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# EXPRESSION OF AVIAN LEUKOSIS VIRUS PROTEINS p27 AND p19 IN UNINFECTED CHICKEN, DRAKE AND QUAIL CELLS

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ABSTRACT: Normal, uninfected animal cells have an unique feature, the presence in their genome of genes (proviruses) identical or closely related to infectious exogenous retrovirus. The level of expression of these genes varies from individual to individual; from complete silence to the production of virus particles. Exogenous and endogenous virus proteins were purified from sonicated viruses passed through a Sepharose 6B column satured with 6M guanidinium hydrochloride. The presence of equivalents of antigens p27 and p19 was analysed by radioimmunoassay. The presence of endogenous virus components was investigated in CEF, heart, brain, tigh muscle and wing muscle of chicken, quail and drake. The function or significance of the proteins are not known. Immunological, hormonal and environmental factors remain to be studied.

DESCRIPTORS: Retrovirus; Endogenous virus; virus endogenous expression; RIA.

## INTRODUCTION

The retrovirus exhibits an unique feature, their complete genome or some of their genes might be present in uninfected, normal cells. This chromosome segment is named endogenous and it is widely distributed in nature, having been characterized not only in birds but also in mammals. Its existence was first reported by DARLINGTON, 1948[15] as provirus, a cellular genetic element activated under special circumstances. More carefull studies concerning the presence of information specifying the genome of an RNA tumor virus in normal cells were carried out by HUEBNER & TODARO[31]. The virus specific DNA was proposed to be vertically transmitted and portions of the genome were proposed to be expressed during certain stages of the development. The authors assumed that in normal adult cells the transcription of the genes would be repressed.

HANAFUSA et al.[20] reported that fibroblasts from certain normal chicken embryos were able to complement the defectiveness of the RSV Bryan strain releasing infectious particles. The fibroblasts contained one component, then called chicken helper factor (chf), later on characterized as an envelope glycoprotein encoded by endogenous viruses present in these cells. VOGT & FRISS[59] found espontaneous release of RAV-O viruses from certain chicken embryo cell lines. This observation led to the conclusion that the endogenous genome by itself is certainly defective: these studies were corroborated by several authors [6,20,42,48,58]. Later on, the presence of this envelope glycoprotein was detected in the majority of commercial flocks of chicken. Due to the ubiquitous presence of the glycoprotein, it was suggested to be harmless to the host organism and even be advantageous in certain cases by protecting the host against exogenous infection[60].

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In regard to the presence of the helper factor and group specific antigen (gs), HANAFUSA et al.[22] and ROBINSON[47] identified three different phenotypes in chicken embryos: gs+chf+ showing both properties; genetic experiments showed the segregation of two single dominant alleles ; $gs^-$ - $chf^+$ or h-e do not show detectable levels of gs but high levels of helper activity; and $gs^-$ $chf^-$ which show either very low or undetectable level of both characters. Hybridization experiments conducted with specific probes showed the absence in the genome of U3-region related to exogenous virus in the chicken fibroblasts analysed[32]. Curiosly, the cells containing env-3 do not express the structural viral component p15. So far, it has been recognized 16 env-endogenous species.

The distribution of the endogenous virus differs tremendously in different species as well as in the same species. Moreover, the phenotype expression covers a wide range, going from complete silence, expression of one component until the production of a full particle. The explanation of this phenomenon is not known, although some hypothesis have been postulated, like the difference in the level of expression might be due to provirus mutation or chemical modification of the provirus genome. Methylation has been shown to be an important modification[12,23,33,54], the relevance of the process was shown by using 5-azocitidine. JONES & TAYLOR[35]; GROUDINE et al.[19] proposed the methylation as a cell mechanism to repress the expression of undesirable genes.

Hybridization experiments have shown the presence of analogous retrovirus genes in germ lines of primates. VARMUS & LEVINE (1983)[56] raised several questions about endogenous viruses. "Do they arise from normal cellular genes? Are they derived in different species from a primordial endogenous provirus in an ancestral vertebrate? Do they find their way into germ lines by intermitent, independent infectious? What are the benefits and dangers to a host organism that carries endogenous proviruses?"

