Two Autonomous *myc* Oncogenes in Avian Carcinoma Virus OK10

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The oncogenic avian retrovirus OK10 has the genetic structure *gag-Δpol-myc-Δenv*. The *myc* sequence is transduced from a cellular gene, proto-*myc*, while *gag*, *pol*, and *env* are essential retrovirus genes. By analogy with other directly oncogenic retroviruses, the specific *myc* sequence of OK10 is thought to be essential for transforming function. However, unlike the specific sequences of all other transforming retroviruses that encode unique transforming proteins, the *myc* sequence of OK10 encodes two potential transforming proteins, p58 and p200. p200 is translated from the *gag-Δpol-myc* region of genomic RNA, while p58 is thought to be translated from the *gag* leader and the open reading frame of *myc* via a subgenomic mRNA. In this paper, we ask whether both *myc* genes of OK10 are autonomous transforming genes. By differentially inactivating the p200 *myc* gene of OK10 provirus in vitro and analyzing transforming function in quail embryo cells, it was found that mutants expressing only p58 transformed like wild-type OK10. Further, it was shown that p58 with and without the *gag* leader had transforming function and that p58 of wild-type OK10 is initiated from the *gag* leader. Mutants expressing only p200 were also transforming but less efficiently than mutants that express only p58. A mutant OK10 virus in which the native frameshift of retroviruses between *gag* and *pol* was deleted expressed a shortened p200 (Δ p200). Although this virus expressed more Δ p200 than wild-type OK10 did, it transformed cells less efficiently. It follows that OK10 expresses two autonomous transforming genes, which is unique among retroviruses with *onc* genes.

The oncogenic avian retrovirus OK10 was isolated from a chicken liver tumor (11). OK10 causes leukemia and endotheliomas and carcinomas of the gut, liver, and testes (11). In vitro, the virus transforms fibroblasts and myeloid cells (6). It belongs to a family of four avian retroviruses that includes MC29, MH2, and CM11, which share the transformation-specific sequence *myc* (2, 8). Retroviruses with *myc* transform multiple cell types, giving rise to carcinomas, sarcomas, and leukemias (22a).

In addition to *myc*, OK10 contains a complete retroviral *gag* gene, a partial *pol* gene, and a partial *env* gene. These elements are linked in the order 5' *gag-Δpol-myc-Δenv* to form an 8-kilobase (kb) RNA genome (2, 8). In addition to genomic RNA, a 4-kb subgenomic RNA is expressed from the OK10 provirus, using the splice donor of *gag* and a splice acceptor near the 5' end of *myc* (4, 8, 19). OK10 is unique among retroviruses with transformation-specific sequences because its *myc* sequence encodes two overlapping genes that encode *myc* proteins of 58,000 and 200,000 daltons, termed p58 and p200 (1, 17). The 58-kilodalton (kDa) *myc* protein is thought to be translated from the *gag* leader and the open reading frame of *myc* via the subgenomic mRNA (4, 8). The 200-kDa *myc* protein is translated from the 8-kb genomic RNA (17).

Using site-directed mutagenesis, we have analyzed whether the two *myc* genes of OK10 are (i) mutually dependent, (ii) one autonomous transforming gene and one non-transforming gene, or (iii) two autonomous transforming genes. Transforming function of mutated proviruses cloned in bacterial plasmids was measured upon transfection in quail embryo fibroblasts. We have found that both viral *myc* genes have autonomous transforming function. However, the spliced *myc* gene that encodes p58 is a stronger transforming gene than the one that encodes p200.

