human c-*raf*-1 gene (5).

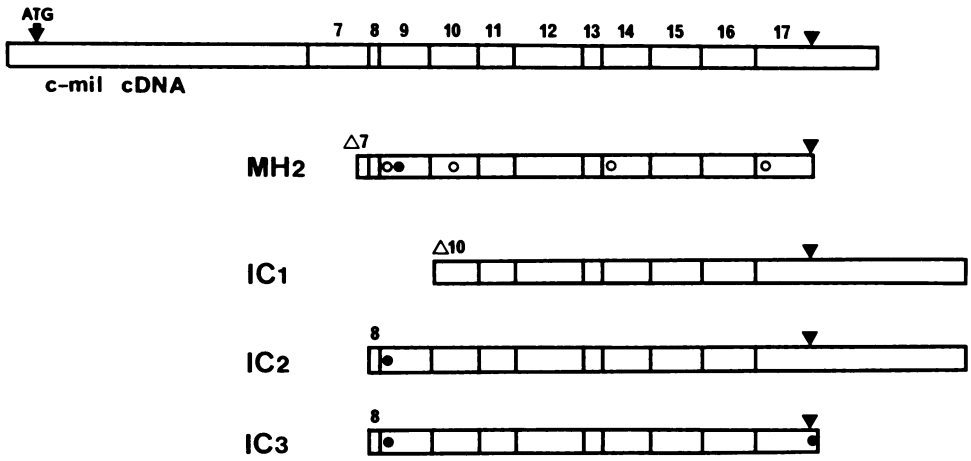

FIG. 2. Molecular organization of c-*mil* sequences transduced in IC1, IC2, and IC3 viruses. The nucleotide sequences of the *mil* genes of the IC1, IC2, and IC3 proviruses are compared with those of the chicken c-*mil* cDNA and of the v-*mil* gene of MH2 virus. Exons are indicated by vertical lines and are numbered according to the human c-*raf*-1 gene. Open and solid triangles indicate incomplete exons and termination codons, respectively. Open circles designate point mutations leading to an amino acid substitution, and solid circles designate silent point mutations.

IC2 and IC3 transduced 1,501 and 1,129 base pairs (bp) of c-*mil* sequences, respectively. In both viruses, transduced sequences begin at nucleotide 1 of exon 8 of the cellular gene, corresponding to nucleotide 907 of the c-*mil* cDNA (26). They both retain the stop codon of the c-*mil* protein and 3′ noncoding sequences of the cellular gene: 391 bp in IC2 (29) and 19 bp in IC3. The transduced *mil* sequences encode a 369-amino-acid protein with a calculated molecular weight of 42,411. This result is in agreement with the detection by immunoprecipitation of a *mil* protein with an apparent molecular weight of 41,000 in NR cells infected with either virus.

IC1 contains 1,342 bp of c-*mil* sequences which begin at nucleotide 4 of exon 10 of the cellular gene, that is, at nucleotide 1066 of the c-*mil* cDNA (26). It also contains the stop codon of the cellular gene and the same 391 bp of downstream noncoding sequences as in IC2. IC1 carries the smallest coding portion of the c-*mil*/c-*raf* gene activated by in vivo amino-terminal truncation (5, 12, 23, 33, 44, 46, 50). This sequence encodes a protein that has a calculated molecular weight of 36,079 and that is expressed as a $P68^{gag\text{-}mil}$ fusion protein in IC1-infected cells.

When compared with the corresponding sequences of the chicken c-*mil* cDNA, the coding portions of the transduced c-*mil* genes in IC viruses did not have base mutations leading to amino acid substitutions. In contrast, the v-*mil* gene of MH2 carries five base mutations corresponding to four amino acid changes (48). Only one silent mutation (C to T) was detected in the coding region of IC2 and IC3 at position 945 relative to the c-*mil* cDNA sequence, and one base change (T to C) was detected in the 3′ noncoding region of IC3 at position 2030 relative to the same sequence. No mutation was found in the *mil* sequence of IC1.

In summary, all three IC viruses, like MH2, transduced the carboxy-terminal part of c-*mil*, which contains the serine/threonine kinase domain (24). Therefore, it is likely that the activation of this gene in retroviruses resulted from its NH_2-terminal truncation. Our data also confirm previous results showing that the induction of NR cell proliferation does not require mutation of the carboxy-terminal portion of c-*mil* (10).

