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Evidence for p15 Cleavage Site in myc-Specific Proteins of Avian MC29 and OK10 Viruses

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Myc-related proteins were precipitated from MC29 virus-transformed cells (PR-2) and from OK10 virus-transformed cells (9C) by anti-gag and anti-myc sera. Immunoprecipitates were cleaved with the avian retroviral protease p15 and the cleavage products analyzed in SDS-PAGE. Cleavage fragments of $p110^{gag\text{-}myc}$ (product of MC29 virus) and $p58^{myc}$ (product of OK10 virus) showed the presence of a p15 cleavage site within the myc-specific region. The site is missing in deletion mutants of MC29 virus.

Key words: avian tumor viruses, myc oncogene, p15 viral protease

Avian acute leukemia viruses contain cell-derived sequences that are responsible for their oncogenic properties [1]. Through the insertion of new genetic material, which replaced some of their genes, these viruses lost their ability to replicate, but acquired their oncogenic potential. The avian myelocytomatosis virus MC29 contains an inserted oncogenic sequence called v-myc. The same region, or a closely related one, has also been found in three other, independently isolated avian leukemia viruses, OK10, MH2, and CMII [2].

The genome of MC29 viruses contains a deleted gag gene, the myc region, and a deleted env gene. In cells transformed by this virus, only one type of genome-length viral mRNA and only one translation product, protein p110, which is a fused product of gag and myc genes ($p110^{gag\text{-}myc}$), have been detected [3].

The genome structure of OK10 virus is different from the other members of the MC29 virus group in that the v-myc sequence is inserted between the pol and env genes, whereas the complete gag gene is retained [4]. Thus this virus is defective in pol and env genes. In addition to a genome-length viral mRNA, in cells transformed by the OK10 virus, a subgenomic mRNA of 3.6 kilobases (kb) is present [5]. Until

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recently only the fused protein $p200^{gag\text{-}pol\text{-}myc}$, a product of genome-length viral mRNA, could be detected in the transformed cells. Owing to the presence of the gag determinants, this protein was detected by means of anti-gag sera, as were other fused proteins.

However, antibodies raised against a product of the molecularly cloned v-myc gene [6] made it possible to detect exclusively myc-specific determinants. In addition to a p200 protein, such a protein with a molecular weight of 58K [6], a product of the subgenomic mRNA, has, in fact, been detected in cells transformed by OK10 virus.

The in vitro cleavage by the p15 viral protease of the fused proteins of defective leukemia viruses, containing at least a portion of the gag-related peptides, has proved to be a promising approach to analyzing the structure of these proteins [7, 8].

In previous studies with MC29 virus-transformed cells we have tried to remove the gag-specific portion of the p110 protein by using in vitro cleavage by the p15 viral protease [7]. The results indicate the presence of two p15 cleavage sites, one localized in a gag portion of gag-myc fusion protein as expected and a new one localized within the v-myc region of p110. The cleavage of an exclusively v-myc-specific protein p58 in cells transformed by OK10 virus [6] provided direct evidence for the presence of a p15 cleavage site in the v-myc-specific product.

METHODS

PR-2 is a cell line derived from a chicken hepatoma induced by MC29 virus [9]. The 10C is a quail cell line transformed by a deletion mutant of MC29 virus [10] and the 9C quail cell line transformed by OK10 virus [11]. The cells were labeled for either 120 min with [^{35}S]methionine (100 μCi/60-mm dish) or 24 hr with [^{14}C]lysine (200 μCi/60-mm dish). Both isotopes were obtained from The Radiochemical Centre (Amersham, England). After labeling, the cultures were washed three times with phosphate-buffered saline, and the cells were lysed by adding 1 ml of lysis buffer (0.02 M Tris-HCL, 0.05 M NaCl, pH 7.3; 0.5% Nonidet P40; 0.5% deoxycholate). Cell lysates were clarified (10,000g, 20 min) and preabsorbed with 3 μl of normal rabbit serum at 4°C overnight. Then an excess of protein A-Sepharose was added, and the precipitates were removed by centrifugation (3,000g, 5 min). Preabsorbed lysates were incubated with 3 μl of immune or control serum, and the immunoprecipitates were isolated by the protein A-Sepharose method as above.

