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Role of Methylation in the Induced and Spontaneous Expression of the Avian Endogenous Virus *ev*-1: DNA Structure and Gene Products

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The endogenous avian provirus *ev*-1 is widespread in white leghorn chickens. Although it has no major structural defects, *ev*-1 has not been associated with any phenotype and is ordinarily expressed at a very low level. In this report, we describe a chicken embryo (Number 1836) cell culture containing both *ev*-1 and *ev*-6 which spontaneously expressed the *ev*-1 provirus. This culture released a high level of noninfectious virions containing a full complement of virion structural (*gag*) proteins but devoid of reverse transcriptase activity or antigen. These virions contained 70S RNA closely related to the genome of Rous-associated virus type 0, but identifiable as the *ev*-1 genome by oligonucleotide mapping. A fraction of the RNA molecules in the 70S complex were unusual in that they were polyadenylated 100 to 200 nucleotides downstream of the usual polyadenylation site. Eight sibling embryo cultures did not share this unusual phenotype with 1836, indicating that it was not inherited. However, an identical phenotype was inducible in the sibling cultures by treatment with 5-azacytidine, an inhibitor of DNA methylation, and the induced expression was stable for more than 10 generations. Analysis of chromatin structure and DNA methylation of the *ev*-1 provirus in 1836 cells revealed the presence (in a fraction of the proviruses) of both DNase I hypersensitive sites in the long terminal repeats and in *gag* and a pattern of cleavage sites for methyl-sensitive restriction endonuclease not found in a nonexpressing sibling. These results lend strong support to the role of DNA methylation in the control of gene expression. Additionally, they explain the lack of phenotype associated with *ev*-1 as due to a combination of its low expression and defectiveness in *pol* and *env*.

The endogenous viruses of chickens, which are inherited in chromosomal DNA, are closely related to the exogenous avian leukosis proviruses (for review, see 26). At least 13 distinct endogenous proviruses have been identified by DNA restriction endonuclease mapping (3, 18). Although closely related to one another, proviruses at different endogenous virus (*ev*) loci differ in DNA content and transcriptional activity. Thus, they may provide a set of genes for the study of elements, both viral and host, that contribute to the control of gene expression.

The nondefective endogenous proviruses have a structure similar to integrated proviral DNA found after exogenous infection. That is, they retain the gene order 5′-*gag-pol-env*-3′ of genome RNA and are flanked by two long terminal repeat (LTR) sequences, consisting of sequences derived from the 5′ (U_5) and 3′ (U_3) ends of viral RNA, as well as a sequence (R) which is itself repeated at the termini of genome RNA. Thus the overall structure is U_3-R-U_5-*gag-pol-env*-U_3-R-U_5 (17, 28; for review, see 6). The U_3 region contains sequences important for regulation of growth rate (39) and appears to include the promoter for viral RNA synthesis (10, 21, 50).

The *ev*-1 provirus is found in more than 99% of white leghorn chickens (38) and does not confer any characteristic virus-related phenotype on cells which contain it. Restriction endonuclease mapping of the provirus has failed to detect an obvious deletion or gross rearrangement of proviral sequences relative to replication-competent endogenous proviruses (18, 35). Cells which contain the inherited *ev*-1 provirus are negative by conventional assays for production of virion core proteins (product of the *gag*

gene), viral reverse transcriptase, viral envelope glycoprotein, and particle production (15). Although no virus-related proteins are detectable in *ev*-1-containing cells, low levels of RNAs resembling complete genome and *env* mRNA are found (4, 15). The estimated number of *ev*-1-encoded RNA molecules is less than one copy per cell; however, this low level of expression alone does not explain the failure of *ev*-1-containing cells to produce infectious virus. The endogenous rate of *ev*-2 expression is lower, yet infectious virus (Rous-associated virus type 0 [RAV-0], reference 2) can be isolated from *ev*-2-containing cells (9).

In the present report, we describe a variant chicken embryo, number 1836, which contained *ev*-1 and *ev*-6 and spontaneously expressed the *ev*-1 provirus. 1836 cells released noninfectious virions containing RAV-0-related 70S RNA and *gag* gene products, but devoid of reverse transcriptase activity or antigens. *ev*-1-encoded particles also lacked envelope glycoproteins.