MATERIALS AND METHODS

Proviral clones of wild-type and mutant OK10: wild-type OK10, pOK10. For in vitro mutagenesis, a complete OK10 provirus in plasmid pBR322 was constructed from several lambda clones carrying complete, and pBR322 clones carrying partial, OK10 proviruses (16). A pBR322 subclone of OK10, pE2.8, containing a 2.6-kb *EcoRI* insert with a short segment of cellular DNA, the 5' long terminal repeat, and most of *gag* was partially digested with *EcoRI*. The staggered ends were blunted with Klenow enzyme and religated to destroy the *EcoRI* site (10). We selected a plasmid, termed pE2.8RI, that had lost the *EcoRI* recognition site in the cellular sequences of the clone, but retained the *EcoRI* site in *gag* (Fig. 1). Next we cloned a 2.1-kb *EcoRI-SacI* fragment of OK10 containing a segment of *env*, the 3' long terminal repeat, and cellular sequences from a lambda clone into pBR322. A 2.0-kb segment of rat-derived DNA was used to link the *SacI* site to the *PstI* site in the ampicillin resistance gene in this pBR322 vector. This clone was termed pE2.1. Both pE2.1 and pE2.8RI were digested with *EcoRI* and *Tth111I* (in pBR322 sequences). The 5' and 3' segments of OK10 were then joined at their unique *EcoRI* and *Tth111I* sites to generate clone pE4.5. This plasmid contains both ends of the OK10 provirus and has a unique *EcoRI* site between *gag* and *env* sequences. The complete pOK10 was formed by ligating the 4.1-kb *EcoRI* fragment containing *pol* and *myc* sequences into the *EcoRI* site between *gag* and *env* of pE4.5.

A frameshift mutant in *pol*, pOK10 fs-*pol*. The unique *BglII* site in the *pol* sequence of the OK10 subclone pE3.3 (a pBR322 clone containing a 4.1-kb *EcoRI* fragment of *pol-myc* sequences [16]) was cut (Fig. 1). The staggered ends of the DNA were then filled in with deoxynucleotides, using the Klenow enzyme (10), and religated to generate pE3.3 fs-*pol*. The filled-in *BglII* site adds four nucleotides (GATC) to *pol*, generates a new *ClaI* site, and shifts the reading frame of p200 such that the protein is truncated because the new

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FIG. 1. OK10 gene expression drawn over the corresponding coding region of the virus genome. p58 myc protein is shown to initiate at the atg codon in *gag*. Pr76 and p200 also initiate translation at the atg codon in *gag*. p200 is translated through the stop codon and frameshift at the junction between *gag* and *pol*. Partial sequence information is shown below the genetic structure of OK10. Specific sequences of the OK10 mutants made are listed below the wild-type OK10 sequence in boldface letters. The code for each mutation is listed on the left (see Materials and Methods). LTR, Long terminal repeat; nt, nucleotides.

reading frame has a stop codon 45 nucleotides downstream of the filled-in *Bgl*II site (Fig. 1). The 4.1-kb *Eco*RI fragment of OK10 was then inserted into pE4.5 to generate pOK10 fs-pol. The truncated p200 protein measures approximately 140 kDa and has the structure *gag*-Δ*pol*.

A splice acceptor mutant, pOK10 sa⁻. The splice acceptor site in the *myc* region of OK10 was mutated from AG to AA (Fig. 1). For this purpose a 1.7-kb *Xba*I-*Sac*I fragment of OK10 spanning the *pol*-*myc* junction was cloned into M13mp19 for site-directed mutagenesis as described by Zoller and Smith (24). A sequence with the desired mutation that is complementary to the splice acceptor of the *myc* region of OK10, namely, 5' GGC GGC TGC TGCT TGC GGG CGG GAG 3', was annealed to the M13 template and extended with Klenow enzyme to form double-stranded phage DNA (24). After transformation of JM109 bacteria with unpurified, primer-extended DNA, 100 plaques were picked and grown for 4 h in 2.0 ml of 2× YT medium plus 20 μl of an overnight culture of JM109 bacteria. A 200-μl portion of phage supernatants from these cultures was directly spotted onto a nitrocellulose filter, using a dot-blot manifold. The filter-bound phage DNA was then hybridized for 2 h with the above primer (end labeled with ³²P by T4 kinase) at 25°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-10× Denhardt solution-0.2% sodium dodecyl sulfate (SDS) (24). After hybridization, the blot was washed with increasing stringency, ranging from 6× SSC-0.05% SDS at 25°C to 0.1× SSC-0.05% SDS at 60°C, until only clones with perfect homology to the primer were detectable by autoradiography. The mutants were verified by dideoxy sequencing, using reagents and protocol from International Biotechnologies, Inc.

