Sequences from Myeloblastosis-Associated Virus MAV-2(O) and UR2AV Involved in the Formation of Plaques and the Induction of Osteopetrosis, Anemia, and Ataxia

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Recombinant viruses were made between myeloblastosis-associated virus MAV-2(O) and UR2AV to examine the relationship between regions of the MAV-2(O) genome and disease induction. The *env*-long terminal repeat (LTR) portion of MAV-2(O), when substituted into UR2AV, was sufficient to induce osteopetrosis identical to that caused by the parent MAV-2(O). When this region was reduced to the gp37 and LTR of MAV-2(O), osteopetrosis more severe than that caused by the parent virus was induced. Recombinant viruses that contained all or part of the MAV-2(O) *env* gene in the absence of the MAV-2(O) LTR induced a severe, chronic anemia and late-onset osteopetrosis, leading to the conclusion that the MAV-2(O) LTR, in addition to *env*, was required for rapid induction of osteopetrosis. A viral recombinant, pEU, which contained the gp85 segment of UR2AV substituted into MAV-2(O), induced an ataxia/cerebellar dysfunction not seen during infection with the other chimeric or parent viruses. In vitro studies of the parent and recombinant viruses demonstrated that the ability to form plaques on chicken embryo fibroblasts correlated with the presence of the MAV-2(O) gp37 and LTR except for construct pEU. When the viruses were inoculated into 10-day-old chickens, chimeras containing the *env*-LTR or gp37-LTR region of MAV-2(O) induced severe regenerative anemia similar to that induced by MAV-2(O). pEU was the exception, suggesting that the unique configuration of this chimera is responsible for its unusual pathogenic properties.

Replication-competent avian leukosis viruses (ALVs) do not carry oncogenes, yet this group frequently induces tumors and nonneoplastic disorders (40). The most common neoplastic disease caused by ALVs is the monoclonal B-cell lymphoma lymphoid leukosis (LL), which originates in the bursa of Fabricius of infected birds and appears after a latent period of 4 to 6 months. Replication-competent ALVs also induce a variety of other diseases, such as osteopetrosis, nephroblastoma, angiosarcoma, and fibrosarcoma (4, 5, 12). ALVs also induce cellular dysfunction and killing, leading to disorders such as stunting (7), acute regenerative anemia (24), and decreased lymphocyte blastogenesis (36).

One of the best-characterized viruses that cause nonlymphoid diseases is MAV-2(O) (35). MAV-2(O) induces an exuberant proliferation of osteoblasts in the long bones when inoculated into chicken embryos at 10 to 15 days of incubation (3, 33). Bone lesions are apparent in 100% of infected chickens at 1 week posthatch. Thickening of the long bones progresses rapidly, and by 4 weeks of age the abnormal bone intrudes into the marrow cavity and contributes to the chickens becoming crippled, anemic, and immunosuppressed. Chickens that survive the osteopetrotic condition often develop nephroblastomas after an 8-week latency (3, 33, 35). When MAV-2(O) is administered to chickens after hatch, severe regenerative anemia (24) and decreased response to mitogen stimulation (10, 25) result. In vivo and in vitro studies attribute the immunosuppression to a macrophage accessory cell dysfunction, possibly resulting from the accumulation of unintegrated viral DNA in these cells (9, 10, 25). A study examining the phenomenon of unintegrated viral DNA in MAV-2(O)-induced osteopetrotic bone shows

that no correlation exists between the appearance of unintegrated viral DNA and the onset of osteopetrosis (1). Instead, unintegrated viral DNA does not appear until after osteopetrotic lesions are well established. The same study (1) also confirms that osteopetrosis is not a clonally derived tumor of bone (27).

In this study, we present experiments which identify the genome region of MAV-2(O) that confers the ability to induce osteopetrosis and severe regenerative anemia. To accomplish this, recombinants were constructed between MAV-2(O) and UR2AV, a subgroup A ALV with a pathogenic spectrum different from that of MAV-2(O). Chickens inoculated with UR2AV as 10-day-old embryos do not develop any diseases with short latency periods, such as osteopetrosis, but develop long-latency diseases such as LL, nephroblastoma, and fibrosarcoma (23, 31). Three regions of the MAV-2(O) genome were selected for study—the MAV-2(O) *gag* gene, the *env* gene, and the viral long terminal repeat (LTR)—because of their involvement in disease induction by other ALVs. The results presented here demonstrate a correlation between segments of the viral genome and pathogenic potential of MAV-2(O).