Examination of DNA from the spontaneously expressing 1836 cells showed that the *ev*-1 provirus contained DNase I hypersensitive sites in both LTRs and its 5′ flanking and *gag* gene sequences and also contained a pattern of methylation different from that of inactive *ev*-1 DNA. These data seem significant in light of recent experiments (13) which have shown that growth of *ev*-1-containing cells in 5-azacytidine leads to induction of *gag* gene expression. 5-Azacytidine treatment decreases levels of 5-methyldeoxycytidine in eucaryotic DNA (20). The finding of specific sites in 1836 *ev*-1 DNA that are differentially methylated only in expressed *ev*-1 DNA suggests that a certain pattern of methylation influences chromosomal activation.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts were prepared from fertilized eggs of embryos 1384 [C/0, *ev*-1, (K(−)×15B)×K28], 1724 (C/0, *ev*-1,3), 1831 (C/0, *ev*-1), 1837 (C/0, *ev*-1,6), and 1836 (C/0, *ev*-1,6). Embryos 1831, 1837, and 1836 are sibling embryos obtained after mating of a K18×K(−) hen (number S107, *ev*-1,6,8) with a K28 rooster (number R3, *ev*-1). Endogenous proviral DNA content was originally determined by S. Astrin (unpublished data). All chickens were maintained at the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Turkey embryo fibroblasts were prepared from fertilized eggs purchased from Orlopp Enterprises, Inc. (Arosi, Calif.). SE21Q1b cells are a line of Rous sarcoma virus-transformed quail cells which produce noninfectious virions apparently due to a small deletion near the 5′ end of the genome (22, 23). These cells were used to provide a convenient set of marker proteins in some experiments.

RAV-0 was isolated from line 100 cells (C/0, *ev*-2) originally provided by L. Crittenden and has been extensively analyzed in this laboratory (8). Prague strain of Rous sarcoma virus subgroup B (Pr-RSV-B) has been described (8). Cells were maintained in Imemzo medium with insulin (Associated Biomedic Systems, Inc., Buffalo, N.Y.) plus 10% tryptose phosphate broth and 5% fetal calf serum.

For induction with 5-azacytidine, freshly plated cells (2×10^6 cells per 100-mm culture dish) were incubated in 3 μM 5-azacytidine (Sigma Chemical Co., St. Louis, Mo.) in growth medium. After 24 h, monolayers were washed once, and growth medium without 5-azacytidine was added. Two days after removal of the analog, cells were trypsinized and replated at 2×10^6 cells per 100-mm culture dish. Three days later, cultures were labeled for analysis. Additional cultures were passaged and analyzed at later times as indicated in the text.

^{32}P labeling and analysis of virion RNA. Labeling and preparation of virion RNA for two-dimensional gel analysis of T_1 oligonucleotides have been described (7). Briefly, 70S virion RNA was purified from culture supernatants after labeling confluent 100-mm cultures of cells with a total of 5 mCi of $^{32}PO_4$ per culture. RNA was digested with RNase T_1, and oligonucleotides were separated by electrophoresis in two-dimensional polyacrylamide gels. 3′ proximal RNA was purified on polyuridylate Sephadex G-10 after partial alkaline hydrolysis to approximately 300-nucleotide fragments before RNase T_1 digestion. Oligonucleotide maps were prepared as described (7).

Labeling and analysis of virus-related proteins. For labeling of virion or cellular proteins, 100-mm plates of confluent cells were washed three times with buffered salt solution, and [^{35}S]methionine was added in modified Eagle medium lacking methionine. Amounts of [^{35}S]methionine used are listed in the figure legends. Cultures to be used for analysis of cell proteins were incubated at 37°C with label for 2 h, washed once with cold buffered salt solution, and stored at −70°C. For virus labeling, cultures were incubated at 37°C for 12 h, and the supernatants were collected, centrifuged at 10,000 rpm for 10 min, and stored at −70°C. Growth medium with methionine was added to cultures, and supernatants were collected two additional times at 12-h intervals. Harvested supernatants were pooled and centrifuged in an SW 60 rotor at 55,000 rpm for 45 min at 4°C through a 25% sucrose cushion. Pellets were stored at −70°C.

Immunoprecipitation and gel electrophoresis. Cell lysis and immunoprecipitation of viral proteins from lysates and viral pellets was done essentially as described (11). Immune complexes were precipitated with IgGsorb (Enzyme Center, Boston, Mass.) which had been resuspended according to the manufacturer's specifications and washed in antibody binding buffer plus 1 mg of bovine albumin per ml before use. Pellets were washed extensively according to published procedures before resuspension in electrophoresis sample buffer and analysis in discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.

The anti-*gag* serum was prepared in a New Zealand rabbit against purified avian myeloblastosis virus *gag* proteins and has been previously described (11). Rabbit anti-*pol* serum, raised against purified avian myeloblastosis virus polymerase (5), was a gift of H. Temin. Anti-gp85 serum was provided by M. Linial (22).