In the present work, we have tried the purification of endogenous virus antigens and their comparison with the profiles obtained with exogenous virus particles. The approach used to measure the expression of these protein was the radioimmunoassay. The expression of endogenous virus components mainly, p27 and p19, were studied in chicken embryo fibroblasts and extracts of chicken tissues and organs. Quail and drake cellular extracts were also analyzed and the identity of p27 and p19 in different systems was investigated.

## MATERIAL AND METHODS

### *Virus purification*

Avian fibroblasts culture supernatants were clarified by low speed centrifugation (3,500 xg/ 30 min./4°C); the supernatants were submitted to ultracentrifugation (100,000 xg/90min/4°C) to separate virus particles. The pellet resuspended in PBS (phosphate saline buffer = 120 mM sodium chloride; 18 mM disodium phosphate and 2.5 mM kalium monobasic phosphate) was layered onto a 20 to 60% sucrose-TRIS (10 mM pH 7.4) gradient. After centrifugation at 100,000 xg/ 10°C for 180 minutes, the fractions of the gradient were collected and these fractions were recentrifuged at 110,000 xg/90min/4°C. The combined pellets were resuspended in PBS and dialysed against buffer containing 40 mM TRIS.HC1 pH 7.4; 20 mM sodium acetate and 1mM EDTA.

### *Isolation and purification of structural proteins*

Virus particles were disrupted in the presence of TNE-TRITON X-100 0.2% by sonication and the sample was suspended in buffer containing 8M guanidine-hydrochloride; 1.5 M beta-mercaptoethanol and 5 mM EDTA pH 8.0. The virus extract was applied to a column of Sepharose 6B-guanidine hydrochloride and eluted with buffer containing 50 mM sodium acetate pH 5.0; 20 mM beta-mercaptoethanol and 6 M guanidine hydrochloride according to the procedure previously described by GREEN & BOLOGNESI[18].

### *Protein determination*

The protein was determined either by LOWRY et al.[38] method or BRADFORD[9] for samples containing guanidine hydrochloride. Bovine serum albumin was used as standard based on $E^{1\%}_{280} = 6.6$.

### *Polyacrylamide sodium dodecyl sulphate gel electrophoresis (PAGE)*

The gel electrophoresis system used were either at 12.5% polyacrylamide concentration or 5-20% gradient according to the technique described by LAEMMLI[36] and MAIZEL[40]. High and low molecular weight standards were used as markers.

### *Cell lines, tissues and extracts preparation*

CEF-commercially available chicken embryo fibroblasts culture; CEF-7.2-chicken embryo fibroblasts line 7 subline 2; brain, heart, tigh and wing muscle from 5 months old chicken (*Gallus gallus domesticus*), 30 days old quail (*Nothura maculosa*) and 30 days old drake (*Anas platyhynchos*).

The tissues and organs were removed immediately after bird killing: the fat was taken out and the tissues and organs were kept in an ice bath. The material was minced and homogenized in the presence of TNE buffer containing 1mM PMSF (phenylmethylsulfonilfluoride) and TRITON X-100 0.2%. The excess of debris was discard and the supernatant centrifuged at 1,500 xg/30min./4°C and then 10,000 xg/60min./4°C. To clean up the extracts, the supernatants were centrifuged at 80,000xg/60min/4°C and the final supernatants were used as "cellular extracts" in the assays.

*Radioimmunoassay*

The viral and to procedures purified proteins were labelled with $^{125}$I-Na according to HUNTER[34], according to technique described elsewhere [27,28]. These techniques allowed the time of reaction to be reduced to 30 seconds.

*Immune serum titre* - The titre was defined as the serum dilution giving 50% precipitation of labelled antigen under specified conditions. Constant amount of labelled antigen plus several dilutions of immune serum plus normal rabbit serum were incubated at 37°C for 3 hours and the reaction completed at 4°C overnight, then the precipitating sheep antibodies or goat anti-rabbit IgG were added. The reaction was allowed to precipitate at 4°C/overnight and subsequently submitted to centrifugation at 3,500 xg/20min: the pellet was washed twice with cold TNE and the radioactivity of the pellet measured in a gamma counter.