MATERIALS AND METHODS

Tissue culture cells. Chicken embryo fibroblasts (CEF) prepared from Line 0 (ev⁻) embryos were used for transfection and propagation of all viruses. CEF prepared from SC embryos (Hyline, Dallas Center, Iowa) were used for plaque assays. SC CEF are classified as C/O, while ev⁻ CEF are classified as C/E. All cells were prepared from 10-day-old embryos by standard techniques (34). Line 0 embryos were provided by L. B. Crittenden (U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich.).

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FIG. 1. Restriction map of viruses MAV-2(O) and UR2AV. S, *SacI*; B, *BamHI*; X, *XhoI*; P, *PstI*; G, *BglII*; E, *EcoRI*; H, *HpaI*; M, *MstII*; A, *XbaI*; K, *KpnI*; L, *SalI*; N, *HindIII*. Numbers represent kilobase pairs. Appropriate gene boundaries are labeled. Enzymes *XhoI*, *XbaI*, and *EcoRI* were used to determine diagnostic restriction fragments during construction of recombinant viruses. Map of UR2AV provided by M. C. Simon (personal communication).

Construction of recombinant viruses. The parental viruses used were a molecular clone of the MAV-2(O) provirus (2) and molecular clones of viruses UR2AV and UR2AV' (Fig. 1). UR2AV' is identical to UR2AV except for a single base change within the U3 region of its LTR which abolishes the *MstII* restriction site found within the U3 region of UR2AV (23, 32). All three viruses were cloned into the *SacI* site of the plasmid vector pUC12 (38). To construct the viral recombinants, sites conserved between the parental viruses were selected (Fig. 1). To obtain the necessary DNA segments, 50 µg of virus or plasmid DNA was digested with the required restriction enzymes. The resulting DNA fragments were separated on 0.8% agarose-TBE gels (22), appropriate bands were excised, and DNA was recovered from the gel by electroelution. After phenol extraction and ethanol precipitation, the viral and plasmid DNA fragments were mixed in equal amounts of approximately 1 µg each and ligated with T4 DNA ligase for at least 8 h at 16°C.

Construct pGPU was made by ligation of the 5.7-kb *SacI*-*KpnI* fragment of pMAV-2(O) containing pUC12-LTR-*env* to the 4.8-kb pUR2AV *SacI*-*KpnI* fragment representing *gag-pol*. pGM was constructed by ligating the 2.5-kb *SacI*-*HpaI* pMAV-2(O) viral DNA representing *gag* with the 8.2-kb pUC12-LTR-*env-pol* fragment of pUR2AV. pEM was generated by ligating the pMAV-2(O) 2.1-kb *SacI*-*MstII* *env* gene with the 8.6-kb fragment of pUR2AV' representing LTR-pUC12-*gag-pol*. For construct pEU, the 1.2-kb *KpnI*-*EcoRI* gp85 fragment of UR2AV was inserted into pMAV-2(O) DNA lacking this region. To generate pLU, the 2.0-kb *SacI*-*EcoRI* fragment of pUR2AV containing gp37-LTR was ligated to pUC12 and the 6.1-kb *SacI*-*EcoRI* partial fragment of pMAV-2(O) containing *gag-pol*-gp85. pLM was made by ligating the 1.7-kb *SacI*-*EcoRI* gp37-LTR portion of pMAV-2(O) with the 6.1-kb *SacI*-*EcoRI* partial fragment of pUR2AV representing *gag-pol*-gp85. After ligation, small samples of DNA (1 µg) were used to transform JM109 bacteria (22, 38). DNA was isolated from positive transformed colonies of JM109, and small samples of DNA were digested with restriction enzymes *XbaI*, *EcoRI*, and *XhoI* (Fig. 1) to verify the presence of the appropriate viral genome segments.

Transfection of CEF and titration of virus stocks. Viral DNA was removed from pUC12 by complete or partial digestion with *SacI* and was purified by agarose gel electrophoresis. Samples (2 to 4 µg) of recovered DNA were briefly ligated and then transfected into Line 0 (ev-) CEF (31).

After transfection, cells were passaged twice and assayed for the presence of reverse transcriptase activity in the tissue culture supernatant (39). To prepare stocks, all viruses were propagated in ev- cells, titer were determined, and stocks were divided into aliquots and placed at -70°C. Approximate viral titers for parental and recombinant virus stocks were determined by reverse transcriptase assay, using known titers of MAV-2(O) (as determined by plaque assay) for the generation of a standard curve. One-milliliter samples of virus stocks were assayed for reverse transcriptase activity, and the values obtained were compared with a standard curve to estimate virus titer (31).

