Common Mechanism of Retrovirus Activation and Transduction of \( c\text{-}mil \) and \( c\text{-}Rmil \) in Chicken Neuroretina Cells Infected with Rous-Associated Virus Type 1

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We previously described the isolation of the IC10 retrovirus which transduced the v-Rmil oncogene, a new member of the \( m\text{-}raf \) gene family. This virus was generated during serial passaging of Rous-associated virus type 1 (RAV-1) in chicken embryo neuroretina (NR) cells and was selected for its ability to induce proliferation of these nondividing cells. IC10 was isolated after six passages of culture supernatants but was not detected in proliferating NR cells during early virus passages. In this study, we molecularly cloned and sequenced another v-Rmil-containing provirus, designated IC11, from NR cells infected at the third virus passage of the same experiment. Both IC11 and IC10 transduced only the serine/threonine kinase domain of c-Rmil. Comparison of v-Rmil and c-Rmil sequences indicated that amino-terminal truncation is sufficient to activate the mitogenic properties of c-Rmil. IC11 and IC10 have identical 3' ends but differ by their 5' RAV-1-Rmil junctions. The 3' ends of both viruses were generated by recombination between Rmil and env genes, involving partial sequence identity. The 5' RAV-1-Rmil junction of IC11 was formed by a splicing process between the RAV-1 leader and a 37-bp c-Rmil exon located upstream of the kinase domain. NR cells infected with this virus synthesize a unique Rmil protein. IC10 contains most of the gag gene recombined with v-Rmil and encodes a gag-Rmil hybrid protein. Serial passaging of IC11 in NR cells led to the formation of a gag-Rmil-containing retrovirus. These results indicate that IC11 represents an early step in transduction and that this virus further recombined with RAV-1 to generate IC10. They confirm our previously proposed model for the multistep generation of v-mil-transducing retroviruses. Therefore, activation and transduction of c-mil and c-Rmil, in NR cells infected with RAV-1, result from a common mechanism.

Oncogenes are eukaryotic cellular genes which have the potential to transform cells in culture and to induce tumors (3). They were initially identified through their association with retroviruses, acting as natural transducing vectors (38). Generation of replication-defective retroviruses carrying transforming genes proceeds through multiple recombination events and results in alteration of both viral and cellular sequences (3, 28, 32, 37, 40). These successive steps during in vivo transduction are difficult to dissect.

We reported that serial in vitro passaging of Rous-associated virus type 1 (RAV-1), an avian retrovirus carrying only replicative genes, on chicken embryo neuroretina (NR) cells reproducibly resulted in retrovirus transduction of cellular oncogenes. These transducing retroviruses were selected for their ability to induce proliferation of these nondividing cells. We characterized four novel retroviruses containing sequences of two related genes. Three viruses, designated Institut Curie 1 (IC1), IC2, and IC3, transduced the catalytic domain of the c-mil gene encoding a serine/threonine protein kinase (23). v-mil is the avian homolog of the v-raf oncogene (18, 39) transduced in murine sarcoma virus 3611 by a procedure which included both in vitro and in vivo experiments (29, 30). IC2 and IC3 were isolated during early passages of RAV-1 on NR cells, whereas IC1 was isolated at later passages of culture supernatants during the same experiment. We were able to demonstrate a generation relationship between these early- and late-passage viruses on the basis of structural analyses of their genomes (9).