5′ RAV-1–*mil* junctions in IC viruses. To determine the structures of recombination sites between c-*mil* and the RAV-1 genome, we sequenced junction fragments in IC viruses and compared these sequences to those of the RAV-1 genome (11) and of the chicken c-*mil* locus (28).

In IC2 and IC3, the splice donor site of the RAV-1 leader sequence (17) was linked to the splice acceptor site of exon 8 of c-*mil* (Fig. 3A). There was no homology in this region between exonic or intronic sequences of c-*mil* and RAV-1, except for the AGG intron-exon consensus sequence (34). Therefore, it is likely that 5′ recombination in IC2 and IC3 genomes involved a splicing process which provided these viruses with all signals important for viral replication and expression (8, 19, 25).

The 5′ recombination site of IC1 was located about 758 bases downstream from the AUG initiation codon of *gag* and around nucleotide 8 of c-*mil* exon 10 (Fig. 3B). This junction allowed an in-frame fusion of the two genes. In this region, we detected a strong homology (8 of 9 bases) between *gag* and *mil* sequences. Furthermore, this junction site did not contain splice boundaries. This result suggested that the 5′ junction of IC1 was generated by homologous recombination between *gag* and *mil* sequences.

3′ *mil*–RAV-1 junctions in IC viruses. We previously reported that IC2 transduced 391 bp of 3′ noncoding sequences belonging to the c-*mil* locus (29). Sequencing data confirmed that these 391 nucleotides were also present in IC1 and that both viruses had identical *mil-env* junctions (Fig. 4A). The recombination site was located about 31 bases upstream from the *env* gene of RAV-1 (6). Since c-*mil* sequences beyond these 391 bp are not known, it is difficult to speculate about the mechanisms of *mil-env* recombination in both viruses.

The sequence of the 3′ recombination site in IC3 was compared with those of the 3′ regions of the c-*mil* locus (24) and of Rous sarcoma virus (43). Recombination took place 19 bases downstream from the c-*mil* termination codon and 5 bases upstream from that of the *gag* gene (Fig. 4B). There was no sequence homology in this region between c-*mil* and the viral *gag-pol* junction, assuming that the latter is well conserved in RAV-1. However, by comparing these sequences around the recombination site, we found a pair of 8-bp inverted repeats in the *gag-pol* junction of Rous sarcoma virus (Fig. 4B). This structure could allow the formation of a hairpinlike configuration reinforced by the T to C

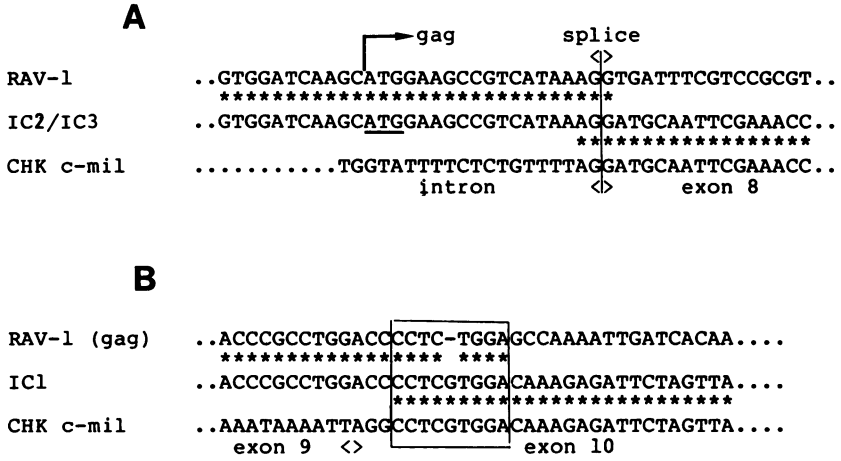

FIG. 3. Nucleotide sequences of 5′ recombination junctions. The sequences of the 5′ RAV-1–c-*mil* junctions of IC2 and IC3 (A) and IC1 (B) proviruses were compared with those of RAV-1 and the chicken (CHK) c-*mil* gene. Nucleotide homologies are indicated by asterisks. The vertical line indicates the recombination site in IC2 and IC3. The initiation codon of the leader sequence of RAV-1 is underlined. The box indicates homology between *gag* and *mil* sequences. A hyphen was added to the RAV-1 *gag* gene to align sequences.

mutation which is located 5 bases upstream from the 3′ recombination site and which introduces a GC pair instead of the expected GU pair. However, we cannot exclude the possibility that this mutation resulted from, rather than contributed to, the recombination between c-*mil* and RAV-1 sequences.