Plaque assays. All parental and recombinant viruses were tested for their ability to form plaques on SC CEF according to a standard procedure (18). Plaque assay data were collected by examining monolayers of virus-infected CEF for uptake of neutral red stain. Stocks of MAV-2(O) with established titers were used as positive controls in these experiments.

Neutralization assay. Antisera against specific viruses were raised by inoculating 6-week-old chickens thrice at monthly intervals with standardized doses of viruses estimated by reverse transcriptase activity and bleeding the chickens 1 month after the last infection. Antisera were prescreened for specificity and titer before use. To perform the neutralization test, a standardized dose of virus estimated by reverse transcriptase activity in 0.1 ml of Tris-buffered saline containing 10% calf serum was incubated for 30 min at 37°C with 0.1 ml of undiluted antiserum. For each virus, a nonneutralized control was provided by using Tris-buffered saline containing 10% calf serum in place of antiserum. After incubation, 0.1 ml of the neutralized or control mixture was used to infect cultures of CEF in 60-mm dishes. The medium was changed at 3 days postinfection, supernatant fluids were collected on day 6, and reverse transcriptase assays were performed on 1-ml samples (39). Neutralization was considered positive when a 10-fold reduction in reverse transcriptase activity was observed.

In vivo characterization of parental and recombinant viruses. Chickens of the SC line were used for a double-blind in vivo study testing the pathogenic potential of parental and chimeric viruses. To ensure that observations made at the time of necropsy remained unbiased, viruses were assigned letter codes by one investigator. The technician who performed the egg inoculations then recoded the viruses, using numbers that defined each virus-infected group for the

duration of the *in vivo* experiments. The number code for the virus groups was not decoded until the *in vivo* experiments were completed. Embryos were inoculated with a standardized dose of virus estimated by reverse transcriptase activity in 0.1 ml of inoculum at 10 days of incubation via a chorioallantoic membrane vein. Chickens infected with each viral group were separated; chicks were hatched in isolated hatcheries and raised in separate rooms. Chickens were sacrificed in groups of 5 at 1, 2, 3, 4, 8, 12, and 24 weeks of age. Each viral group contained at least 35 chickens. Those chickens appearing most ill were selected for sacrifice at the various time points. Morbidly ill chickens were immediately sacrificed, and chickens that died spontaneously were subjected to necropsy and examination. Gross observations were recorded, and tissues were collected from the lung, liver, bursa, kidney, spleen, and femur of each chicken and placed in a phosphate-buffered saline–10% Formalin solution. Formalin-fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin-and-eosin stain (Colorado Histo-Prep, Fort Collins, Colo.), and examined for the presence of microscopic lesions.

In vivo study of acute anemia induction. SC Hyline chickens were infected intravenously with a standardized dose of virus estimated by reverse transcriptase activity in 0.25 ml of inoculum at 10 days posthatch. Blood samples were drawn into heparinized capillary tubes following puncture of a wing vein. Following centrifugation, hematocrit values from infected chickens were obtained and compared with those from age-matched uninfected chickens. Blood samples were collected at 3-day intervals beginning at 3 days postinfection through 24 days postinfection. Seventeen to twenty chickens were tested per virus group. The average of individual hematocrit values was calculated for each virus group at each time interval. Statistical evaluation was performed by an analysis of variance, using Student's two-tailed *t* test.

RESULTS

Construction of recombinant viruses. Six recombinant viruses were constructed: pGPU, pGM, pEM, pEU, pLU, and pLM (Fig. 2). These and the three parental viruses, MAV-2(O), UR2AV, and UR2AV', were propagated in *ev*-CEF and inoculated into SC Hyline embryos to yield nine experimental groups of chickens.

Plaque and neutralization assays. Since subgroup B ALVs induce plaques on CEF while subgroup A viruses do not (18, 19), plaque assays were performed to determine which of the chimeric viruses resembled MAV-2(O) in this attribute. Parent virus MAV-2(O) and recombinant viruses pGPU and pLM induced plaques on SC CEF (Fig. 3). Viruses UR2AV, UR2AV', pGM, pEU, pEM, and pLU did not form plaques on these cells. Neutralization assays with polyclonal chicken antisera specific for subgroup A and B ALVs showed that recombinants pGM and pEU were neutralized by a subgroup A antiserum, recombinants pGPU and pLM were neutralized by a subgroup B antiserum, and recombinants pEM and pLU appeared to be neutralized by both antisera (Table 1).