Analysis of cell DNA. To determine the endogenous provirus content of cells from embryos 1384, 1831,

1837, and 1836, DNA was purified from cell cultures by the following procedure. Monolayer cultures were washed two times with buffered salt solution, and 2 ml of NTE (0.15 M NaCl, 20 mM Tris-hydrochloride [pH 7.5], 5 mM EDTA) per 100-mm culture dish plus 250 μg of Pronase per ml and 1% SDS were added, and the dishes were rocked gently and then incubated at 37°C for approximately 60 min. The viscous lysate was extracted 5 to 10 times with an equal volume of phenol–chloroform (1:1). Total nucleic acid was precipitated at −70°C with the addition of 0.1 M sodium acetate (pH 5.4) and 2.5 volumes of ethanol and collected by centrifugation at 10,000 rpm for 10 min at 4°C in an SS34 rotor. After lyophilization, pellets were suspended overnight in 20 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA and treated with RNase T_1 (10 to 15 U/10^7 cells) and pancreatic RNase A (4 μg/10^7 cells). After phenol extraction and ethanol precipitation as described above, the DNA was suspended in 10 mM Tris-hydrochloride (pH 7.5), and the DNA concentration was determined by absorption at 260 nm. A 5-μg amount of cellular DNA from each cell type was digested with 10 U of *Sac*I or 8 U of *Bam*HI (New England Biolabs, Beverly, Mass.) in the manufacturer's recommended buffer for 90 min at 37°C. Digested DNA was precipitated with ethanol as described above, collected by centrifugation, and suspended in 10 μl of 1× SB3 buffer (40 mM Tris base–5 mM sodium acetate–1 mM EDTA; pH 7.9 with glacial acetic acid) plus 2.5 μl of 5× sample buffer (1× sample buffer = 4% Ficoll, 16 mg each of bromophenol blue and xylene cyanol per ml). Electrophoresis was in a 0.8% horizontal agarose (Seakem) gel in SB3 buffer until the bromophenol blue dye marker was 11 cm from the origin. After ethidium bromide staining (0.5 μg/ml for 15 min) and UV photography to verify complete digestion of 250 ng of bacteriophage λ DNA added to each sample, the gel was treated with acid and alkali as described by Wahl et al. (45). DNA was transferred to nitrocellulose (Schleicher & Schuell Co., Keene, N.H.; 0.45 μM) which had been soaked in water and equilibrated with 10× SSC (1× SSC = 150 mM NaCl–15 mM sodium citrate). After 20 h, the nitrocellulose was rinsed for 5 min in 10× SSC before baking for 2 h at 80°C in a vacuum oven. The nitrocellulose filter was then rinsed for 15 min in 3× SSC, sealed in a plastic bag, and soaked for 60 min at 65°C in 3× SSC, 0.1% SDS, and 10× Denhardt solution (1× Denhardt = 0.02% [wt/vol] each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll). This solution was removed and replaced with 3× SSC, 10× Denhardt solution, 50 μg of denatured salmon sperm DNA per ml, 10 μg of polyadenylate per ml, 0.1% SDS, and 10% dextran sulfate (prehybridization solution), which was incubated for 3 h at 65°C. Hybridization was in 10 ml of fresh pre-hybridization solution with a [^{32}P]cDNA probe. RAV-2 70S RNA was used for cDNA synthesis as described (8) except [^{32}P]dTTP (New England Nuclear Corp., Boston, Mass.) was used at 700 Ci/mmol to obtain a high specific activity preparation. After hybridization at 65°C for 24 h, the nitrocellulose was washed at 65°C for 15 min with 3× SSC, 10× Denhardt solution, and 0.1% SDS, and four times with 2× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate. A final rinse for 15 min at room temperature was done with 3× SSC before autoradiography.

Preparation of nuclei, digestion with DNase I, and analysis with methyl-sensitive restriction endonucleases were described by Stalder et al. (36) and in the legend of Fig. 7.

RESULTS

An embryo with a novel endogenous virus phenotype. In the course of analyzing endogenous virus expression in fibroblast cultures from sibling chicken embryos, we encountered an unusual embryo, number 1836, which released noninfectious particles that had the density expected for C type viruses (data not shown). This finding was unexpected since preliminary experiments showed that DNA from embryo 1836 contained the endogenous proviruses *ev*-1 and *ev*-6 (S. Astrin and H. Robinson, unpublished observations), neither of which has been associated with spontaneous virus production (3). Specifically, the *ev*-6 provirus contains a 2.5-kilobasepair (kbp) deletion which encompasses the 5′ LTR and most or all of the *gag* gene (15, 18), and therefore cannot encode the *gag* gene products or full-size viral genome RNA. It is, however, associated with high-level production of *env* mRNA and expression of biologically active