In another experiment, we molecularly cloned a fourth provirus, IC10, from NR cells infected at the sixth passage of culture supernatants. IC10 contains a new member of the \( m\text{-}raf \) gene family, designated Rmil (24). The transduced v-Rmil oncogene is the avian homolog of the activated human B-raf gene (15). The IC10 provirus carries Rmil sequences inserted between gag and env sequences (10, 24). It was detectable in RAV-1-infected NR cells only after the fourth passage of culture supernatants. In this study, we show that proliferation of NR cells infected at earlier virus passages during the same experiment was correlated with the presence of another Rmil-containing provirus, designated IC11, which represents an early step in Rmil transduction. We compared the extent of Rmil sequences in IC11 and IC10 and characterized the structure of RAV-1-Rmil junctions of the two viruses. We report that generation of IC11 involves a splicing process between RAV-1 leader and activated Rmil sequences corresponding to the serine/threonine kinase domain. This early virus structure is unstable and acquires substantial amounts of gag sequences by further recombination with the RAV-1 genome. These results extend our proposed model for the generation of oncogene-transducing retroviruses and show that activation and transduction of c-mil and c-Rmil in NR cells initially infected with RAV-1 result from a common mechanism.

MATERIALS AND METHODS

Cell cultures and viruses. NR cultures were prepared from 8-day-old brown leghorn chicken embryos (gs\textsuperscript{+} chf\textsuperscript{+}) of the Edinburgh strain, as previously described (25). Cultures
were maintained and passaged in Eagle basal medium supplemented with 5% fetal calf serum.

RAV-1 is a subgroup A lymphomatosis virus routinely grown in chicken embryo fibroblasts. Virus purified by two cycles of end-point dilution on chicken embryo fibroblasts was used to generate transducing retroviruses, as previously reported (23, 24). Helper pRAV-1 DNA was used as a source of helper virus in transfection experiments.

DNA purification and restriction enzyme analysis. High-molecular-weight DNA was purified from cells by standard procedures (33). DNA of Xgt11 phages grown in Escherichia coli Y1a55 was prepared as described previously (22). Plasmid DNAs were purified by the cleared-lysate method and by centrifugation in cesium chloride-ethidium bromide gradients. DNAs were digested to completion with restriction endonucleases under conditions recommended by the suppliers (New England Biolabs and Appligene), 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 (35). Hybridization was performed under stringent conditions described by Wahl et al. (42) with probes radioactively labeled by nick translation (31).

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated by the guanidium thiocyanate-cesium chloride method (6). The RNAs were denatured at 60°C in a formamide-formaldehyde mixture (22), fractionated by electrophoresis in 1% agarose-2.2% formaldehyde gels (21), transferred to nitrocellulose filters in 20× SSC (41), and hybridized to 32P-labeled probes.

Molecular probes. We used the following probes: (i) the 35-bp EcoRI fragment (Eco D) of long terminal repeat (LTR) sequences from the Rous sarcoma virus molecular clone pSR2A (7); (ii) the 735-bp PstI-NsiI 5’ v-Rmil-specific fragment of an IC10 molecular clone subcloned into the PstI site of Bluescript plasmid vector (see Fig. 3B); (iii) the 370-bp 3’ v-Rmil-specific fragment of an IC10 molecular clone (see Fig. 3B) subcloned into the Bluescript vector.

Molecular cloning. High-molecular-weight DNA was digested to completion with EcoRI and fractionated by centrifugation through a sucrose gradient (22). The 2- to 3-kb EcoRI DNA fragments were ligated to EcoRI-digested arms of Xgt11 (44) and packaged in vitro with the Gigapack Plus packaging system (Stratagene). Recombinant plasmids were selected by plaque hybridization (2) with 32P-labeled LTR and v-Rmil probes and further purified and amplified.

DNA sequencing. Restriction fragments of IC11 provirus and of chicken genomic clones were subcloned into appropriate sites of Bluescript (Stratagene) or M13tig131 vectors (19). The nucleotide sequence was determined on both strands by the dideoxy-chain-termination method (33).