OK10 sa⁻ was constructed from this M13 clone (M13/1.7 sa⁻) by replacing the 1.7-kb *Xba*I-*Sac*I fragment from pE3.3

with the mutant fragment from the double-stranded replicative form of the phage to give pE3.3 sa⁻. The 4.1-kb *Eco*RI insert in pE3.3 sa⁻ was then cloned into pE4.5 to give pOK10 sa⁻. The native Gln (CAG) codon of p200 in the splice acceptor site OK10 remains a Gln in mutant pOK10 sa⁻ with the triplet CAA.

A mutant of OK10 without splice acceptor and methionine initiation codon in *myc*, pOK10 sa⁻met⁻. The first met codon in the *myc* sequence of pOK10 sa⁻ was mutated to a Glu codon (GAG) by site-directed mutagenesis (Fig. 1). For this purpose the above M13 clone (M13/1.7 sa⁻) was hybridized with the complementary synthetic primer sequence 5' GCT GAG CGG CTC CGC GGC GGC T 3' that carried the desired mutation in the first methionine codon of *myc*. The mutant virus pOK10 sa⁻met⁻ was reconstructed, using the mutated M13 DNA as described above for pOK10 sa⁻.

A mutant of OK10 with a frameshift in p200 and without splice acceptor and initiation codon in *myc*, pOK10 fs-pol sa⁻met⁻. The *Bgl*II site in *pol* of pOK10 sa⁻met⁻ was cut with *Bgl*II, filled in with deoxynucleotides, and religated as described above for pOK10 fs-pol.

An OK10 mutant with a stop codon preceding the first methionine of *myc*, pOK10 amb. To construct pOK10 amb, the M13 clone carrying the 1.7-kb *Xba*I-*Sac*I fragment of pOK10 was mutated with the complementary primer sequence 5' CAT CGC GGC GGC TCA TGC CTG CGG GCG. The M13 mutant was isolated, and the 1.7-kb *Xba*I-*Sac*I fragment was reinserted into pE3.3. The 4.1-kb *Eco*RI fragment from pE3.3 was cloned into pE4.5 to give pOK10 amb. In this mutant the wild-type Ala codon (GCA) was changed to an amber stop codon (TGA) between the *myc* splice acceptor and the first methionine codon of *myc* (Fig. 1).

An OK10 mutant with a frameshift in p200 and without the

first methionine codon of *myc*, pOK10 fs-pol met⁻. pOK10 fs-pol met⁻ was constructed by mutating the first methionine codon of *myc* to a Glu codon, using the *Xba*I-*Sac*I fragment of pOK10 cloned in M13 phage as described above. After constructing a mutant pOK10 met⁻, the *Bgl*III site in *pol* was cut, filled in with deoxynucleotides, and religated as described above for pOK10 fs-pol, to give pOK10 fs-pol met⁻.

An OK10 mutant with a deletion that places *gag* and *pol* in the same reading frame and point mutations in *myc* that inactivate p58, pOK10 fs-gag/pol sa⁻ met⁻. A linker was constructed to span an *Eco*RI-*Hind*III region between *gag* and *pol* of pOK10 (sequence of linker shown in Fig. 1). After cutting pE3.3 sa⁻ met⁻ (precursor of pOK10 sa⁻ met⁻) with *Hind*III and *Eco*RI, the 3.6-kb *pol*-*myc* fragment was ligated to the synthetic linker shown in Fig. 1 and then digested with *Eco*RI. The 3.6-kb *Eco*RI fragment was then purified and cloned into the *Eco*RI site of pE4.5 to give pOK10 fs-gag/pol sa⁻ met⁻. This linker puts *pol* in the same reading frame as *gag* and deletes the stop codon between *gag* and *pol*. In addition, it deletes 40 *gag* codons and 120 *pol* codons from the *Eco*RI site in *gag* to the *Hind*III site in *pol* (Fig. 1).

Cell culture and transfections with viral DNA. Primary Japanese quail embryo fibroblasts were grown in Ham F10 medium (GIBCO Laboratories) supplemented with 5% tryptose phosphate broth, 4% calf serum, and 1% chicken serum.

Transfections with viral DNA were done as follows: 3 µg of OK10 proviral DNA cloned in pBR322 together with 1 µg of MAV-1 DNA (a lambda clone of myeloblastosis-associated virus, subgroup A) (14) and 20 µg of sheared salmon sperm DNA were coprecipitated as described by Zhou et al. (23). Precipitates were added to cell media, and after 6 h cells were treated for 45 s with 20% dimethyl sulfoxide in medium to enhance transfection efficiency (22). Three days after transfection, cells were reseeded after a threefold dilution.