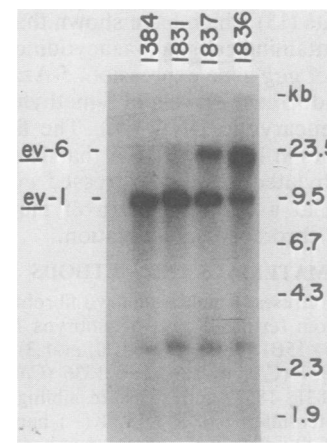

FIG. 1. Endogenous virus loci in 1836 cells and in cells from sibling embryos. DNA was extracted from monolayer cultures, digested with *Sac*I, and subjected to electrophoresis in 1% agarose gels. The DNA was transferred to nitrocellulose and hybridized with RAV-2 [^{32}P]cDNA (2.1 × 10^6 cpm). The filter was autoradiographed with a Cronex intensifying screen at −70°C for 10 days. The DNA was from embryos 1384 (lane 1), 1831 (lane 2), 1837 (lane 3), and 1836 (lane 4). Bacteriophage λ DNA digested with *Hin*dIII, visualized after ethidium bromide staining of the gel, was used for size markers. The band of approximately 3 kb is an artifactual fragment usually observed in hybridizations with viral RNA or cDNA but not with cloned DNA probes. This band may represent annealing to tRNA coding regions.

env gene products (4, 14, 15). *ev*-1 has not previously been shown to confer a virus-related phenotype on cells which contain it (4, 15).

To verify the endogenous virus content in 1836 cells and two sibling embryos which did not have this phenotype, DNA was digested with *Sac*I restriction endonuclease, subjected to electrophoresis in agarose gels, transferred to nitrocellulose paper, and hybridized to a ^{32}P-labeled probe. Figure 1 shows the result of this experiment with sibling embryos 1831, 1837, and 1836, as well as an unrelated embryo 1384, previously found to contain only *ev*-1 (Astrin and Robinson, unpublished observations). DNA of embryo 1831 as well as the 1384 control contained only *ev*-1, marked by a band of approximately 10.7 kbp (16, 18). Embryos 1837 and 1836 contained, in addition to *ev*-1, a band of about 18 kbp, corresponding to the *ev*-6 locus (3, 18). Digestion of DNA from these four embryos with *Bam*HI and *Eco*RI confirmed these assignments (data not shown). No differences in digestion products with these three enzymes were detected in the two embryos that contained *ev*-1 and *ev*-6, indicating that the presence of additional retrovirus-related information was not responsible for particle production from 1836 cells. For this reason and others (see Discussion), we conclude that the particles produced by 1836 cells were due to spontaneous expression of the usually silent *ev*-1 provirus.

Analysis of *ev*-1 expression. To analyze *ev*-1 expression, cultures of 1836 cells and particle preparations purified from culture supernatant fluids were assayed for the presence of *gag*, *pol*, and *env* gene products. To compare spontaneous and induced *ev*-1 expression and to determine what role, if any, the *ev*-6 provirus played in the 1836 phenotype, cultures of cells from sibling embryos 1831 (*ev*-1) and 1837 (*ev*-1, 6) were analyzed in parallel, before and after growth in 5-azacytidine. It has recently been shown that this cytidine analog induces expression of the *ev*-1 provirus in transformed chicken lymphocytes (13). Figure 2A shows the *gag*-related proteins immunoprecipitated from partially purified supernatant fluids of [^{35}S]methionine-labeled cultures. As expected, untreated cultures of 1831 (*ev*-1; Fig. 2A, lane 3) and 1837 (*ev*-1,6; Fig. 2A, lane 4) cells did not produce any material precipitable with anti-*gag* serum. 1836 cultures (*ev*-1,6; Fig. 2A, lane 5) produced significant amounts of virus-like particles that contained the virion internal structural proteins p27, p19β, p12, and p15. Particles containing the same content of *gag*-related proteins were also