In vivo characterization of parental and recombinant viruses. All nine groups of virus-infected chickens were compared for the incidence of osteopetrosis and other diseases (Table 2). As expected, chickens inoculated with the parent virus, MAV-2(O), had a 98% incidence of osteopetrosis which was first apparent at 1 week of age. The severity of osteopetrosis increased with age and was accompanied by stunting, crippling, and the appearance of blood cysts. At 4 weeks posthatch, the incidence of angiosarcoma rose to

FIG. 2. Restriction maps of UR2AV, UR2AV', MAV-2(O), and recombinant viruses. Symbols: —, sequence from UR2AV; ■, sequence from MAV-2(O); ---, sequence from UR2AV'. S, *SacI*; H, *HpaI*; K, *KpnI*; E, *EcoRI*; M, *MstII*. Approximate gene boundaries are labeled. The molecular clone of MAV-2(O) contains one viral LTR, while UR2AV and UR2AV' contain two copies of the viral LTR.

26%, followed by an increase in the incidence of nephroblastoma (15%). By 12 weeks, crippling due to osteopetrosis made it necessary to sacrifice the remainder of the chickens.

Chickens inoculated with parent virus UR2AV appeared healthy until 10 weeks of age with the exception of sporadic cases of LL, anemia, and angiosarcoma. After 10 weeks of age, the incidence of LL rose to a total of 19%. Nephroblastomas were observed in several chickens older than 20 weeks. UR2AV'-infected chickens manifested lesions similar to those caused by UR2AV. There were single cases of osteopetrosis at ages 12 and 16 weeks. Cases of LL rose after 10 weeks of age to a final incidence of 26%, and 2 chickens at 24 weeks of age had nephroblastomas. The incidence of blood cysts and angiosarcoma was low.

The chimeric virus pGM was designed to test the role of the *gag* gene in osteopetrosis induction. Four chickens inoculated with virus pGM developed osteopetrosis after 19 weeks of age but, as with the UR2AV- and UR2AV'-infected chickens, the most frequent lesion seen in this group was LL (24%) appearing after 12 weeks posthatch. There were low incidences of anemia, blood cysts, and fibromatosis in this group.

Viral construct pGPU was prepared to examine the involvement of the *env*-LTR region of MAV-2(O) in osteo-

FIG. 3. Summary chart comparing viral gene segments and incidence of disease. Diagrams of the viral genomes are presented at the right. Symbols: ■, MAV-2(O)-derived sequence; —, UR2AV-derived sequence; ---, UR2AV'-derived sequence. Plaques, Formation of plaques on CEF monolayers; Osteo, macroscopic or microscopic lesions of osteopetrosis; Chronic Anemia, pallor, weight loss, depressed hematocrit count, enlarged heart, and ascites; Acute Anemia, transient depression in mean hematocrit count within 2 weeks after infection of 10-day-old chickens; Angiosarc, macroscopic and microscopic lesions of angiosarcoma in the lung; LL, lymphoid leukosis; Ataxia, congenital ataxia/cerebellar dysfunction. +, Disease incidences occurring in more than 15% of chickens; ++, incidence rates of greater than 40%; +++, incidence rates of greater than 70%. The severity of acute anemia is reported as ++ for viruses that caused a mean hematocrit value of <21% and as + for viruses that caused hematocrit values significantly less than ($P < 0.001$) those for chickens infected with UR2AV.

petrosis induction. All chickens infected with viral recombinant pGPU exhibited osteopetrosis at 1 week of age. The severity of osteopetrotic lesions increased rapidly such that by 2 weeks of age, virtually 100% of the chickens had severe osteopetrosis and were stunted and crippled. All chickens infected with pGPU were sacrificed by 8 weeks of age because of their condition. The incidence of angiosarcoma (8%) in pGPU-infected chickens was low, occurring in chickens older than 6 weeks. There were also single cases of LL and nephroblastoma in chickens older than 6 weeks.

Chickens infected with pEU exhibited a new lesion not seen with any other virus group in this study. This virus contained the gp85 portion of UR2AV substituted into MAV-2(O). Virtually 100% of chickens infected with pEU were unable to stand or walk correctly upon hatch. Many had splayed legs and could not walk, while those that could walk did so by staggering backward or by lunging forward. Eating and drinking were difficult for pEU-infected chickens since they did not have the balance needed to swallow. The majority of hatched chickens died or were sacrificed before 1 week of age. Those chickens surviving the initial 3- to