Transfection of NR cells. To obtain a provirus with two complete LTRs, the IC11 EcoRI fragment was subcloned into the pLTR(E) vector which contains Rous sarcoma virus LTR sequences with a single EcoRI site within the LTR region (kindly provided by P. Dezéée). Eight-day-old chicken embryo NR cells were transfected with cloned DNA by the calcium phosphate method of Graham and Van der Eb (12). Thirty micrograms of pLTR/IC11 plasmid DNA coprecipitated with 5 μg of pRAV-1 plasmid DNA was added in a 0.5-ml volume to 35-mm dishes containing 4×10⁶ 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. NR cultures were labeled with [35S]methionine and cell extracts were prepared as previously described (26). The Rmil protein was immunoprecipitated with either rabbit anti-gag serum or rabbit anti-Rmil serum. The latter was prepared by immunizing rabbits with a v-Rmil-specific protein expressed in E. coli (1a). Immunoprecipitates were processed as previously described (26) and analyzed by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels (20).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been given GenBank accession number M62407.

RESULTS

NR cells induced to proliferate by RAV-1 contain two forms of Rmil-transducing retroviruses. The experimental procedure which led to the isolation of IC10, a novel retrovirus containing the v-Rmil oncogene, is summarized in Fig. 1. As previously reported, infection of nondividing NR cells from 8-day-old chick embryos with RAV-1 resulted in the occasional appearance of foci of dividing cells after 4 to 6 weeks. Medium from proliferating cells was serially passaged on fresh NR cultures. Cell proliferation was observed upon each virus infection. Culture fluids collected after the fifth passage of supernatants on NR cells were able to induce cell multiplication within 1 week. DNA extracted from multiplying cells at each virus passage was digested with EcoRI and analyzed by Southern blotting and hybridization with an LTR probe (Fig. 2A). All infected cells contained the 2.4- and 1.3-kb DNA fragments corresponding to the 5’ and 3’ portions, respectively, of the RAV-1 genome. An additional 4.2-kb fragment, corresponding to IC10 provirus, was detected only in NR cells infected at the fifth and sixth virus passages. Molecular cloning and sequencing of this provirus led to the identification of v-Rmil sequences inserted between gag and env sequences (10).

To identify the mitogenic component(s) responsible for NR proliferation during earlier virus passages, we hybridized the same DNA blots with v-Rmil probes (Fig. 2B). All cells contained the 13.0-, 7.0-, and 2.7-kb EcoRI fragments of the endogenous c-Rmil gene (24). Cells infected at the third, fourth, and fifth passages of virus supernatants contained an additional DNA fragment of 2.4 kb which could not have been detected by the LTR probe because of the size similarity with that of the 5’ RAV-1 fragment. Hybridization signals of the IC10 provirus were barely visible in cells.
infected at the fourth virus passage and steadily increased during further passaging. In contrast, those of the 2.4-kb fragment progressively decreased during passages and were no longer detected at the sixth virus passage. Northern blot analysis of RNAs extracted from NR cells at each virus passage and hybridized with v-Rmil (Fig. 2C) and LTR (data not shown) probes showed that the 2.4-kb fragment corresponded to a v-Rmil-containing retrovirus of about 2.5 kb.

These results indicated that proliferation of NR cells induced by serial passaging of RAV-1 was correlated with the generation of two Rmil-containing retroviruses, the relative amounts of which varied during the course of the experiment. Therefore, it was likely that the 2.5-kb virus, the first detected in RAV-1-infected cells, represented an early step in Rmil transduction.

Comparison of the genetic structures of IC11 and IC10 proviruses. High-molecular-weight DNA was extracted from NR cells infected at the third passage of virus-containing supernatants and digested with restriction enzyme EcoRI. DNA fragments of between 2 and 3 kb were ligated to purified arms of λgt11 DNA and packaged in vitro. A recombinant clone hybridizing to both v-Rmil and LTR probes was selected, further purified, and amplified. This clone harbored the 2.4-kb EcoRI fragment of the v-Rmil-containing provirus designated IC11.