Mitogenic and transforming properties of IC viruses. We previously reported that PA200, a spontaneous mutant of MH2 with a large deletion in the v-*myc* gene, efficiently induced NR cell proliferation but caused only limited in vitro transformation (2, 3). This virus encodes a *gag-mil* fusion protein. The availability of viruses, such as IC2 or IC3, which express the *mil* gene product as a unique protein made it possible to study the mitogenic and transforming properties of this gene in the absence of *gag* sequences.

NR cells infected with IC1 or IC2 were passaged twice to select for growing cells and were subsequently seeded at a low density for a study of their growth curves (Fig. 5B). Growth kinetics of NR cells infected with either virus were similar, indicating that the addition of *gag* sequences to c-*mil* did not influence the growth rate of infected NR cells. However, the presence of *gag* sequences strikingly increased the rate of IC virus replication. IC1 replicated with a much higher efficiency than did IC2, since virus titers produced by chronically infected NR cells usually reached 10^7 mitogenic units per ml, as compared with about 10^4 mitogenic units per ml for IC2.

Proliferating NR cells infected with either IC1 or IC2 remained flat and adherent to the substrate. They were indistinguishable by morphology from PA200-infected cells. Cultures of NR cells expressing only the *mil* gene were devoid of round refringent cells, unlike those infected by MH2, which expressed both the v-*mil* and v-*myc* oncogenes (data not shown). NR cells transformed by MH2 formed large and numerous colonies in soft agar-containing medium (Fig. 5A, panel a), whereas NR cells infected with IC1 or PA200 gave rise to much smaller colonies (Fig. 5A, panels b and c). In contrast, NR cells infected with IC2 were unable to grow in soft agar (Fig. 5A, panel d).

Our data on the transforming properties of retrovirally activated *mil* protein in the absence of *gag* sequences strengthen our previous conclusions that the *mil* oncogene is

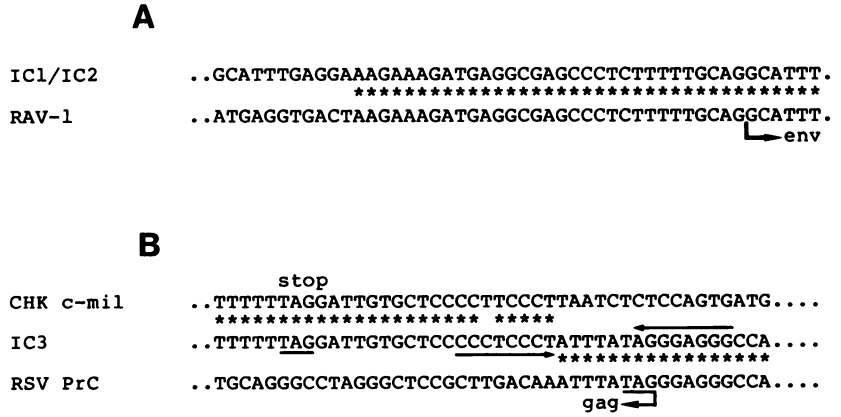

FIG. 4. Nucleotide sequences of 3′ recombination junctions. The sequences flanking the 3′ c-*mil*–RAV-1 junctions of IC1 and IC2 (A) were compared with that of the *env* region of RAV-1. The sequence of the 3′ c-*mil*–RAV-1 junction of IC3 (B) was compared with those of the chicken (CHK) c-*mil* gene and the *gag-pol* region of the Rous sarcoma virus (RSV) Prague C (PrC) strain. The termination codons of the *mil* and *gag* genes are underlined. Nucleotide homologies are indicated by asterisks. Arrows indicate the complementary inverted sequences.