4-week period following hatch (approximately 5% of pEU-infected hatchlings) exhibited signs of persisting neurological problems such as swaying, shaking, and falling down. This condition was labeled congenital ataxia/cerebellar dysfunction. Histological analysis of the central nervous system of pEU-infected chickens revealed mild involvement of the cerebrum and spinal cord, with extensive lesions throughout the cerebellum. An in-depth description of this neurological condition will be presented elsewhere. This group of chickens also exhibited delayed-onset osteopetrosis which appeared after 4 weeks of age (41%) and was accompanied by stunting and the development of numerous blood cysts that were much larger than blood cysts induced by MAV-2(O). These blood cysts occurred subcutaneously, within muscle tissue, and in the abdomen and thorax and were often actively hemorrhaging at the time of death. Concurrent with the increase in incidence and severity of the blood cysts was the increase in incidence and severity of angiosarcoma. Severe, chronic anemia also appeared at an increased rate after 10 weeks of age (11%), accompanied by enlargement of the heart.

pEM was designed to test the entire *env* gene of MAV-2(O) for the ability to induce osteopetrosis in the absence of the MAV-2(O) LTR. Chickens inoculated with pEM exhibited a delayed-onset osteopetrosis, with mild lesions appearing at 2 weeks of age. At 4 weeks posthatch, the incidence of osteopetrosis was 100%, but lesions still appeared mild. Between 4 and 8 weeks of age, pEM-infected chickens began to exhibit severe, chronic anemia which had not been observed in chickens infected with either parent virus. This anemia was not due to occlusion of the marrow space, but gross observation showed the marrow to be extremely pale. Chickens also had accompanying symptoms of chronic anemia, such as enlarged hearts and accumulation of ascites fluid, and many chickens appeared to die of congestive heart failure. There were low incidences of angiosarcoma, LL, fibromatosis, and blood cysts.

Virus pLU contained *gag-pol-gp85* of MAV-2(O) substituted into UR2AV. Chickens infected with pLU exhibited

TABLE 1. Neutralization of parent and recombinant viruses as measured by reverse transcriptase analysis of supernatant fluids after treatment with subgroup-specific chicken antisera^a

Virus	Neutralization (cpm)		
	A	B	None
pMAV-2(O)	67,638	377	114,013
pUR2AV	362	23,433	30,761
pGPU	78,007	224	118,847
pGM	274	29,479	30,815
pEU	446	42,783	70,622
pEM	445	1,175	38,538
pLU	461	586	7,931
pLM	112,031	480	112,650

^a Subgroup-specific antisera: A, MAV-1; B, MAV-2(O); none, buffer substituted for antiserum.

TABLE 2. Summary of necropsy results from all virus groups^a

Virus	No. (%) of chickens that developed:						Comments, including disorders occurring at low frequency
	Osteopetrosis ^b	Chronic anemia	Angiosarcoma ^c	Nephroblastoma	LL	Ataxia ^d	
pMAV-2(O)	46/47 (98)	1/47 (2)	12/47 (26)	7/47 (15)	0/47 (<3)	0/47 (<3)	Blood cysts, stunting, fibrosarcoma; onset of osteopetrosis within 1 week
pUR2AV	0/42 (<3)	2/42 (5)	1/42 (2)	4/42 (10)	8/42 (19)	0/42 (<3)	Blood cysts, fibromatosis, fibrosarcoma
pUR2AV'	2/43 (5)	1/43 (2)	1/43 (2)	2/43 (5)	11/43 (26)	0/43 (<3)	Blood cysts, fibromatosis, fibrosarcoma; onset of osteopetrosis after 12 weeks
pGPU	37/37 (100)	2/37 (5)	3/37 (8)	1/37 (3)	1/37 (3)	0/37 (<3)	Blood cysts, stunting, fibrosarcoma; onset of osteopetrosis within 1 week
pGM	4/38 (10)	3/38 (8)	0/38 (<3)	0/38 (<3)	9/38 (24)	0/38 (<3)	Fibromatosis, enlarged heart, blood cysts; onset of osteopetrosis after 16 weeks
pEU	25/61 (41)	7/61 (11)	21/61 (34)	0/61 (<2)	1/61 (2)	27/61 (44)	Blood cysts, enlarged heart, fibromatosis, fibrosarcoma, stunting; onset of osteopetrosis after 4 weeks
pEM	25/37 (67)	14/37 (38)	3/37 (8)	0/37 (<3)	2/37 (5)	0/37 (<3)	Stunting, enlarged heart, blood cysts, fibromatosis; onset of osteopetrosis after 2 weeks
pLU	26/53 (49)	22/53 (42)	3/53 (6)	2/53 (4)	2/53 (4)	0/53 (<2)	Stunting, enlarged heart, blood cysts, fibromatosis, fibrosarcoma; onset of osteopetrosis after 8 weeks
pLM ^e	41/42 (98)	0/42 (<3)	10/42 (24)	1/42 (2)	0/42 (<3)	0/42 (<3)	Stunting, hemorrhages, blood cysts; onset of osteopetrosis within 1 week

^a Virus samples were administered to 10-day-old SC Hyline chicken embryos. All embryos were given a standardized virus inoculum based on reverse transcriptase activity.