Preparation of virus-specific proteins from infected cells. A 60-mm culture dish of 80% confluent cells seeded the previous day was rinsed with Tris-buffered saline and starved for 1 h at 37°C in 4 ml of methionine-free, Dulbecco modified Eagle medium containing 4% calf serum dialyzed against Tris-buffered saline. Cells were labeled for 1 h at 37°C with 200 µCi of [³⁵S]methionine and then lysed in 1 ml of RIPA buffer (50 mM NaCl, 20 mM Tris hydrochloride, pH 8.2, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% pure bovine serum albumin). The lysate was passed through a 26-gauge needle and clarified by centrifugation at 13,000 × *g* at 4°C for 10 min. A 200-µl amount of protein A-Sepharose plus 50 µl of normal rabbit serum were added to the supernatant, and incubation was for 1 h at 4°C with rocking. After centrifugation at 13,000 × *g* for 5 min at 4°C, the supernatant was diluted with RIPA buffer, usually to three 1-ml aliquots. Typically, each 1-ml aliquot was incubated with either 5 µl of anti-*gag*, 1 µl of anti-*myc*, or 15 µl of normal serum for immunoprecipitation. Anti-*gag* serum was a gift from William Phares. Anti-*myc* serum (pan-*myc*) was the generous gift of Gerard I. Evan (Ludwig Institute for Cancer Research, Cambridge, England). Normal rabbit serum was obtained from GIBCO. Incubation with antibody was for 30 min at room temperature and then for 2 to 12 h at 4°C with rocking. Subsequently, 40 µl of protein A-Sepharose was added to each 1-ml aliquot. After incubation for 1 h at 4°C with rocking, the Sepharose-bound immune complex was pelleted and washed extensively at 4°C with RIPA buffer lacking serum albumin. The pellet was then suspended in 50 µl of SDS sample buffer and incubated for 20 min at room temperature. After centrifugation for 8 min in a microcentri-

TABLE 1. Transforming activity of OK10 mutants^a

OK10 clone	<i>myc</i> -related protein	Foci/µg	% Wild-type efficiency
Wild type	p58, p200	600	100
fs-pol met ⁻	p58 + leader	550	92
amb	p58 - leader	150	25
sa ⁻ met ⁻	p200	30	5
fs-gag/pol sa ⁻ met ⁻	Δp200	250	42
fs-pol sa ⁻ met ⁻	none	0	0

^a A 3-µg amount of proviral OK10 and 1 µg of proviral MAV-1 DNA (approximately 10:1 molar ratio) were transfected in quail embryo cells. Cells were passaged for 2 days posttransfection, reseeded after a threefold dilution, and overlaid with agar after cell reattachment. Foci were scored 15 to 18 days posttransfection. These results are representative of the efficiencies determined in four other experiments in which all clones were tested together.

fuge, the supernatant was boiled for 2 min. Samples were electrophoresed in polyacrylamide gel containing 0.1% SDS and 0.4 M Tris hydrochloride, pH 9.0, with ¹⁴C-labeled protein molecular weight standards from Amersham Corp. (9). After electrophoresis, gels were soaked in a scintillation fluid (Enlightening; Amersham) for 30 min at 25°C, prior to gel drying and fluorography.

RESULTS

Infectious proviral clone of OK10. For the intended deletion analyses of OK10, an infectious provirus termed pOK10 was constructed from existing lambda clones and from subclones of OK10 in pBR322 (16) (see Materials and Methods; Fig. 1). The biological activity of pOK10 was tested by transfection into quail embryo cells in the presence of MAV-1 helper virus DNA cloned in lambda phage (14). Within 9 days of transfection, we observed 600 foci per µg of pOK10 DNA (Table 1). Foci consisted of refractile, round, and multilayered cells (Fig. 2). Immunoprecipitation with anti-*gag* serum detected the viral p200 *gag*-Δ*pol*-*myc* protein in addition to the helper viral proteins Pr76 and Pr180 (Fig. 3B, lane 2). Immunoprecipitation of proteins from transformed cells with anti-*myc* serum, followed by electrophoresis, detected the viral p58 *myc* protein. The p200 *myc* protein was not seen under the conditions of the experiment (Fig. 3A, lane 1), presumably because too little of it was precipitated to stand out against the background of other large proteins that were also precipitated by our anti-*myc* serum (not shown).