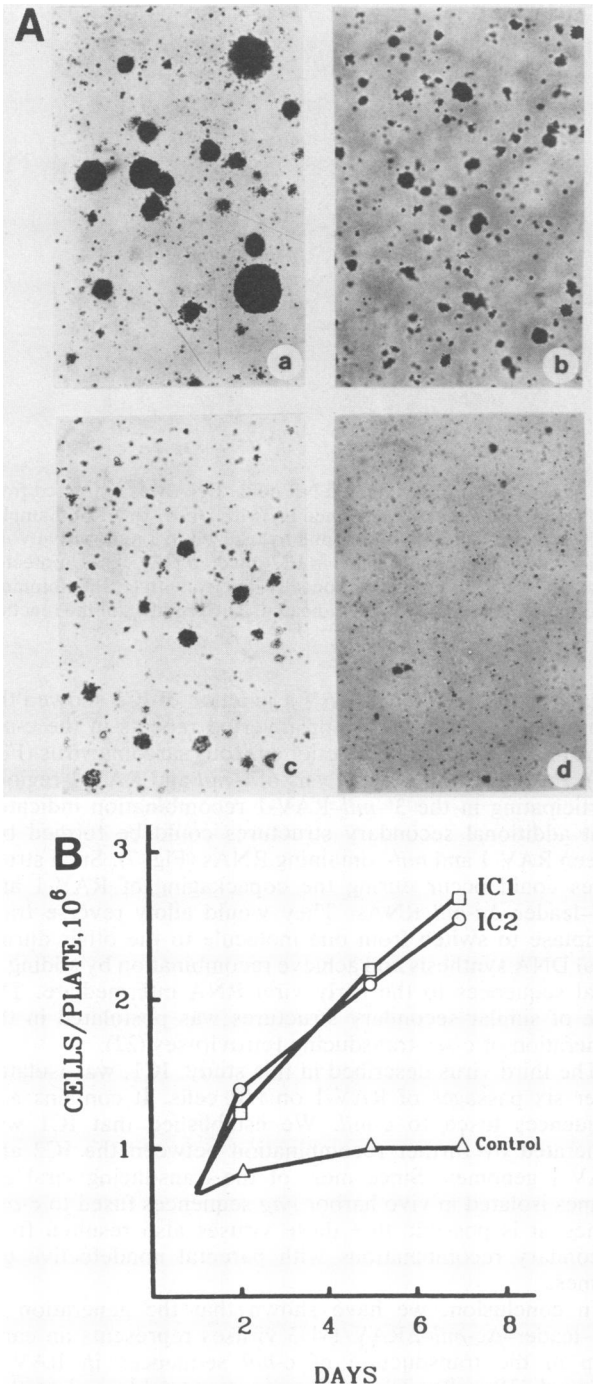

FIG. 5. Mitogenic and transforming properties of IC viruses. (A) Anchorage-independent growth of virus-infected cells. NR cells were infected with MH2 (a), PA200 (b), IC1 (c), and IC2 (d). Cultures were passaged twice and subsequently seeded in soft agar-containing medium. Colonies were observed 10 to 14 days later. (B) Growth kinetics of virus-infected NR cells. Control and IC virus-infected NR cells were subcultured once and then seeded at a density of 6×10^5 cells per 60-mm dish. Medium was renewed daily, and cells were counted at the indicated times.

a weakly transforming gene in avian cells (3). Fusion with *gag* sequences not only increased the replicative ability of IC viruses but also enhanced their transforming capacity. The added *gag* sequences provide these viruses with an additional packaging signal located 150 bp downstream from the ATG start codon of the virus (39) and with *cis*-acting enhancer elements located about 900 bp downstream from the site of transcription initiation (1). While the presence of these signals should obviously improve the rate of virus replication, their role in increasing the transforming properties of *mil*-containing viruses is unclear.

Generation of *gag-mil*-containing IC viruses. IC2 and IC3 were isolated during early passages of RAV-1 on NR cells, whereas IC1 was isolated after six virus passages. To investigate the relationship between these early and late isolates, we serially passaged virus produced by NR cells cotransfected with a molecular clone of IC2 and RAV-1 DNA on fresh cultures. DNA extracted from cells infected at each virus passage was digested with *Eco*RI, blotted, and hybridized to a v-*mil*-specific probe (Fig. 6A). All infected NR cells contained, besides the chicken c-*mil* fragments, the 3.1-kbp *Eco*RI fragment corresponding to the 5′ portion of the IC2 genome (29). However, *Eco*RI digestion of DNA from NR cells infected at the sixth passage of culture supernatants generated an additional 4.3-kbp fragment which also hybridized to a *gag*-specific probe (data not shown).