^b Macroscopic or microscopic lesions of osteopetrosis.

^c Macroscopic or microscopic lesions of angiosarcoma.

^d Characterized by inability to stand or walk correctly.

^e This group was given half of the standard dose of virus per chicken embryo.

the same lesions as did chickens of group pEM but with different times of onset. Osteopetrosis took somewhat longer to develop in pLU-infected chickens than in chickens infected with pEM, appearing after 8 weeks of age. Severe chronic anemia occurred after 6 weeks of age and was also accompanied by enlargement of the heart. In addition, this group of chickens had low incidences of blood cysts, angiosarcoma, nephroblastoma, fibrosarcoma, fibromatosis, and LL.

The final group of chickens, infected with pLM, contained the gp37-LTR portion of MAV-2(O), with the remainder of the genome derived from UR2AV. This group of chickens exhibited severe osteopetrotic lesions at hatch when inoculated with the standard dose of virus. Many chicks in this group did not hatch, while those that hatched (<10%) survived for only 1 or 2 weeks. A second group of chicken embryos was inoculated with a twofold dilution of the standard dose of pLM virus. These chickens hatched successfully and by 1 week of age developed obvious osteopetrotic lesions. The lesions increased in severity with time, and the chickens were severely osteopetrotic and crippled by 4 weeks of age. In addition, this group of chickens demonstrated a marked stunting syndrome such that chickens older than 4 weeks were comparable in size to 1- and 2-week-old chicks. This group also showed a high incidence of angiosarcoma (24%) beginning at 5 weeks of age, a slightly faster onset time than seen in MAV-2(O)- and pGPU-infected chickens. All chickens infected with this dose of pLM were sacrificed by 7 weeks of age.

In vivo study of acute anemia induction. All virus-infected chickens exhibited hematocrit values lower than those of uninfected controls (Fig. 4), especially at 9 and 12 days postinfection. Only two recombinant virus groups resulted in marked decreases in hematocrit values, similar to MAV-2(O)

values, indicating anemia. These groups, pGPU and pLM, are also the recombinant viruses that induced rapid-onset osteopetrosis similar to that induced by the MAV-2(O) parent. Virus groups pEM, pLU, and pEU induced small decreases in hematocrit values relative to UR2AV, but these values were only slightly below 30%. By 18 days postinfection, hematocrit values for all infected chickens returned to normal.

DISCUSSION

Genome regions associated with plaque formation and neutralization. In vitro studies of all viruses were performed to determine the influence of the genetic substitutions on the ability to form plaques. Since pGPU contains the entire MAV-2(O) *env* and LTR regions (Fig. 3), it was not surprising that this virus formed plaques similar to those formed by MAV-2(O). Like viruses UR2AV and UR2AV', virus pGM did not form plaques. This can be attributed to the fact that pGM derives its *env*-LTR segment from UR2AV. The plaque assay data for the remaining four constructs were not expected, however. Viral construct pLM formed plaques on CEF even though it contained only gp37-LTR of the subgroup B virus MAV-2(O). It is a widely held belief that the gp85 glycoprotein of ALVs is the determinant of cytopathic effect (CPE) induction (13, 14); therefore, pLM, which derives most of its gp85 gene from UR2AV, was expected to act as a subgroup A virus. However, it is obvious from the in vitro studies of pLM that there must be other factors which govern the ability to induce plaques on CEF monolayers. One explanation for the ability of pLM to induce plaques may be the presence of a fusion epitope on MAV-2(O) gp37 which causes syncytium formation. Such a fusion epitope has been described for the retrovirus human immunodeficiency

FIG. 4. Induction of severe regenerative anemia by MAV-2(O), UR2AV, and recombinant viruses. Hematocrit values are expressed as percentage of packed cell volume. (A) Parent viruses and recombinants that induced severe anemia; (B) recombinant viruses that induced mild anemia. Symbols: ○, hematocrit values significantly less than ($P < 0.001$) that of chickens infected with UR2AV; △, Hematocrit values significantly different from ($P < 0.001$) that of MAV-2(O)-infected chickens.

ciency virus type 1, in which it is found at the amino terminus of gp41, the transmembrane protein of the AIDS virus (17, 20). The simian immunodeficiency virus SIV_{mac} also has a fusion epitope at the amino terminus of its transmembrane protein, gp32 (6). It is possible that cells which fused to form syncytia appeared as plaques on the red monolayer of neutral red-stained CEF. We have not been able to conclude whether pLM was inducing syncytium formation or true CPE. The role of gp85 in CPE induction is further challenged by the inability of viruses pEM and pLU to form plaques on CEF even though both viruses contain gp85 sequences from MAV-2(O). The absence of the MAV-2(O) LTR in these two recombinant viruses suggests that the MAV-2(O) LTR is also necessary for CPE induction.