Transformation by the p58 *myc* protein of OK10. To determine whether the p58 *myc* protein of OK10 has transforming function by itself, we inactivated the p200 *myc* gene of OK10 by introducing a frameshift mutation into *pol*. Further, we asked whether the p58 protein of OK10 is initiated in the *gag* leader sequence or at the first methionine of the *myc* sequence. Therefore, a double mutant, pOK10 fs-pol met⁻, was constructed in which the first methionine of *myc* was changed to glutamine by site-directed mutagenesis in addition to the frameshift in *pol*. Such a mutant should express p58 initiated at the *gag* leader from the subgenomic OK10 mRNA but should not express a functional p200. When quail embryo cells were transfected with this OK10 mutant and cloned MAV-1 helper virus DNA, about 550 foci per µg of cloned pOK10 fs-pol met⁻ DNA appeared 9 days after transfection (Table 1). These foci were indistinguishable from those generated by wild-type pOK10 (Fig. 2).

Immunoprecipitation with anti-*myc* serum detected in OK10 fs-pol met⁻ infected cells a 58-kDa protein that migrated with the same mobility as the p58 of wild-type

FIG. 2. Foci of OK10 virus-transformed quail cells, under agar, 12 days posttransfection with cloned proviral OK10 and MAV-1 DNA. (1) OK10 wild type (p58 and p200); (2) OK10 fs-pol met^- (p58); (3) OK10 amb (p58 minus *gag* leader); (4) OK10 $\text{sa}^- \text{met}^-$ (p200); (5) OK10 fs-gag/pol $\text{sa}^- \text{met}^-$ (Δ p200); (6) OK10 fs-pol $\text{sa}^- \text{met}^-$ (no *myc*-related protein).

OK10 (Fig. 3A, lanes 1 and 2). Antibody to *gag* proteins detected the OK10 and helper virus-encoded *gag* precursor Pr76 and the helper virus-specific *pol* precursor Pr180. As expected, no p200 *myc* protein was detected in OK10 fs-pol met^- -infected cells. Instead, a *gag*-related p140 protein was detected, which is thought to be a truncated p200 protein due to the frameshift mutation in *pol* (Fig. 3B, lane 7). We conclude that the p58 *myc* protein of OK10 is initiated in the *gag* leader sequence and is sufficient to transform quail embryo fibroblasts.

To confirm that transformation by OK10 fs-pol met^- was due to p58, the p58 *myc* gene was inactivated by eliminating the splice acceptor of the *myc* sequence (Fig. 1). As expected, the resulting OK10 fs-pol $\text{sa}^- \text{met}^-$ mutant clone (Materials and Methods) did not transform quail embryo fibroblasts (Fig. 2, part 6) (see below). This was not due to lack of infectivity of the clone, because OK10 RNA was detectable by hybridization with *myc* probes in virus released into the growth medium.

Virus-specific leader sequence not essential for transforming function of p58. Although p58 of OK10 appears to be initiated from the methionine codon in *gag* 15 nucleotides upstream

from the splice donor, a methionine codon in *myc* 15 nucleotides downstream from the splice acceptor in *myc* could also function to initiate p58 translation (Fig. 1). This methionine is thought to be the translation initiation codon of the cellular *myc* protein. To determine whether the first methionine codon of *myc* could function to initiate p58 protein with transforming function, a mutant, pOK10 amb (see Materials and Methods), was constructed which has an amber stop codon between the splice acceptor and first methionine of *myc* (Fig. 1). This mutant should make either no p58 or p58 without the *gag* leader sequence. In addition, this mutant should not synthesize a functional p200 because of the stop codon at the 5' end of *myc*. Instead, it should make a truncated p200 protein of 160 kDa with the structure *gag*- Δ *pol* (Fig. 1).