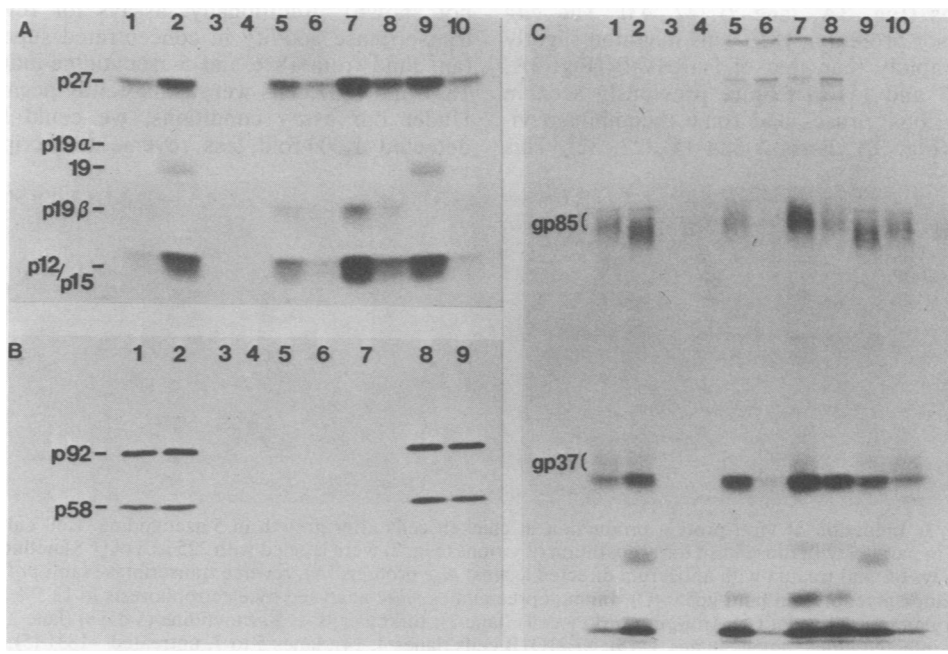

FIG. 2. Virion proteins in particles from 5-azacytidine-treated cells. Cell cultures were grown in 3 μM 5-azacytidine for 24 h. Five days after removal of the analog, the cultures were labeled with 225 μCi of [^{35}S]methionine for 20 h, and harvests were prepared as described in the text. Aliquots of each preparation were immunoprecipitated with anti-*gag* (A), anti-*pol* (B), and anti-*env* (C) serum. (A) SE21 (lanes 1, 10), RAV-0 (lanes 2, 9,), 1831 (lane 3), 1837 (lane 4), 1836 (lane 5), 1831 + 5-azacytidine (lane 6), 1837 + 5-azacytidine (lane 7), and 1836 + 5-azacytidine (lane 8). (B) SE21 (lanes 1, 9), RAV-0 (lanes 2, 8), 1831 (lane 3), 1837 (lane 4), 1836 (lane 5), 1831 + 5-azacytidine (lane 6), 1837 + 5-azacytidine (lane 7). (C) As in (A).

produced by 1831 and 1837 cells 5 days after 5-azacytidine treatment (Fig. 2A, lanes 6 and 7). The production by 5-azacytidine-induced 1831 cells of particles with an identical *gag* protein content shows that the *ev*-1 provirus alone was sufficient for this phenotype.

With the exception of p19, the *ev*-1-encoded virion structural proteins were identical in mobility to those of RAV-0 (Fig. 2A, lanes 2 and 9). The more rapidly migrating form of p19, called p19β, has previously been identified in some recombinants involving endogenous viruses (31, 32), and was found in all *ev*-1-expressing cultures. The identity of this protein in the virions produced by 1836 cells was confirmed by tryptic peptide analysis (data not shown). Virions which contain p19β have also been found to contain varying amounts of a related protein called p19α (32). p19α was also found in particles obtained from all *ev*-1-expressing cell cultures, although it is visible only in lane 7 of the experiment shown in Fig. 2A.

Lysates of [^{35}S]methionine-labeled cell cultures were also treated with anti-*gag* serum, and the immunoprecipitates were analyzed by electrophoresis in SDS-polyacrylamide gels. As expected from the compositon of particles produced by 1836 cultures, these cells contained significant levels of the *gag*-encoded precursor proteins (Fig. 3A, lane 7) (42, 43). The *gag* precursor protein in 1836 cells migrated slightly more rapidly than that of Pr-RSV-B (Fig. 3A, lanes 3 and 15), a feature previously seen in endogenous viruses and some recombinant viruses (Fig. 3A, lanes 4 and 14) (27, 32). This faster-migrating form is called ΔPr76. Tryptic peptide maps of ΔPr76 immunoprecipitated from 1836 cells were prepared and confirmed its identity (data not shown). Although uninduced cultures of 1831 (*ev*-1; Fig. 3A, lane 5) and 1837 (*ev*-1,6; Fig. 3A, lane 6) did not produce any *gag*-related proteins, significant amounts of ΔPr76, which was identical in mobility to that obtained from 1836 cells, were produced 5 and 10 days after growth in 5-azacytidine (1831 is shown in Fig. 3A, lanes 8 and 11, and 1837 is shown in lanes 9 and 12). Note that a significant increase in the level of ΔPr76 was not detected in 1836 cells after treatment with 5-azacytidine (compare lane 7 with lanes 10 and 13, Fig. 3A). As expected, turkey cells which do not contain avian retrovirus-related DNA (34) did not produce virus-related proteins before or after growth in 5-azacytidine (Fig. 3A, lanes 1 and 2). The variability in the content of lower-molecular-weight *gag* products in some cells (Fig. 3A, lanes 8, 11, and 13) probably reflects somewhat variable rates of processing of ΔPr76; this phenomenon is of uncertain significance.