The most puzzling result from the in vitro fibroblast experiments occurred with virus pEU, which did not induce CPE even though it contained the same MAV-2(O)-derived gp37-LTR fragment as did viral recombinant pLM. The presence of the MAV-2(O) *gag-pol* region in this recombinant suggests several possible reasons for its behavior, including an altered splicing pattern and altered ratios of spliced products, leading to altered expression of MAV-2(O) and UR2AV envelope glycoproteins.

Neutralization with subgroup-specific antisera showed that four of the recombinants were neutralized in a pattern consistent with that of one or the other parent virus. pGM

and pEU were neutralized by a subgroup A-specific antiserum, and pGPU and pLM were neutralized by a subgroup B-specific antiserum (Table 1). However, two recombinants, pEM and pLU, appeared to be neutralized by both antisera, suggesting that the polyclonal chicken antisera recognized neutralizing determinants from both subgroups on each of these two recombinants.

The reverse transcriptase assay performed in the neutralization test also revealed information about the replication of the viruses used in this study. Viruses containing an LTR derived from MAV-2(O) [pMAV-2(O), pGPU, pEU, and pLM] appeared to contain more reverse transcriptase activity in the 1 ml of supernatant fluid tested than did viruses containing an LTR derived from UR2AV or UR2AV' (pUR2AV, pGM, pEM, and pLU) (Table 1). This experiment does not allow for the adherence of virus particles to the cell surface, which may contribute to a decrease in the titer of virus in the supernatant fluid, a problem associated with subgroup A viruses. However, it is interesting that the recombinant pEU, which has a MAV-2(O) LTR but was neutralized with a subgroup A antiserum, appeared to grow to a higher titer than did any of the other viruses containing a UR2AV or UR2AV' LTR (Table 1).

Genome regions associated with osteopetrosis induction. The osteopetrosis-inducing ability of each virus was examined (Fig. 3). Virus pGM induced disease in a manner similar to that of parent viruses UR2AV and UR2AV', leading us to conclude that the *gag* gene of MAV-2(O), which was present in pGM, does not contain DNA sequences sufficient for osteopetrosis induction.

Similar to the MAV-2(O) parent, two recombinant viruses, pGPU and pLM, induced rapid-onset osteopetrosis. The most important observation made from pGPU-infected chickens was the requirement of the MAV-2(O)-derived *env*-LTR gene segment in osteopetrosis induction. A low incidence of angiosarcoma in pGPU-infected chickens may have occurred because all of the chickens were sacrificed by 8 weeks of age, whereas MAV-2(O)-infected chickens were sacrificed by 12 weeks of age. Therefore, cases of angiosarcoma may have risen to the same level seen in the MAV-2(O) group had the pGPU chickens lived longer.

In vivo observations of chickens infected with the recombinant virus pLM enabled us to conclude that an even smaller segment of MAV-2(O) DNA, the gp37-LTR portion, is sufficient to induce rapid-onset osteopetrosis. It was interesting that pLM appeared to be more virulent than either MAV-2(O) or pGPU, causing a higher incidence of osteopetrosis, angiosarcoma, and stunting, despite a reduced viral inoculum. These results suggested that removal of the MAV-2(O) gp85 sequence from pGPU yielded a more pathogenic virus. The reason for this enhanced pathogenicity is unknown but may be due to altered splicing patterns or, perhaps, the removal of viral regulatory sequences present in the MAV-2(O) gp85 gene.

Viral constructs pEM and pLU demonstrated the importance of the MAV-2(O) LTR in rapid osteopetrosis induction, since removal of this sequence in pEM and pLU resulted in osteopetrosis with a longer time of onset. Because of its importance in viral replication, the MAV-2(O) LTR may be better suited for rapid induction of osteopetrosis than the UR2AV LTR.