NR cells infected at each virus passage were labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with either *gag*- or *mil*-specific antiserum, and immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6B). All infected cultures contained the p41^{mil} protein, whereas only lysates of cells infected at the sixth virus passage contained a 93-kilodalton hybrid protein immunoprecipitated by both anti-*mil* and anti-*gag* antibodies.

These results showed that recombination between IC2 and RAV-1 genomes took place during multiple cycles of virus replication in NR cells and generated a new *gag-mil*-containing virus encoding a *gag-mil* hybrid protein. Since IC1 and IC2 contain the same 3′ *env-mil* junction site and were isolated during the same experiment, we conclude that IC1 was generated by recombination between IC2 and RAV-1 genomes.

Mechanism of c-*mil* transduction. There have been several reports on in vivo transduction of proto-oncogenes, including *src* (13, 18, 22), *fps* (35), *erbB* (21, 32), and *fos* (36), by ALV. A general model for a multistep transduction of proto-oncogenes by ALV has been proposed (49). It relies upon the study of retroviruses which achieved their final and stable structure through multiple passages in vivo and in vitro. Our experimental system of in vitro transduction enabled us to determine the genetic structure of c-*mil*-containing viruses generated at different stages during serial passaging of RAV-1 on NR cells.

IC2 and IC3, which are the first viruses detected in RAV-1-infected NR cells, could represent early forms in the transduction process. The 5′ ends of these viruses were generated by joining the regular splice donor site of the RAV-1 leader sequence to the acceptor site of c-*mil* exon 8. Similar structures were recently described in the 5′ ends of two viruses which transduced the c-*src* gene (13, 22). It is possible that the transduction of c-*mil* proceeded by the integration of RAV-1 provirus upstream of exon 8 of c-*mil* and the cotranscription of viral and c-*mil* sequences. These cotranscripts could be formed by either the deletion of 3′ viral sequences (49) or the generation of readthrough transcripts (20) escaping cleavage and polyadenylation at the normal site in the 3′ long terminal repeat and extending into the truncated c-*mil* gene. A splice junction could then occur

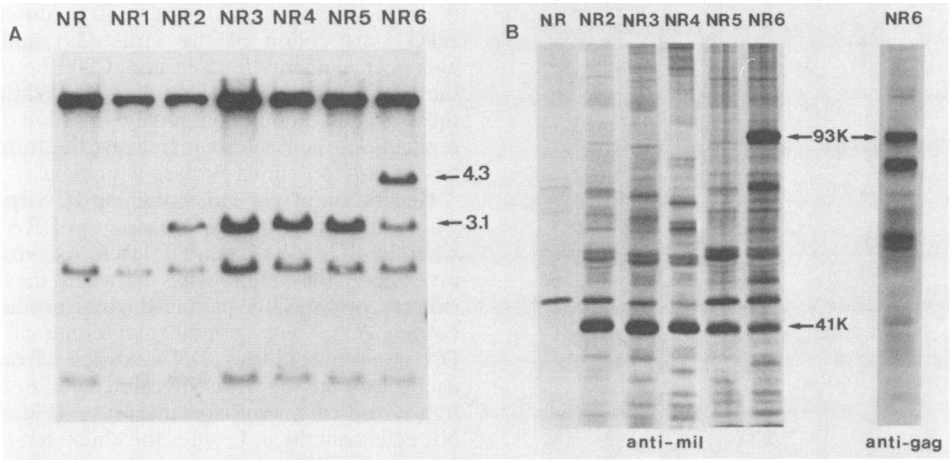

FIG. 6. Generation of *gag*-containing viruses. (A) Southern blot analysis of DNAs from IC2-infected NR cells. DNA was extracted from uninfected NR cells (NR) and from proliferating NR cells infected with serially passaged molecularly cloned IC2 virus (NR1 to NR6). Samples (10 μg) were digested with *Eco*RI, electrophoresed on 1% agarose gels, blotted on nitrocellulose filters, and hybridized to a radioactive v-*mil* probe. The sizes of the DNA fragments are expressed in kilobases. (B) Synthesis of *mil*-specific proteins in IC2-infected NR cells. Uninfected NR cells (NR) and NR cells infected with serially passaged cloned IC2 virus (NR2 to NR6) were metabolically labeled with [^{35}S]methionine. Cell extracts were immunoprecipitated with either anti-*gag* or anti-*mil* antiserum prepared in rabbits as indicated. The products of the reaction were run on an 8.5% polyacrylamide gel.