*Competition assay* - The competition assay of the proteins of cellular extracts were performed in a system similar to the precipitation reaction, with the addition of cellular extracts. The amount of the equivalent of antigen present in the cellular extract was calculated by comparison with a standard competition curve prepared by using known amounts of unlabelled protein as competitor in the above experimental conditions.

## RESULTS

*Purification of viruses particles*

The virus particles separeted from culture fluids when submitted to sucrose gradient ultracentrifugation showed different banding patterns depending whether the virus, was exogenous or endogenous. The exogenous viruses formed only one layer around the sucrose concentration of 35%, which is equivalent to a density of 1.16 - 1.18g/ml[7,8]. The endogenous viruses presented besides the main layer at the concentration of 35%, second band at around 20% and a third layer (lower) between the sucrose concentration of 50 and 60%. In addition, both endogenous viruses 15I5 and 7.2 presented similar protein profiles in PAGE on a gel gradient 5 to 20% (figure 1). Compared with the molecular weights markers, 200 K = myosin; 150 K = immunoglobulin; 90 K = phosphorilase B; 45 K = ovalbumin; 30 K = carbonic anydrase; 20 K = trypsinogen and 15 K = beta-lactoglobulin, we could detect the presence of several components gp85, gp35, p27, p19, p15, p12 and probably a reverse transcriptase line in the 90 K region. The electrophoresis profiles showed that the layer at 35% contained the complete virus or intact virus particles.

The table 1, presents the protein content of the virus samples obtained after sucrose gradient centrifugation. The viruses concentrated in the main layer were used for further processing.

*Radioimmunoassay*

The purified antigens p27, p19 and p15 from exogenous ALV were sucessfully labelled with $^{125}$I-Na according to HUNTER[34] procedure, giving specific activities between 2 to 3 x $10^4$ cpm/ng protein.

*Immune serum titration* - Each immune serum was titrated in a system containing 0.02 ml $^{125}$I-p (40 - 50,000 cpm); 0.02 ml Anti-p in serial dilu-

TABLE 1

Sucrose gradient virus purifications: protein determination

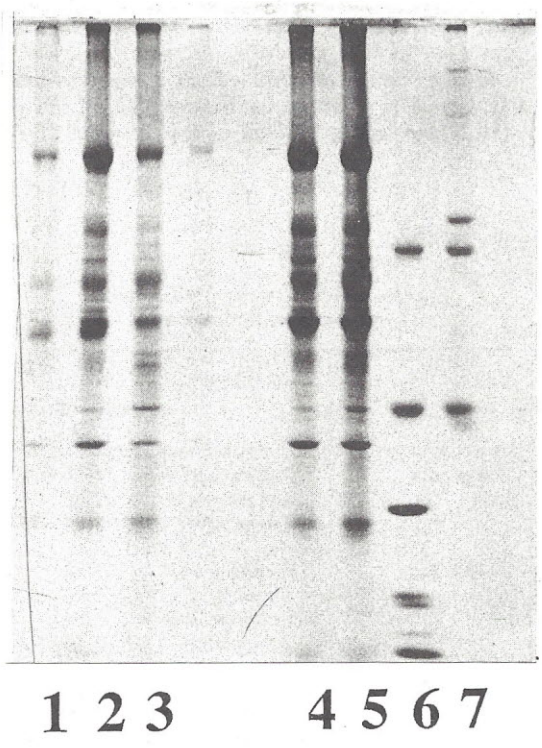
| VIRUS | SAMPLE[a] | TOTAL PROTEIN[b] mg |
|---|---|---|
| Endogenous 1515 | Crude extract | 4.50 |
| | Upper layer | 2.00 |
| | Lower layer | 1.94 |
| Endogenous 7.2 | Crude extract | 9.00 |
| | Upper layer | 1.30 |
| | Intermediate layer | 3.84 |
| | Lower layer | 2.76 |
| Avian leukosis Virus (exogenous) | Crude extract | 4.50 |
| | Virus layer | 4.00 |

(a) Virus purification carried out by sucrose gradient ultracentrifugation at 110,000g/90min/10°C of the virus suspension.