The assignment of osteopetrosis and anemia induction to the gp37-LTR portion of the MAV-2(O) genome is in contrast to the requirement of *gag* and *env* sequences necessary for osteopetrosis induction by subgroup E recombinants BR-21 and NTRE-2 (29, 30). However, a direct comparison

of the osteopetrotic potentials of these viruses, using the same chicken strains, routes of infection, and ages at infection, has not been performed. In addition, the osteopetrotic potential of Br-21 sequences were evaluated in the context of Rous-associated virus type 0 (RAV-0) sequences (29, 30), whereas the MAV-2(O) sequences were tested in the context of UR2AV sequences.

While we can conclude that the MAV-2(O) LTR is necessary for rapid induction of osteopetrosis, we cannot exclude the possibility that the MAV-2(O) LTR is, by itself, sufficient to induce osteopetrosis. The induction of osteopetrosis by recombinants pEM, pLU, and pEU demonstrates that a complex interaction of genome segments must be required, since these three viruses have no single MAV-2(O) gene segment in common (Fig. 3). We also cannot infer from the present experiments whether the MAV-2(O) gp37-LTR segment is involved in inducing other long-term MAV-2(O)-associated diseases such as nephroblastoma. This region may not be involved in causing long-latency diseases, since similar studies examining lymphoma induction by RAV-1 demonstrated that the gp37-LTR region, when substituted into RAV-0, was not sufficient to induce lymphoma (28).

Genome regions associated with anemia and blood cyst induction. Infection of 10-day-old chickens with MAV-2(O) results in the induction of a severe regenerative anemia due to destruction of erythrocyte precursors (11, 24). Interestingly, all virus-infected groups of chickens displayed hematocrit values less than those of uninfected, control chickens (Fig. 4), suggesting that ALV infection influences hematopoiesis. The data presented in this study clearly attribute this anemia induction to the same region of the MAV-2(O) genome which induces rapid-onset osteopetrosis. Because recombinants pGPU and pLM induce regenerative anemia similarly to the MAV-2(O) parent, we conclude that the gp37-LTR portion of MAV-2(O) is sufficient to cause this disease.

pEM and pLU also induced chronic anemia similar to that seen in chickens infected with MAV-2(O) at 10 days post-hatch. Under such conditions, chickens experience a transient anemia due to the destruction of erythrocyte precursors (11, 24). Several months later, these same chickens develop a chronic anemia from which they do not recover. It is possible that chickens receiving MAV-2(O) as embryos, rather than as 10-day-old chicks, succumb to osteopetrosis before they can develop chronic anemia. With the delayed onset of osteopetrosis in pEM- and pLU-infected chickens, chronic anemia is exhibited. It is also possible that the chronic anemia is a novel disease caused by constructs pEM and pLU, since MAV-2(O) isolates that induce late-onset osteopetrosis do not induce a high incidence of chronic anemia (33a). It will be important to determine whether the chronic anemia observed in pEM- and pLU-infected chickens is identical to that caused by MAV-2(O).

While blood cysts and angiosarcoma were observed with infection by viruses MAV-2(O), pGPU, and pLM, which all contain MAV-2(O) gp37-LTR, none of these viruses induced these lesions with the severity of pEU. The cause of blood cysts is unknown. One possibility is that blood cysts arise because of a clotting dysfunction, perhaps as a result of viral infection of cells of the thrombocyte series. The examination of thrombocytes for evidence of viral infection was beyond the scope of this study. The correlation between appearance of blood cysts and appearance of angiosarcoma makes it more likely that defects in vasculature due to viral infection and possibly neoplasia are the cause of blood cysts. An ALV recently described in Israel, AHV, induces vascular prolif-

eration resulting in lesions labeled hemangiomas (26), indicating that ALV may have the potential to induce this pathological manifestation.

Genome region associated with ataxia. The pEU virus caused an array of unexpected and unusual *in vivo* manifestations. The congenital ataxia/cerebellar dysfunction seen in newly hatched chicks infected with pEU is unlike any previously described neuropathology described for ALVs. A recent report describes RAV-1-induced cerebellar lesions (16) which appeared much less severe than those described in here. Previous observations of brains from RAV-7-infected chickens concluded that an inflammatory infiltrate was much more prominent (41). While neuropathological lesions seen in the central nervous systems of AIDS patients (described as vacuolar myelopathy) are unlike the lesions that we observed in pEU-infected chickens, studies on human immunodeficiency virus type 1 isolates from these patients demonstrate a highly conserved transmembrane glycoprotein, gp41 (8, 15, 21, 37).

The induction of osteopetrosis by the recombinant viruses presented here demonstrates the complex nature of viral pathogenesis. In addition, the appearance of novel diseases (congenital ataxia and chronic anemia) induced by viral recombinants provides an enlarged spectrum of lesions that can be used to further explore the molecular basis of retrovirus-induced disease.

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