Although particles produced spontaneously by 1836 cells and by 5-azacytidine-induced 1831 and 1837 cells had an apparently normal complement of *gag* gene products, we could detect no significant infectivity associated with them (data not shown). Additionally, assays for reverse transcriptase activity in concentrated supernatant fluid from 1836 and 5-azacytidine-induced 1831 and 1837 cells were consistently negative. Under our assay conditions, we could have detected 1,000-fold less reverse transcriptase

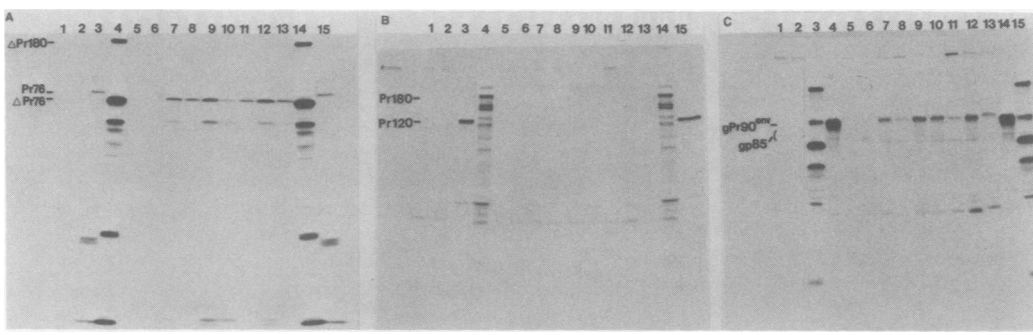

FIG. 3. Induction of viral protein production in chicken cells after growth in 5-azacytidine. Cell cultures treated in parallel with those used for preparation of virions (Fig. 2) were labeled with 225 μCi of [^{35}S]methionine for 2 h, lysed, and treated with antiserum directed against *gag* proteins (A), reverse transcriptase (anti-*pol*) (B), or envelope glycoprotein (anti-gp85) (C). Immunoprecipitates were analyzed by electrophoresis in 12.5% SDS-polyacrylamide slab gels. (A) Anti-*gag*: turkey cells (lane 1); turkey cells + 5-azacytidine (5 days) (lane 2); Pr-RSV-B-infected chicken cells (lanes 3, 15); SE21Q1B cells (lanes 4, 14); lanes 5 to 7, untreated—1831 (5), 1837 (6), 1836 (7) cells; lanes 8 to 10, + 5-azacytidine (5 days)—1831 (8), 1837 (9), 1836 (10) cells; lanes 11 to 13, + 5-azacytidine (10 days)—1831 (11), 1837 (12), 1836 (13) cells. (B) Anti-*pol*: turkey cells (lane 1); turkey cells + 5-azacytidine (5 days) (lane 2); chicken embryo 1724 (*ev*-3) (lanes 3, 15); SE21Q1b cells (lanes 4, 14). Lanes 5 to 13 as in (A). (C) Anti-gp85: turkey cells (lane 1); turkey cells + 5-azacytidine (5 days) (lane 2); SE21Q1b cells (lanes 3, 15); Pr-RSV-B-infected cells (lanes 4, 14). Lanes 5 to 13 as in (A). The apparent lower mobility of gPr90 in lane 13 is an artifact due to a defect in the gel. The antiserum used in this experiment also had variable reactivity with *gag* proteins as well as with gp37.

activity than that obtained from an equal volume of supernatant from RAV-0-producing cells.

To determine whether cells that contained an active *ev*-1 provirus produced reverse transcriptase-related polypeptides, cell lysates and particle preparations were treated with anti-*pol* serum, and precipitates were analyzed in SDS-polyacrylamide gels. Figure 2B shows the complete absence of the 92- and 58-kilodalton (kd) β and α chains of reverse transcriptase in *ev*-1-encoded virions, and Fig. 3B shows the absence of the 180-kd *gag-pol* precursor protein in *ev*-1-expressing cells. These proteins are clearly visible in virion preparations and cells lysates obtained from virus-infected control cultures (Fig. 2B, lanes 1, 2, 8, and 9, and Fig. 3B, lanes 4 and 14). Furthermore, no *pol*- or *gag*-related protein larger than 76 kd which would represent a defective *gag-pol* gene product was detected in lysates prepared from *ev*-1-expressing cells, although we could precipitate the 120-kd *gag-pol*-related protein produced in *ev*-3-containing cells (reference 12, and Fig. 3B, lanes 3 and 15).