(b) Protein determination according to LOWRY et alii 38 refered to in Methods.

tions; 0.03 ml NRS (dil.1:3) TNE buffer to complete the volume to 0.250 ml; a first incubation 37°C/3hr was followed by an overnight incubation at 4°C; the addition of 0.03 ml GAR or SAR (dil. 1:36) and then a precipitation to completion at 4°C/overnight. The pellet was collected by centrifugation at 3,500 xg/20min., washed twice and the radioactivity was measured in the gamma counter. Under the experimental conditions used, the following titre were obtained: Anti-p27 = 1:2,000, Anti-p19 = 1:4,000 and Anti-p15 = 1:5,000 (table not shown).

The standard competition curve was used to determine the concentration of the equivalents of antigen present in the system. It was prepared by using as standard competitor unlabelled purified antigen in several concentration. It was allowed to compete with fixed amounts of labelled antigen, in the combination with the antibody present in limited concentration under defined experimental conditions. The figure 2 shows the standard curves of antigens p27, p19 and p15 in the system $^{125}$I-p vs Anti-p respectively.




PICTURE 1 - Polyacrylamide sodium dodecyl sulphate gel electrophoresis (5-20% gradient) of virus extracts. Lanes 1: Endogenous 15I5-sucrose gradient upper layer; 2: E-15I5 - lower layer; 3: E-15I5 - supernatant; 4: Endogenous 7.2 - upper layer; 5: Endogenous 7.2 - lower layer; 6 and 7: Molecular weight standards.

The purification of proteins from exogenous virus particles was carried out Sepharose 6B-satured with 6 M guanidine - hydrochloride under very special conditions (see Methods). The components were nicely separated exactly as shown by GREEN & BOLOGNESI[18]. Unfortunatelly, the purification of endogenous virus components could not be done in the same experimental conditions, since the behaviour is not alike, giving an indication of the difference in the properties of the components. Instead of giving distinct peaks in the Sepharose column as in the case of exogenous virus, the collected fractions from endogenous contained all three major antigens, detected by radioimmunoassay. As shown in the