Upon transfection into quail embryo cells, this mutant produced 150 foci per μg of pOK10 amb DNA in the presence of helper virus DNA (Table 1). Foci of pOK10 amb were heterogeneous; some looked like foci of wild-type OK10, while others were less developed (Fig. 2). Immune precipitates with *myc* antibody from cells transformed by pOK10 amb detected a p58 protein that migrated faster than

FIG. 3. Immunoprecipitation of OK10 clones. (A) Proviral OK10 clones were transfected into quail cells, and transformed foci were picked and grown into mass cultures. An 80% confluent 60-mm dish of cells was labeled with 200 μ Ci of [35 S]methionine for 1 h. Cell extracts were prepared as described in Materials and Methods. After preabsorption with normal rabbit serum and protein A-Sepharose, 2×10^7 cpm of 35 S-labeled extract was incubated with anti-*myc* serum. Samples were analyzed on a 9% polyacrylamide gel containing 0.1% SDS. After electrophoresis, gels were treated with Enlightening fluor, dried, and autoradiographed 14 days. Lanes: 1, OK10 wild-type; 2, OK10 fs-pol met⁻; 3, OK10 amb; 4, OK10 sa⁻ met⁻; 5, OK10 fs-gag/pol sa⁻ met⁻; 6, uninfected quail embryo primary cells. (B) Immunoprecipitation was done as described for part A, except anti-*gag* serum was used for all samples, samples 1 to 4 were electrophoresed in a 6.5% polyacrylamide gel, and samples 5 to 7 were electrophoresed in an 8% gel. Gels were autoradiographed for 14 days. Lanes: 1, OK10 sa⁻ met⁻; 2, OK10 wild type; 3 and 6, OK10 amb; 4, uninfected quail embryo primary cells; 5, OK10 fs-gag/pol sa⁻ met⁻; 7, OK10 fs-pol met⁻.

either p58 of pOK10 or p58 of pOK10 fs-pol met⁻ upon electrophoresis in polyacrylamide gels (Fig. 3A, lanes 1, 2, and 3). That the level of p58 *myc* protein in pOK10 amb-infected cells (Fig. 3A, lane 3) is barely higher than that of the endogenous p58 *myc* suggests that the expression of p58 *myc* is not very efficient. This, in turn, could explain the lower transforming potency of the OK10 amb mutant. It now appears that it might have been better to mutate the AUG codon of the *gag* gene, leaving the first AUG in *myc* as the first initiation site in the spliced mRNA. pOK10 amb also encoded a *gag*-related protein of 160 kDa, which is the expected truncated p200 protein, and helper virus proteins Pr76 and Pr180, which were detected with anti-*gag* sera (Fig. 3B, lanes 3 and 6). We conclude that the *gag* leader of viral p58 is not essential but important for the transforming function of OK10.

Transformation by the p200 *myc* protein of OK10. To determine whether the p200 *myc* protein of OK10 is an autonomous transforming protein, we constructed an OK10 mutant that would not express p58. To this end, the splice acceptor in *myc* was inactivated by mutation. This mutant, OK10 sa⁻ (see Materials and Methods), transformed quail cells. However, contrary to expectation, the transformed cells expressed a subgenomic 4-kb mRNA as well as p58 (not shown). Thus, an alternative splice acceptor must be present in OK10 that allows expression of p58 despite the absence of the canonical *myc* splice acceptor.

To prevent translation of p58 from the aberrantly spliced subgenomic RNA of OK10 sa⁻, we introduced a second mutation in OK10 sa⁻ that prevents translation from the first methionine of *myc* (Fig. 1). For this purpose, the first methionine codon in *myc* of OK10 sa⁻ was changed to a glutamine codon. The resulting double mutant, pOK10 sa⁻ met⁻ (see Materials and Methods; Fig. 1), induced 30 foci per μ g of DNA in quail fibroblasts upon transfection with MAV-1 helper virus DNA (Table 1). These foci appeared 12 days posttransfection. Cells transformed by this mutant were less refractile than cells transformed by wild-

type OK10 (Fig. 2). Immune precipitates with anti-*gag* serum from cells transformed by OK10 sa⁻ met⁻ detected a p200 protein in addition to helper viral proteins (Fig. 3B, lane 1). The level of p58 detected with anti-*myc* serum was very low, but possibly above the p58 background of uninfected cells (Fig. 3A, lane 4). To determine whether the transforming function of this mutant was due to p200 *myc* protein or to a low level of p58 (due to leakiness of the mutant), a frameshift mutation in the *pol* region of the *gag*- Δ *pol*-*myc* p200 gene of OK10 sa⁻ met⁻ was prepared. This mutant, OK10 fs-pol sa⁻ met⁻ (Fig. 1), failed to transform fibroblasts (Fig. 2, part 6; Table 1). We conclude that p200 is an autonomous transforming protein.