between the donor site of RAV-1 and the acceptor site of c-*mil* exon 8, together with normal splicing between the remaining 3′ exons. cDNA clones corresponding to read-through transcripts have been isolated during activation of c-*erbB* in ALV-induced erythroblastosis (14). Transcription of truncated c-*erbB* mRNAs was initiated in the 5′ long terminal repeat of the integrated RAV-1 provirus and was processed through a series of remarkable splicings in which the splice donor site of the RAV-1 genome was linked to the acceptor site of the truncated c-*erbB* mRNA. According to this model, the early intermediate in the generation of IC2 and IC3 viruses could be a U5–leader–Δc-*mil* RNA.

The generation of a complete retrovirus genome requires the acquisition of a U3 region. Analysis of the sequencing

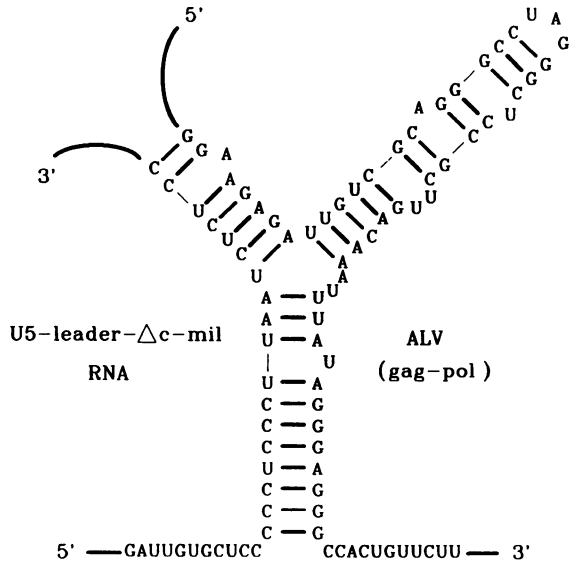

FIG. 7. Proposed model for 3′ recombination in IC3. Secondary structures that could be formed between the *gag-pol* region of RAV-1 and transduced c-*mil* sequences are shown.

data around the 3′ c-*mil*–RAV-1 junction of IC3 showed the presence of a stretch of 8-bp inverted repeats in the c-*mil* gene and in the *gag-pol* junction of Rous sarcoma virus (Fig. 4B). Further computer analysis of c-*mil* and RAV-1 regions participating in the 3′ *mil*–RAV-1 recombination indicated that additional secondary structures could be formed between RAV-1 and *mil*-containing RNAs (Fig. 7). Such structures could occur during the copackaging of RAV-1 and U5–leader–Δc-*mil* RNAs. They would allow reverse transcriptase to switch from one molecule to the other during viral DNA synthesis and achieve recombination by adding 3′ viral sequences to the early viral RNA intermediate. The role of similar secondary structures was postulated in the generation of c-*src* transducing retroviruses (22).

The third virus described in this study, IC1, was isolated after six passages of RAV-1 on NR cells. It contains *gag* sequences fused to c-*mil*. We established that IC1 was generated by further recombination between the IC2 and RAV-1 genomes. Since most of the transducing viral genomes isolated in vivo harbor *gag* sequences fused to c-*onc* genes, it is possible that these viruses also resulted from secondary recombinations with parental nondefective genomes.

In conclusion, we have shown that the generation of U5–leader–Δc-*mil*–ΔRAV-1–U3 viruses represents an early step in the transduction of c-*mil* sequences in RAV-1-infected NR cells. These viruses are unstable and further recombine with RAV-1 to generate virions encoding *gag-mil* fusion proteins. The newly acquired *gag* sequences are not required for the expression of the mitogenic property of transduced c-*mil* sequences. However, they confer upon the viruses a replicative advantage and enhance their transforming capacity.

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