Cleavage of immunoprecipitates with the p15 protease and subsequent SDS-PAGE have been described in detail previously [12]. A [^{14}C]-methylated protein mixture (The Radiochemical Centre), containing 14.3K, 30K, 46K, 69K, 92.5K, and 200K proteins, was used as a molecular weight marker. For immunoprecipitation, anti-myc serum [6] and anti-gag serum [7] were used.

RESULTS

Figure 1 shows an in vitro cleavage by p15 of immunoprecipitates prepared from 9C cells labeled with [^{35}S]methionine. When an antiserum against the v-myc product was used for immunoprecipitation, only the v-myc-containing proteins, p200 and p58, were found (lane 2). These two proteins were cleaved by the p15 protease, but the cleavage of the myc-specific proteins is obscured by the cleavage products of the gag-pol portion of the p200 protein (lane 3).

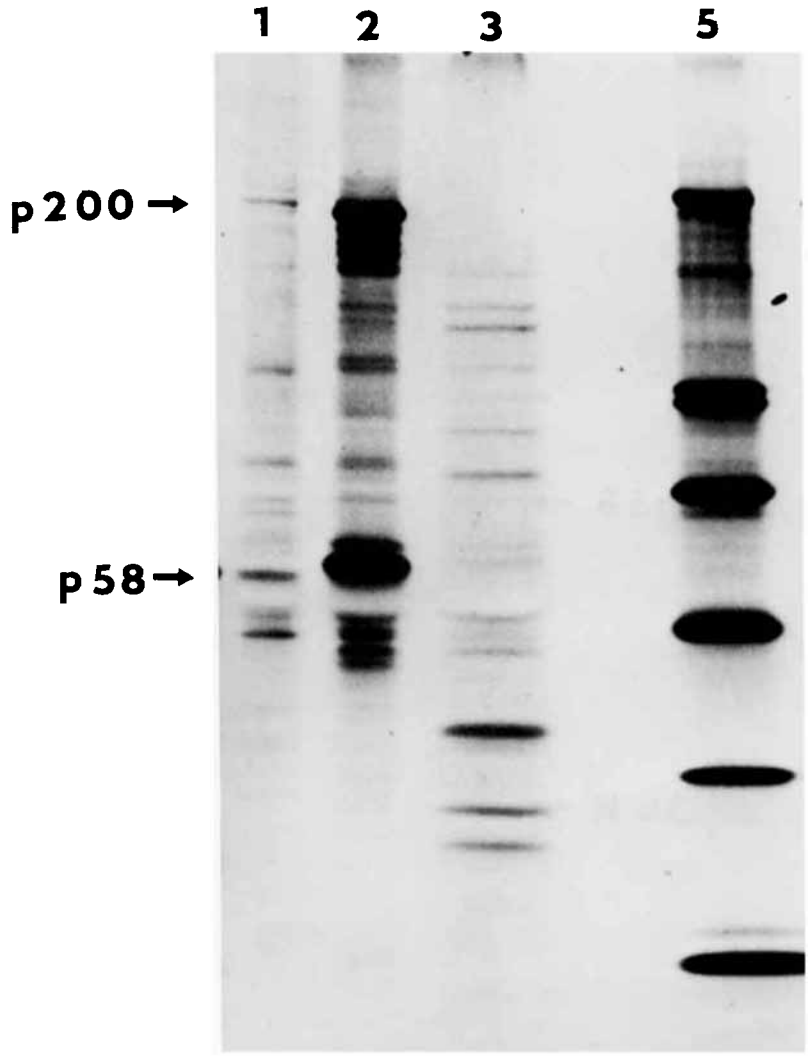

Fig. 1. In vitro cleavage of $p200^{gag\text{-}pol\text{-}myc}$ and $p58^{myc}$ by p15 protease. v-myc-Related proteins were immunoprecipitated from [^{35}S]methionine-labeled 9C cells with normal rabbit serum (lane 1) or anti-myc serum (lanes 2, 3). Samples were incubated for 37°C for 3 hr without (lane 2) or with (lane 3) p15 protease. Lane 4 (unmarked) is empty, and lane 5 contains [^{14}C]protein markers. Analysis was done in 6–18% gradient SDS-PAGE followed by fluorography.