As expected for *ev*-6-containing cells (14, 29), both uninduced and induced 1836 and 1837 cells contained the glycosylated *env* precursor protein, gPr90 (Fig. 3C, lanes 6, 7, 9, 10, 12, and 13), as detected with antiserum prepared against the gp85 cleavage product. This protein was also seen in control infected cell cultures. Varying levels of gp85 were also found in these cells as well as in virions from 1836 and 5-azacytidine-induced 1837 cells (Fig. 2C, lanes 5, 7, and 8). No *env*-related proteins were found in particle preparations from uninduced 1837 cells (Fig. 2C, lane 4), indicating that the *ev*-1 *gag* gene expression was necessary to produce sedimentable *env* protein-containing particles. To allow determination of *env* gene expression from the *ev*-1 provirus, it was necessary to analyze 1831 cells which contained only *ev*-1. Although *env*-related proteins were not detectable in untreated 1831 cells (Fig. 3C, lane 5), a band comigrating with gPr90 was evident in 1831 cells 5 and 10 days after growth in 5-azacytidine (Fig. 3C, lanes 8 and 11). However, no gp85 could be found in induced 1831 cells or in virions obtained from these cells (Fig. 2C, lane 6). The reduced amount of gPr90 in induced 1831 cells compared to the other cells was probably due to the absence of *ev*-6. Note that the amount of gPr90 attributable to *ev*-1 was greater than that due to *ev*-6 alone as seen in the noninduced 1837 cells (Fig. 3C, lane 6), where a substantial amount of gp85 could be detected. Furthermore, no gp85 was visible in the lanes from 1831 cells even after a fivefold longer exposure than that shown in Fig. 3C (data not shown). We therefore consider it highly improbable that the failure to detect gPr90 cleavage products in 1831 cells was due to the reduced amount of gPr90, and conclude that *ev*-1 does encode the envelope glycoprotein precursor but that this protein is not processed normally.

The virion RNA. A number of experiments were undertaken to determine the RNA content of *ev*-1 encoded particles. ^{32}P-labeled virion RNA was prepared as described (7) from 1836 as well as from 5-azacytidine-induced 1831 and 1837 particle-producing cultures. All samples contained RNA that cosedimented in sucrose gradients with 70S RNA from RAV-0 virions (data not shown). This RNA was digested with RNase T_1 and analyzed by two-dimensional gel electrophoresis (7). Figure 4 shows fingerprints of RNA isolated from RAV-0 virions (Fig. 4A) and from particles from 1836 (Fig. 4B) and from 5-azacytidine-treated 1831 (Fig. 4C) cells. Only those oligonucleotides that differed from RAV-0 are labeled in Fig. 4B and C. Fingerprints of particle RNA produced by 1836 cells before and after growth in 5-azacytidine were identical and indistinguishable from those obtained from either 5-azacytidine-treated 1831 (Fig. 4C) or 1837 cultures (data not shown). It is apparent from these fingerprints that the particles contained a single major species of RNA closely related to that of RAV-0.

The relationship between the genomes of RAV-0 and *ev*-1 is displayed in the oligonucleotide maps shown in Fig. 5. Although the *ev*-1 RNA was closely related to that of RAV-0 (24 common oligonucleotides), we can identify it as the product of the *ev*-1 provirus. Three oligonucleotides, 617, 613, and 612, have been identified as unique markers for the *ev*-1 transcripts as judged by fingerprints of recombinant viruses isolated from cells which contain this provirus (J. M. Coffin, P. N. Tsichlis, K. F. Conklin, and H. L. Robinson, manuscript in preparation). In addition, the DNA sequences which code for oligonucleotide 612, which maps near the 3′ end of the genome RNA, and 617, located at the 5′ end of the genome, have been located at the appropriate sites in cloned *ev*-1 DNA (16). Two oligonucleotides, labeled 617* and h1 were present in less than molar yield in Fig. 4B and C. The composition of 617* was identical to that of 617, except that it did not contain the capping group normally found on this oligonucleotide. All other oligonucleotides were in approximately equimolar yield, indicating a relatively pure class of RNA. RNA attributable to the *ev*-6 provirus was not detectable in these fingerprints, as determined by the absence of any oligonucleotides in the RNA from *ev*-1, 6 cells not present in the RNA from cells containing only *ev*-1. In addition, if *ev*-6-encoded RNA had been present, the yield of the *gag* oligonucleotides should have been decreased relative to *env* and *pol* markers