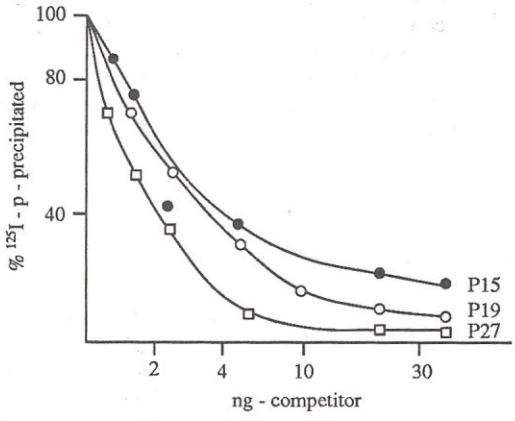
TABLE 2

Protein concentration of fractions eluted from Sepharose 6B-GuHCL column

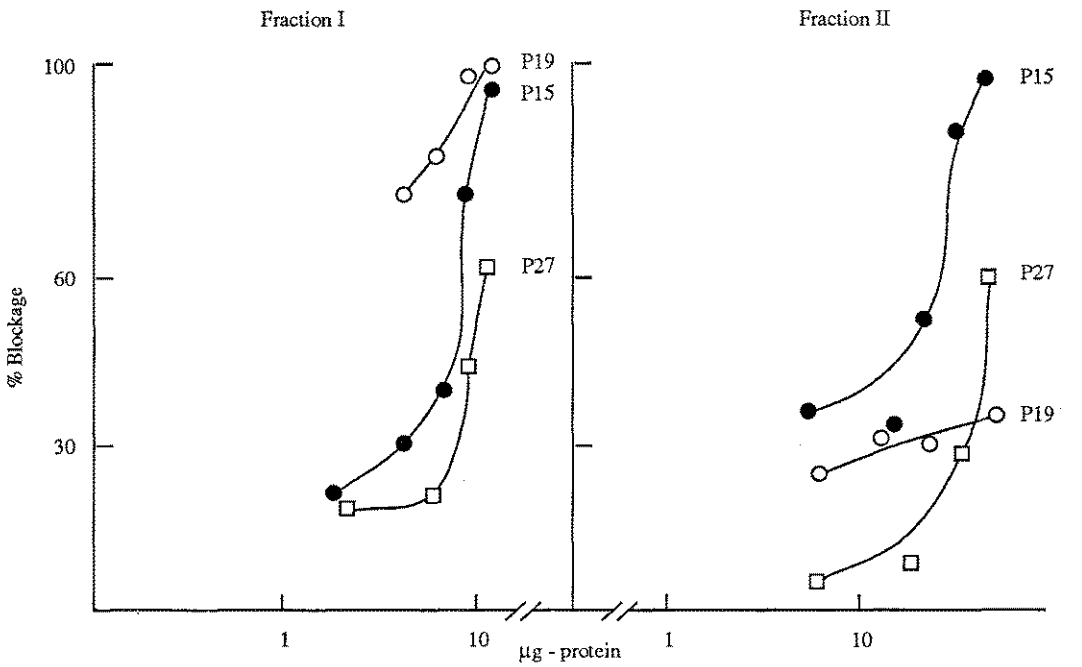
| SAMPLE[a] | TOTAL PROTEIN[b] mg |
|---|---|
| Crude extract | 8.00 |
| Fraction I | 0.93 |
| Fraction II | 4.68 |
| Fraction III | 1.72 |

(a) 9.0 mg of virus extract disrupted and applied to Sepharose 6B-GuHCL column (1.4 x 100 cm) eluted with 20 mM sodium phosphate buffer ph 6.5 containing 0.1% beta-mercaptoethanol and 6M-GuHCL.

(b) Protein content evaluated by BRADFORD method as refered to in Methods.




PICTURE 2 - Standard competition radioimmunoassay: Assay carried out according to description in Materials and Methods. $^{125}$I-p vs Anti-p in dilution equivalent to serum titre vs unlabelled protein, amount indicated in the Figure; precipitation with second antibody and collected pellet radioactivity measured in gamma counter.




PICTURE 3 - Detection of components in the fractions I and II eluted from Sepharose 6B-GuHCl column: Competition assay according to procedure described in "Methods". $^{125}$I-p vs Anti-p vs different amount of fraction I and II; precipitation with second antibody; radioactivity of the pellets determined in gamma counter.

TABLE 3

Competition assay: $^{125}$I-p27 vs Anti-27 vs chicken embryo fibroblasts

| Competitor[a] µg | Precipitation[b] % | Blockag[c] % |
|---|---|---|
| 10 | 98.9 | 1.1 |
| 30 | 88.7 | 11.3 |
| 60 | 76.4 | 23.6 |
| 80 | 72.2 | 27.8 |
| 180 | 54.4 | 45.6 |

(a) Chicken embryo fobroblasts suspension sonicated in the presence of TRITON X-100 0.2% in the presence of 1mM PMSF. Suspension centrifuged at 1,500 xg/20min/4°C. Further centrifugations at 10,000 xg/60min/4°C. The supernatant was used as "extract".

(b) The competiton assay was carried out in a system containing 0.03 ml NRS dil 1:3; 0.02 ml Anti-p27 dil 1:2,000; 0.02 ml$^{125}$ I-p27; competitor in the indicated concentration, TNE . 2 to volume 0.25 ml and 0.03 ml GAR dil 1:250. Pellet collected by centrifugation and the amount of radiactivity of the pellet measured in gamma counter. 100% precipitation is the cpm obtainned in a tube without any competitor.

(c) The percentage of blockage is calculated by subtracting from 100 the percentage of precitation in the column[b].

figure 3, both fractions contained similar amounts of p27 and p15 although the p19 was present mainly in the first fraction. The distribution is indicative of the difficulties of having the antigens purified by Sepharose 6B-guanidine - hydrochloride procedure, for sure more careful studies of the nature of the endogenous virus proteins should be done.