To analyze the fragments of the myc product only (p58), the fused protein $p200^{gag\text{-}pol\text{-}myc}$ had to be removed by extensive preabsorption of cell lysates with anti-gag serum (4°C overnight). The supernatant from the cell lysates was then precipitated with anti-myc serum and p15 cleavage was carried out (Fig. 2). The results (Fig. 2) indicate that the 58K protein is cleaved by p15 and that the only detectable fragment is approximately 34K (lane 1). This fragment must be derived from the N-terminal part of the v-myc protein, in which the carboxy-terminal domain does not contain any methionine. To detect the carboxy-terminal fragment, we repeated the

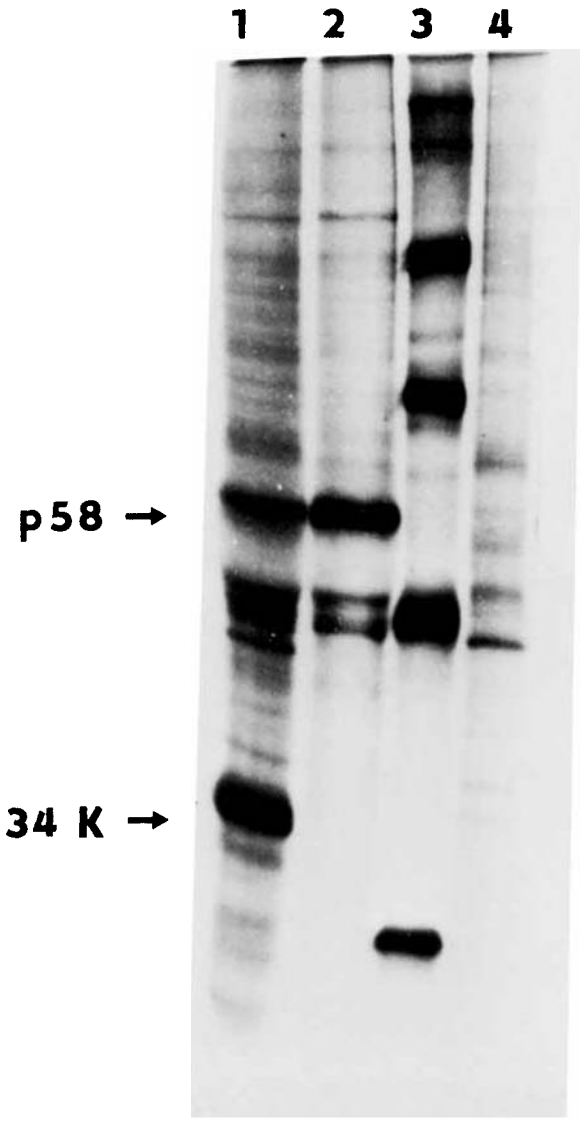

Fig. 2. Fluorogram of 10% SDS-PAGE analysis of p58 protein cleaved by p15. 9C cell lysates were preabsorbed with anti-gag serum and precipitated with anti-myc serum (lanes 1, 2) or normal rabbit serum (lane 4). Immunoprecipitates were incubated with (lane 1) or without p15 (lane 2). Lane 3 contains [^{14}C]protein markers.

p15 cleavage of p58 from 9C cells labeled with [^{14}C]lysine. The cleavage of both [^{14}C]lysine- (lanes 2 and 3) and [^{35}S]methionine- (lanes 4 and 5) labeled 9C cell lysates, which were immunoprecipitated with anti-myc serum, is presented in Figure 3. Besides the 34K cleavage fragment found both in lysine- and methionine-labeled cells (lanes 3 and 5), a smaller 24K fragment was detected in the sample labeled with [^{14}C]lysine only (lane 3).