We subcloned the 2.4-kb insert into the EcoRI site of the Bluescript plasmid vector and determined its entire nucleotide sequence (Fig. 3A). This sequence was compared with those of the IC10 provirus and of a cDNA clone containing a partial sequence of c-Rmil (8a). The 2.4-kb fragment of IC11 provirus contains 2,326 nucleotides. A single long open reading frame of 450 amino acids extends from nucleotide 453 to nucleotide 1802. It encodes a protein with a calculated molecular weight of 50,313. This open reading frame contains the six N-terminal amino acids of gag and 377 amino acids of v-Rmil. The last 67 amino acids and the stop codon are provided by RAV-1 env sequences.

Comparison of the IC11 nucleotide sequence with that of IC10 provirus (Fig. 3B) showed complete identity between the 3' portions of the two viruses, corresponding to nucleotides 501 to 2326 of IC11 and nucleotides 2388 to 4213 of IC10. Both viruses transduced the carboxy-terminal portion of c-Rmil, which contained the serine/threonine kinase domain. We did not find amino acid differences between the v-Rmil genes of IC11 and IC10 and the corresponding sequence of the c-Rmil cDNA (data not shown). In contrast, we observed major differences in the 5' portions of the two viruses. Coding nucleotides 471 to 500 of IC11, which belong to the c-Rmil gene, were not present in the IC10 sequence. They were linked to the splice donor site of the RAV-1 leader sequence (14). Moreover, IC10 contains most of the gag gene, whereas IC11 contained only the first 18 nucleotides of gag, which are included in the leader sequence.

5' RAV-1-Rmil junctions in IC11 and IC10 viruses are generated by different mechanisms. To confirm that the 5' RAV-1-Rmil recombination in IC11 involved a splicing process between the donor site of the RAV-1 leader sequence and the acceptor site of an Rmil exon, we cloned and sequenced a portion of the c-Rmil gene containing the 30 nucleotides present in IC11 but not in IC10. Therefore, the 0.2-kb PstI fragment of IC11 containing these nucleotides (Fig. 3B) was subcloned into the M13tg131 vector and used...
as a probe to screen a chicken genomic library. We characterized a recombinant clone harboring a 2.1-kb NsiI-PstI DNA fragment which hybridized to this probe. Sequence analysis and comparison with v-Rmil cDNA showed that this fragment contained an exon of 37 bp flanked by consensus sequences for splice acceptor and donor sites (Fig. 4A). The first nucleotide of this exon corresponds to the first nucleotide of the v-Rmil gene in IC11 (Fig. 4B). This indicated that the 5′ recombination of the IC11 genome resulted from a splice junction between viral and oncogene sequences. The 5′ recombination site of IC10 was located about 1,930 nucleotides downstream from the AUG initiation codon of gag within the 37-bp Rmil exon present in IC11 (Fig. 4C). This junction allowed an in-frame fusion of the two genes. Comparison of the sequence around the recombination site with those of the gag genes of Rous sarcoma virus (34) and
of c-Rmil cDNA showed a strong similarity (9 of 10 bases) between gag and Rmil sequences. Therefore, it is likely that the 5' junction of IC10 was generated by illegitimate recombination between gag and Rmil sequences, involving sequence similarity between the two genes.

3' Rmil-RAV-1 junctions in IC11 and IC10 proviruses are identical. We compared the nucleotide sequences around the 3' recombination sites of IC11 and IC10 with those of a chicken genomic clone containing the 3' coding region of the c-Rmil gene and of the env gene of RAV-2 (4). We detected a sequence similarity (five of six bases) between env and Rmil sequences (Fig. 4D) located within the recombination site. This result suggested that the 3' junctions of IC11 and IC10 resulted from illegitimate recombination between Rmil and env genes.