The low transforming efficiency of OK10 sa⁻ met⁻ may reflect a poor intrinsic transforming quality of p200 or a low level of p200 expression. p200 expression may be low due to a native retroviral frameshift between *gag* and *pol* (20). We have confirmed by sequence analysis of our OK10 clone that the frameshift between the *gag* and *pol* genes of retroviruses is also present in OK10 (sequence in Fig. 1). p200 expression from OK10 is probably as inefficient as the synthesis of Pr180, the precursor of the viral polymerase. The polymerase precursor Pr180 is expressed at a 20-fold-lower level than the *gag* precursor protein Pr76 (12).

In an effort to increase p200 expression, we have eliminated the native frameshift between *gag* and *pol* by deletion of 480 nucleotides and insertion of an appropriate linker between *gag* and *pol*. p58 synthesis was blocked by two mutations that inactivated the splice acceptor and the *myc* methionine codon. The resulting mutant, OK10 fs-gag/pol sa⁻ met⁻ (see Materials and Methods; Fig. 1), generated 250 foci per μ g of DNA compared with 30 foci per μ g of DNA for OK10 sa⁻ met⁻ (Table 1). Foci appeared 10 days after transfection. These foci were phenotypically similar to those generated by OK10 sa⁻ met⁻. Transformed cells were not as refractile and piled up as cells transformed by wild-type OK10 (Fig. 2). Immunoprecipitation with anti-*gag* serum from cells transformed by this virus show a truncated p200

(Δ p200) of approximately 190 kDa (Fig. 3B, lane 5). This shortened p200 is expressed at considerably higher levels than p200 in cells transformed by wild-type OK10 (cf. Fig. 3B, lanes 2 and 3). We tentatively conclude that p200 is an intrinsically less efficient transforming protein than p58, even when it is overexpressed as a truncated variant. A definitive assessment of the relative transforming efficiencies of the p200 and p58 *myc* proteins would require a better quantitation of these proteins than was obtained here.

DISCUSSION

OK10 only retrovirus to encode two autonomous transforming proteins. Our experiments show that OK10 expresses two *myc* proteins, p58 and p200, that can each function as autonomous transforming proteins. Mutants expressing only p58 transform quail embryo cells as efficiently as wild-type OK10. However, OK10 mutants that express only p200 or internally deleted p200 (Δ p200) transform quail embryo cells less efficiently than mutants expressing only p58. OK10 is thus the only retrovirus to express two distinct proteins with transforming function in the same cell type.

Our conclusion that the p58 *myc* gene of OK10 is an autonomous transforming gene is supported by previous experiments showing that the p58 *myc* gene of MH2, which is closely related to that of OK10 (23), and those of MC29 and CMII (2, 3) are also autonomous transforming genes. The lower transforming efficiency of OK10 mutants expressing only p200 compared with those expressing only p58 corresponds to a lower level of expression of p200. It also appears to reflect a lower specific transforming efficiency of p200 compared with the transforming efficiency of p58. This is deduced from an OK10 mutant that expressed a mutant p200, called Δ p200, at levels similar to p58 (Fig. 3A, lane 1, and B, lane 5), yet failed to transform like mutants expressing only p58 (Fig. 2, panels 2 and 5).

A probable answer to the question of why OK10 encodes two transforming proteins, although one (p58) is sufficient for transforming function, emerges if the origin of retroviral *onc* genes is considered. Retroviral *onc* genes are products of rare, illegitimate, and, hence, random recombinations between retroviruses (without *onc* genes) and proto-*onc* genes (5, 5a). Thus, sequences that are not relevant to transforming function may be transduced together with sequences necessary for transformation. One of the two redundant *onc* genes of OK10, the nontransforming *mht* sequence of MH2 (23), and the nontransforming *erba* sequence of avian erythroblastosis virus (21) are all examples of sequences that are not essential for either transformation or replication. In view of this, the two transforming genes of OK10 simply appear as accidents of the transduction process that generated OK10.

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