The expression of the equivalents to epitopes of antigens p27, p15 and p19 in chicken embryo fibroblasts were measured in the system analogous to the standard competition assay except in this case the competitor was the extract of chicken embryo fibroblasts. We found very high level of analogous to p27 and also expression of components analogous to p15, indicating the presence of endogenous provirus in the so called normal cells. As shown in the table 3, 30 µg of chicken embryo fibroblasts blocked the precipitation reaction in about 11,3%, what is equivalent to 5 ng of p27. On the other hand, 40 µg contained about 3 ng of p15.

Several authors[1,6,11,39] had shown the expression of certain viral genomic region in the embryogenesis but their activity seems to be repressed in the adulthood. There are difference in

TABLE 4

Cmpetition of normal cells from chicken, quail and drake in the system $^{125}$I-p Anti-p

| Competitor | | ng of equivalents p19 | ng of equivalents p27 |
|---|---|---|---|
| Brain | Chicken | 5.0 | 17.5 |
| | Quail | 36.8 | 33.08 |
| | Drake | 32.3 | — |
| Heart | Chicken | 4.8 | 10.0 |
| | Quail | zero | zero |
| | Drake | 58.8 | 44.3 |
| Wing muscle | Chicken | 2.1 | 9.3 |
| | Quail | 24.5 | 8.3 |
| | Drake | 39.5 | 8.7 |
| Tight muscle | Chicken | 3.0 | 4.0 |
| | Quail | 46.5 | 10.5 |
| | Drake | — | 29.9 |

SYSTEM: Cellular or tissue extracts in several protein concentration: 0.06 ml NRS dil. 1:3; 0.02 ml of $^{125}$I-p (40-45,000 cpm); 37°C/3h; 4°C overnight; 0.06 ml SAR: 4°C/overnight; collected and counted. Table calculated by normalizing all competitor protein concentration to 5.0 mg in order to compare the level of competition.

the level of repression depending on the tissue differentiation level. In our experiments there were a variation of equivalent to viral antigens, depending not only on the tissues but also on the source of chicken tested, giving a clue of the importance of the enviromental conditions and the species of bird used. Very little variation in the amount of p27 and p15 was found in tigh muscle using competitor in the range of 75 to 300 mg. On the other hand, brain and heart extracts showed considerable level of p27 (table 4). The reaction was highly specific, there were no inespecific precipitations, in all assays individual controls were carried out.

Cellular extracts from quails were tested in a system similar to chicken cells, in other words, brain, heart, tigh and wing muscles were used as competitor. Higher expression of p27 analogous was found in tigh muscle and then wing muscle, but no competition was found in heart and brain tissues (table 4).

When drake cells extracts were used, there were no significant variation in the level of p19 in brain, heart and wing muscle. But, the heart muscle showed the blockage of 28.73% when 394.4 µg of tissues was used in the system $^{125}$I-p27 vs Anti-p27 vs heart muscle extract (table 4).

## DISCUSSION

Several attempts have been made in order to set the viral andogenous structural components of avian retrovirus in the purified form, without much success. GREEN & BOLOGNESI[18] improved the separation procedure for viral exogenous antigens by using agarose gel column in the presence of 6M-guanidine hydrochloride. This approach was used previously in order to determine the protein molecular weight through a simple relationship of chain lenght and elution volume in the solvent.

We used Sepharose 6B column satured with 6 M-Gu-HCl, which made the gel system very viscous, being so, it was necessary to adjust the flow rate in order to get adequate separation. The protein diffusion coefficient in 6 M-GuHCl is usually very low, close to the native protein. The protein renaturation was achieved by dialysis against a solution containing beta-mercaptohetanol and EDTA (see Methods). The viral exogenous antigens were nicely separated by this procedure (see Results).

In order to standardized the abbreviations used in the field AUGUST et al.[4] suggested a standard nomenclature for virus components: p for protein; gp for glycoprotein; pp for phosphoprotein and Pr for precursors because of the diversity of abbreviations by several authors.