Expression of Rmil-transducing proviruses in NR cells. To generate a provirus with complete LTRs, we subcloned the IC11 EcoRI fragment into the pLTR(E) plasmid. This plasmid contains Rous sarcoma virus LTR sequences with a single EcoRI site within the LTR region. Transfection of NR cells with this clone (pLTR/IC11) (Fig. 5), in association with

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**FIG. 4.** RAV-1-Rmil junctions in IC11 and IC10 proviruses. (A) Nucleotide sequences of the 37-bp c-Rmil exon transduced in IC11 virus and adjacent intronic sequences. Consensus sequences are indicated according to the method of Breathnach and Chambon (5). (B) 5' recombination junction in IC11. The sequence of the 5' RAV-1-Rmil junction in IC11 provirus was compared with those of RAV-1 and the c-Rmil genes. (C) 5' recombination junction in IC10. The sequence of the 5' RAV-1-Rmil junction in IC10 provirus was compared with those of RSV PrC gag and c-Rmil genes. (D) 3' recombination junctions in IC11 and IC10. The sequences of the 3' Rmil-RAV-1 junctions in IC11 and IC10 were compared with those of RAV-2 env and c-Rmil genes. Nucleotide similarities are indicated by asterisks (panels B through D). The vertical line indicates the 5' recombination site in IC11. The initiation codon of the leader sequence of RAV-1 is underlined (panel B). Boxes indicate homologies between gag and Rmil sequences and between env and Rmil sequences at the recombination sites of IC11 and IC10 viruses (panels C and D).

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**FIG. 5.** Structure of the pLTR/IC11 plasmid. pLTR/IC11 was obtained by subcloning the EcoRI fragment of the IC11 provirus into the EcoRI (E) site of the pLTR(E), a pBR322-derived vector.
helper pRAV-1 DNA, resulted in efficient cell proliferation.

To characterize the v-Rmil proteins, we labeled proliferating NR cells containing IC11 or IC10 provirus with [35S]methionine. Cell lysates were immunoprecipitated with either gag- or Rmil-specific antisera, and immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6). NR cells infected with IC11 virus contain a 44-kDa protein immunoprecipitated only by the anti-Rmil serum (Fig. 6A). In contrast, NR cells infected with IC10 virus synthesize a 122-kDa fusion protein recognized by both anti-Rmil and anti-gag antibodies (Fig. 6B).

To investigate the possibility of a generation relationship between IC11 and IC10 viruses, we serially passaged virus, produced by NR cells cotransfected by pLTR/IC11 and pRAV-1 DNAs, on fresh cultures. Lysates of [35S]methionine-labeled cells, prepared at each virus passage, were immunoprecipitated with anti-Rmil and anti-gag antibodies and analyzed on SDS-polyacrylamide gels. All NR cultures infected with IC11 contain the 44-kDa protein immunoprecipitated only by anti-Rmil serum. However, lysates of cells infected at the fifth and sixth passages of culture supernatants contained an additional 118-kDa hybrid protein recognized by both anti-Rmil and anti-gag antibodies (Fig. 6A). The synthesis of the 118-kDa gag-Rmil protein was correlated with the presence of an EcoRI provirus fragment of 4.1 kb hybridizing to both gag and v-Rmil probes (data not shown). These results showed that recombination between IC11 and RAV-1 genomes took place during subsequent replicative cycles in NR cells and generated a new virus encoding a gag-Rmil hybrid protein.

**DISCUSSION**

We have described an experimental model for studying the mechanisms of retrovirus activation and transduction of cellular oncogenes. By serially passaging culture supernatants of chicken embryo NR cells initially infected with RAV-1 on fresh cells, we repeatedly isolated retroviruses containing sequences of two related proto-oncogenes. This model proved useful in two ways. First, it enabled us to identify the c-Rmil oncogene, a new member of the milti-rat gene family, transduced in retroviruses IC11 and IC10. Second, by comparing the genetic organization of viruses generated at different steps of transduction, we established a precursor-product relationship between viruses isolated during early and late passages of culture supernatants on NR cells.