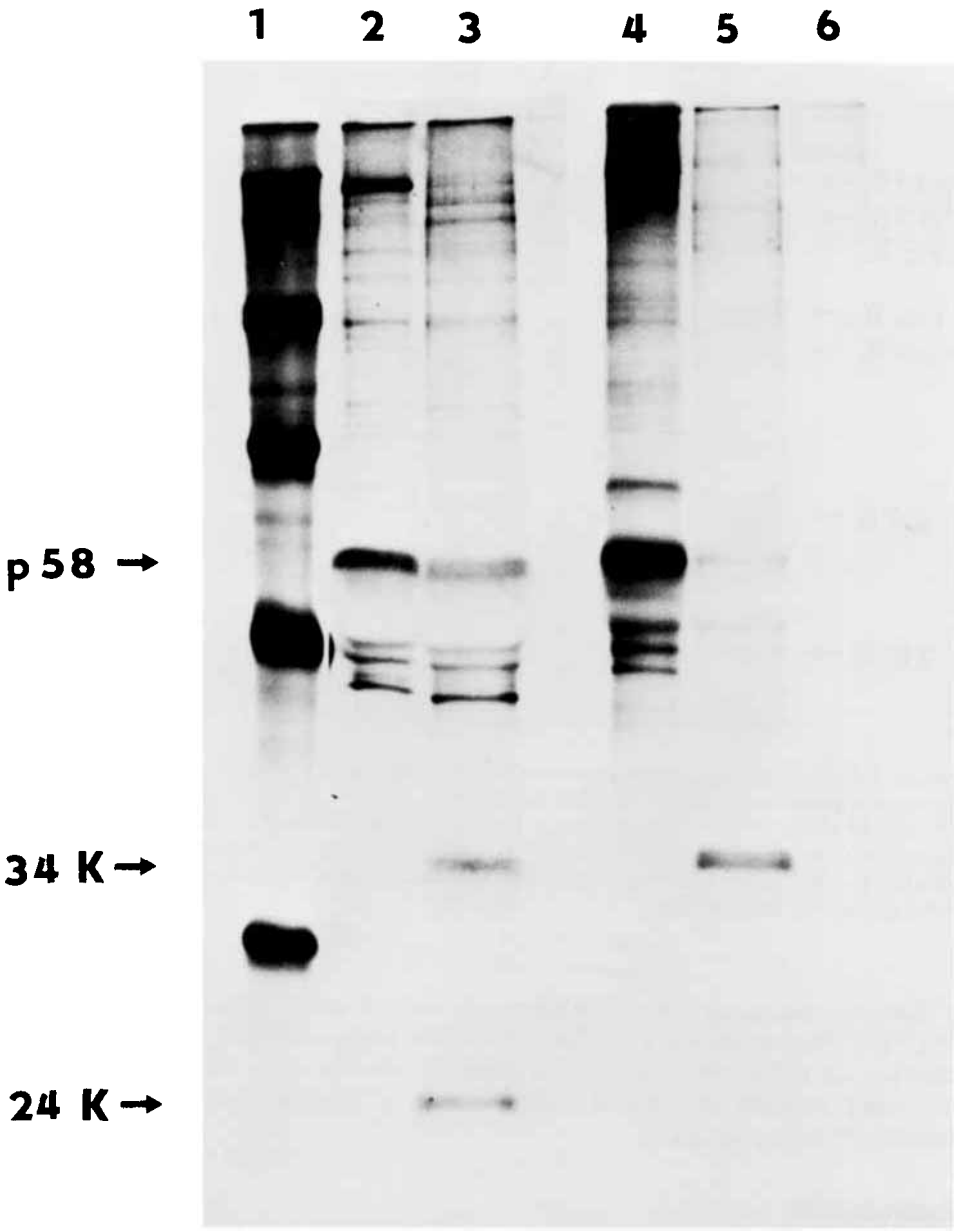

Fig. 3. p15 cleavage of p58 protein precipitated with anti-myc serum from 9C cells labeled with either [^{14}C]lysine (lanes 2, 3) or [^{35}S]methionine (lanes 4–6). Immunoprecipitates were incubated with (lanes 3, 5) or without p15 protease (lanes 2, 4). Lane 1 contains [^{14}C]protein markers, lane 6 contains precipitate with normal rabbit serum. Analysis in 10% SDS-PAGE followed by fluorography.