HIGUCHI[26], HIGUCHI & AUGUST[27,28] tried to correlate the appearance of detectable group specific antigen and cellular transformation, surprisingly at that time they were simultaneous, the appearance of group specific antigen seemed to be essential for exogenous virus replication but not enough to start cell transformation. The importance of p27 of avian system and p30 of murine virus was extensively studied by Higuchi[26] and HIGUCHI & AUGUST[27,28].

Several groups of investigators[8,21,26,27,28,30,43,49,51,52] developed the radioimmunoassay in order to detect minimal amount of viral antigen or its equivalent in a system, and also to investigate the expression of viral genes present in the host cell.

We used the competition radioimmunoassay in order to detect the expression of antigens or its equivalents. We investigated the expression and the level of the presence of equivalents to p27 and p19 in several tissues and organs of adult chickens as well as in chicken embryo fibroblasts. Since the ability to detect them depends on the amount of competitor used no competition was found at protein concentration in the range of 0.1 to 0.2 mg. However, when the

amount of competitor, in the experimental conditions was increased, epitopes of p27 and p19 were detected[17] as shown in table 4 and also of p15 (date no shown).

When the system $^{125}$I-p27 vs Anti-p27 vs cellular extracts were used, 3.85 mg of chicken brain material blocked 50% the precipitation reaction, it was equivalent to 28 ng of p27. On the other hand, almost twice as much 5.52 mg of heart extract was necessary to give the same inhibition. Surprisingly, tigh muscle and wing muscle did not shown significant competition for any of labelled antigens.

The p19 is associated with genomic RNA and is linked to lipids and the outer core. Sometimes it is phosphorilated. It might be important for RNA packaging[16,37,45,50].

A different pattern of expression was found for p19 expression; all sample analysed showed competition, which might be due to some similarity between the p19 and some cellular components, although the control values were taken into account.

When the same tissues and organs extracts from quail and drake were analysed, similar competition pattern were found, although at different protein concentrations. The experimental results suggest more similarity between the endogenous proteins of chicken and drake than with the quail proteins. Moreover, the radioimmunoassay results showed satisfactory immunological reaction with the exogenous virus antigens p27, p19 and p15 and the endogenous components, which strenghens the assumption that the *gag* genes are conserved among avian retrovirus.

Making several crosses among white Leghorn chickens, CRITTENDEN[14] isolated cell strains, 5.7 and 17 carrying a dominant gene predisposing the cell to spontaneous activation of endogenous viruses genomes. The line 7.2 is homozygous for the gene V-E7 and lacks the dominant host gene Gs and H-E. The line 15 is inducible it produces non infectious particles by BrdU treatment[47] and it is an unique line.

MARTIN et al.[39] reported the recovery of endogenous retrovirus from baboon, stump-tail macaque and colobus, but, so far, endogenous retroviral DNA has not been detected in preparations with normal human DNA probes.

There are considerable amount of speculations about the mechanisms used for integrative recombination of retroviral DNA in the cellular chromosome. Surely, there must be an endogenous control, that could be broken by one or combination of immunological, hormonal, enviromental factors; mutagenic elements[10,57] promotion of transcription of cellular DNA[41,44,46], oncogenes[25,41] and transpossons[1,13,33]. It seems to be particularly important the region of insertion because it might promote or activate the full expression of relevant genes.

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RESUMO: Células animais normais, não infectadas, apresentam uma característica única; a presença em seu genoma de genes (provirus) idênticos ou estritamente relacionados a retrovirus exógenos infecciosos. O nível de expressão destes genes varia de indivíduo para indivíduo: do silêncio completo até a produção de partículas virais. Proteínas de vírus endógenos e exógenos foram purificadas a partir de vírus sonicados e passados em coluna de Sepharose 6B saturada com GuHCl 6M. A presença de equivalentes dos antígenos p27 e p19 foi analisada por radioimunoensaio. A presença de componentes de vírus endógenos foi investigada em CEF, coração, cérebro, músculo de asa e músculo de coxa de galinha, codorna e marreco. A função ou significado destas proteínas é desconhecida. Fatores ambientais, hormonais e imunológicos necessitam ser estudados.

DESCRITORES: Retrovírus; vírus endógeno; expressão de vírus endógenos; RIA.

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