The cellular allele of the v-Rmil gene, c-Rmil, is the avian homolog of the human B-raf gene, identified in NIH 3T3 cells transfected with human Ewing sarcoma DNA (15). It is believed that the transforming properties of B-raf were activated during transfection by fusion of its serine/threonine kinase domain with foreign DNA sequences. By comparison with the c-Rmil cDNA, we showed that IC11 and IC10 transduced only the carboxy-terminal coding portion of the gene including the catalytic domain. Characterization of IC11 retroviruses established that the protein encoded by this domain is capable of inducing NR cell proliferation, as previously shown for the homologous portion of the c-mil gene (8). When compared with the corresponding sequences of the c-Rmil cDNA, the coding portions of c-Rmil, transduced in IC11 and IC10, did not have base mutations leading to amino acid changes. Therefore, it is likely that activation of the oncogenic properties of the Rmil/B-raf gene resulted from its NH2-terminal truncation. Oncogenic activation of the c-raf protein was obtained by alteration of a region located immediately upstream of the kinase domain (17, 36, 43). Taken together, these results suggest that the NH2-terminal portions of these two genes are essential in negatively regulating their transforming potential.

The genetic structures of c-mil- and c-Rmil-transducing Institut Curie viruses are presented in Fig. 7. IC2 and IC11
show similar genetic organization and represent early virus forms in the transduction process. Their 5′ ends were generated by joining the splice donor site of the RAV-1 leader to the acceptor sites of c-mil exon 8 (9) and of the 37-bp c-Rmil exon, respectively. Partial analysis of exon-intron organization of the chicken c-Rmil gene showed that only this 37-bp exon allows an in-frame junction between the six gag amino acids in the leader sequence and the entire c-Rmil kinase domain. Similar structures at the 5′ ends of two retroviruses, S1 and PR2257, which transduced the c-src gene in vivo, were described previously (11, 16). Therefore, constitution of 5′ ends by a splice process could represent a general mechanism in retrovirus transduction of proto-oncoproteins. We previously proposed that a precursor of these early virus forms could be an alternatively spliced U5-leader-Aq onc RNA (9). Obtaining a complete retrovirus requires the acquisition of 3′ sequences including a U3 region. Analysis of the sequencing data around the 3′ Rmil-RAV-1 junction of IC11 and IC10 suggested that the 3′ portions of early virus forms were acquired by illegitimate recombination between c-onc and env sequences. This recombination could take place during reverse transcription of copackaged RAV-1 and U5-leader-Aq onc RNAs and could be favored by sequence similarities, such as those found between c-Rmil and env genes.

The late-passage viruses IC1 and IC10 also show similar genetic organization. Their 5′ ends were generated by recombination between gag and oncogene sequences, leading to in-frame fusion of gag sequences with activated c-mil and c-Rmil. Moreover, IC2 and IC1 viruses and IC11 and IC10 have identical 3′ ends. It is, therefore, possible that IC1 and IC10 were generated by secondary recombination between their corresponding early virus forms and parental nondefective genomes. This possibility was supported by the generation, during serial passaging of IC2 (9) and IC11 on NR cells, of viruses encoding gag-onc fusion proteins. We previously showed that acquisition of gag sequences provided c-mil-transducing retroviruses with a replicative advantage (9). The added gag sequences contain an additional packaging signal located 150 bp downstream from the ATG start codon of the virus (27) and cis-acting enhancer elements located about 900 bp downstream from the site of transcription initiation (1). The presence of these signals should improve the replication rate of gag-mil- and gag-Rmil-containing viruses.

Institut Curie viruses transduced homologous domains of two genes that are both expressed in NR cells. However, the major c-Rmil transcript is greater than 10 kb in length (24), whereas c-mil is transcribed into a 3.4-kb mRNA (23). We have shown that generation of retroviruses containing these two genes, which markedly differ in their complexity, proceeds through similar steps. We conclude, therefore, that a common mechanism must be responsible for the retrovirus activation and transduction of the c-mil and c-Rmil proto-oncogenes in NR cells induced to proliferate by RAV-1 infection.

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REFERENCES