On the basis of these results we repeated the cleavage of MC29-specific proteins labeled with [^{14}C]lysine. We analyzed the cleavage fragments of p110$^{gag\text{-}myc}$ and of p95, a protein of the MC29 deletion mutant 10C. Figure 4 shows the cleavage fragments of the proteins labeled with [^{14}C]lysine (lanes 8–11) and with

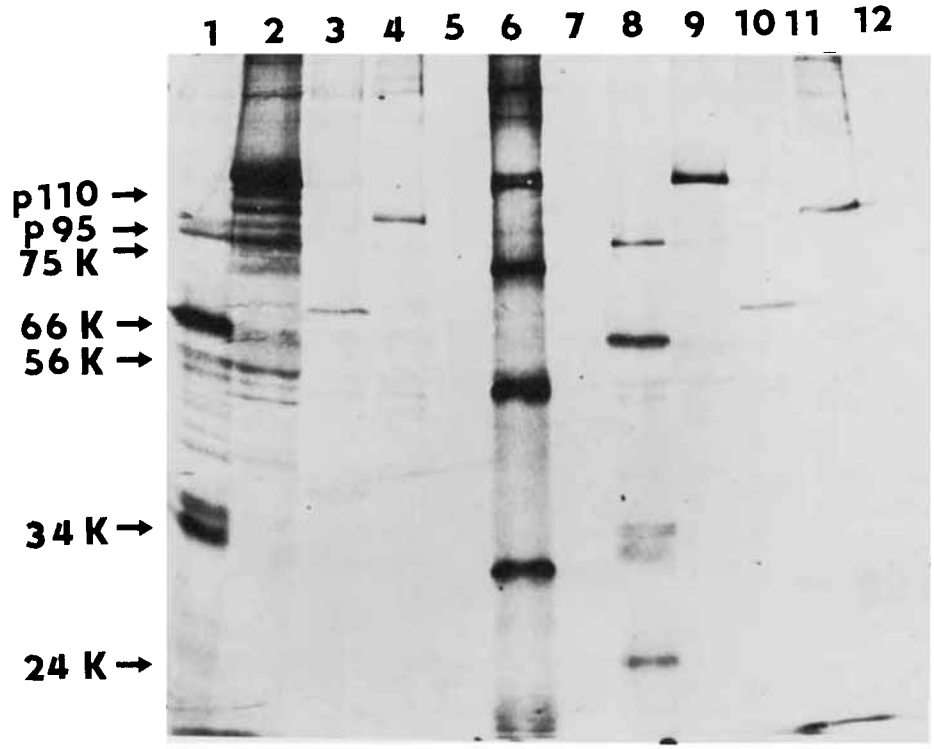

Fig. 4. Cleavage of myc-specific proteins of MC29 virus and its deletion mutant 10C by p15. Immunoprecipitates with anti-myc serum of p110 (lanes 1, 2, and 8, 9) and of p95 (lanes 3, 4 and 10, 11) proteins labeled with [^{14}C]lysine (lanes 8–12) or [^{35}S]methionine (lanes 1–5) were incubated with (lanes 1, 3, 8, 10) or without (lanes 2, 4, 9, 11) p15 protease. Lanes 5 and 12 contain precipitates of p95 with normal rabbit serum; lane 6 contains [^{14}C]lysine protein markers. Analysis was done in 10% SDS-PAGE followed by fluorography.

[^{35}S]methionine (lanes 1–4). In addition to the three fragments detected after cleavage of [^{35}S]methionine-labeled p110 (lane 1), another fragment of 24K was obtained after cleavage of [^{14}C]lysine-labeled p110 (lane 8). On the other hand, there was no difference between the cleavage fragments of [^{14}C]lysine- and [^{35}S]methionine-labeled p95 (lanes 10 and 3).

DISCUSSION

In vitro cleavage of Pr76 has shown that the p15 cleavage sites are localized at both termini of the p27 protein [12]. In an attempt to obtain the myc protein free of gag antigenic determinants for raising antibodies against the v-myc protein, the p15 cleavage strategy was used. The portion coding for the COOH terminus of the p27 protein is missing in p110 [13]. Therefore, a gag-protein-free fragment was not obtained, because the COOH terminus of p27 with the corresponding p15 cleavage site is deleted in p110. However, the size of the major fragment (56K) indicated the presence of another p15 cleavage site within the v-myc region of p110 [14]. It was assumed that this site was missing in the protein products, p110, p95, and p90, of the

three MC29 mutants with partial deletions in the myc region [15]. This assumption was based on the unexpected length of the major fragments after the p15 cleavage (66K from p100, 60K from p95, and 56K from p90).

According to the nucleotide sequence of v-myc of MC29 virus [13], the carboxy-terminal part of the v-myc polypeptide contains no methionine residues, and thus, it escapes detection when [^{35}S]methionine label is used for analysis. Therefore we have used the [^{14}C]lysine labeling of MC29- and OK10-transformed cells in this study. The cleavage pattern of p58 protein of OK10-transformed cells, which protein is exclusively v-myc-specific, provided direct evidence for the presence of a p15 cleavage site in the v-myc-specific product.

Some uncleaved 58K protein was always present even when the conditions of cleavage were modified in an attempt to achieve a complete cleavage. The presence of a v-myc-unrelated protein of the same mobility was excluded by control precipitation (Fig. 2, lane 4). The incomplete cleavage of the p58 could be explained by differences in the spatial configuration between myc-containing proteins of MC29 ($p110^{gag\text{-}myc}$) and OK10 ($p58^{myc}$) viruses. However, a c-myc gene product of the same molecular weight [6] can also comigrate. Further analysis is needed to determine the possible cleavage of c-myc products.

It has not been definitely clarified whether the nucleotide sequences of the v-myc genes of OK10 and MC29 viruses are identical. Nevertheless, the v-myc gene structure and the length of both viruses are similar according to restriction endonuclease mapping and heteroduplex analysis [16]. The localization of the cleavage site for p15 in the myc product of OK10 virus also seems to be the same as that in the MC29 protein. The size of cleavage fragments of the p58 protein is in agreement with our hypothesis on the presence of a p15 cleavage site within the myc-specific region of the $p110^{gag\text{-}myc}$ protein [14]. After cleavage of [^{14}C]lysine-labeled p110, four fragments were generated. The 75K fragment is probably an intermediate product of p15 cleavage in vitro (unpublished observation). The 56K fragment contains the N-terminal portion of the v-myc product and an incomplete p27 protein [14]. The 34K fragment contains the remaining N-terminal portion of p110, ie, the p19 and p10 moieties. This cleavage product is identical with that resulting from p15 cleavage of $Pr76^{gag}$ [12]. The smallest fragment of about 22K represents the C-terminal portion of p110 and is not detected in cleavage experiments with [^{35}S]methionine-labeled p110 (Fig. 4, lane 1). On the other hand, no difference was found between cleavage fragments of [^{14}C]lysine- and [^{35}S]methionine-labeled p95, a product of the MC29 deletion mutant. These results again confirm the presence of a cleavage site in the v-myc-specific region of the p110 molecule and indicate the absence of this site in the protein products of MC29 deletion mutants, as has been suggested in earlier work [14]. A study is in progress for locating the p15 cleavage products from [^{14}C]lysine-labeled proteins.

The presence of a p15 cleavage site in the position deleted in the transformation-defective mutants would suggest a possible posttranslational processing of the gag-myc-fused protein of MC29 virus in vivo. Such processing might affect the mechanism of transformation by MC29 viruses. Since there is no coding sequence for the p15 protein in MC29 virus, such cleavage might be performed by a cellular protease with similar specificity as that of p15. It remains to be elucidated, whether a cellular protease with this specificity does exist, as has been suggested for the processing of $Pr76^{gag}$ [17].

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