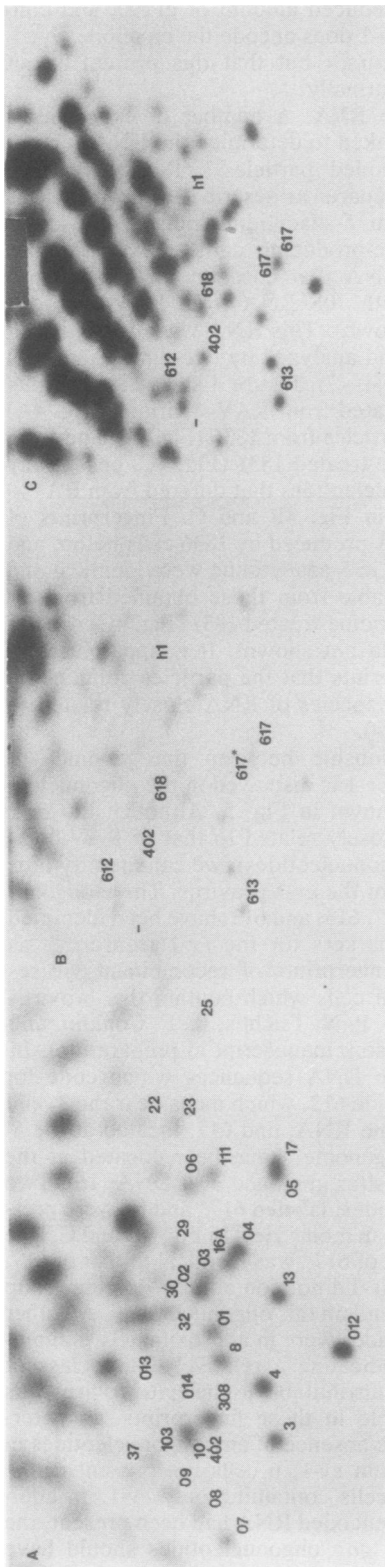

FIG. 4. Fingerprints of RAV-0 and *ev*-1-encoded RNA isolated from virions. ^{32}P-labeled 70S RNA of RAV-0 (A), 1836 (B), and 5-azacytidine-induced 1831 (C) particles was digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis as described (7). The numbering is according to our previous convention (8). The identity of all oligonucleotides was confirmed by RNase A digestion (not shown). Only those oligonucleotides which differ from RAV-0 are labeled in (B) and (C).

because *ev*-6 does not contain the *gag* gene (15, 18).

Fingerprint analysis of short (about 300 nucleotides long) polyadenylated RNA molecules obtained by partial alkaline hydrolysis of the 70S RNA isolated from RAV-0 (Fig. 6A) and 1836 (Fig. 6B) particles showed that there were five oligonucleotides present in the 3′ proximal portion of *ev*-1 RNA not found in RAV-0 RNA. Oligonucleotide 08 in RAV-0 is allelic to oligonucleotide 612 from *ev*-1 (Coffin et al., manuscript in preparation). The other four oligonucleotides (617*, h1, h2, and h3) appeared to derive from RNA molecules which were longer than usual at the 3′ end. Oligonucleotide 617 contains about 20 nucleotides of the genome repeat sequence (R), and 3 nucleotides of the 5′ unique sequences (U_5). The R and U_5 regions are within the LTR sequences and are therefore present twice in proviral DNA, once at each end of the provirus (see Fig. 5). Genome RNA molecules typically contain the R sequences at both ends but U_5 sequences only near the 5′ end. The presence of 617* in the 3′ terminal region of the *ev*-1-encoded particle RNA suggests that a fraction of the *ev*-1 RNA was polyadenylated further downstream than normal and therefore included some U_5 sequences at the 3′ end. Analysis of the h1, h2, and h3 oligonucleotides confirmed the aberrant nature of the 3′ polyadenylation of the *ev*-1 transcripts. Comparison of the composition of these three oligonucleotides (data not shown) with the DNA sequence of the host cell DNA flanking the right-hand end of the LTR of *ev*-1 (16) disclosed DNA sequences which could code for these oligonucleotides. The approximate lengths and amounts of these longer RNA species could be estimated from the fingerprints shown in Fig. 4 and 6. Since the 5′ oligonucleotide 617 had a greater intensity in the whole genome than the 3′ oligonucleotide 617* (Fig. 4B and C), not all of the RNA molecules contained the unusual 3′ end. Since the yield of 617* was roughly equal to that of h1 and h2 and much greater than that of h3, the large majority of these molecules must have had a 3′ terminus between 120 and 200 nucleotides from the usual polyadenylated site. From the presence of a very small amount of oligonucleotide h3 (not visible in the reproduction), perhaps 10% or fewer of the molecules were more than 220 nucleotides longer than usual.

The RNA used for 3′ end analysis was originally isolated as 70S virion RNA, suggesting that the host cell sequences were on extended *ev*-1 transcripts and do not represent independent molecules, although we cannot at this time exclude the possibility that additional smaller transcripts cosedimented with *ev*-1 70S RNA.

To confirm that the RNA identified as the

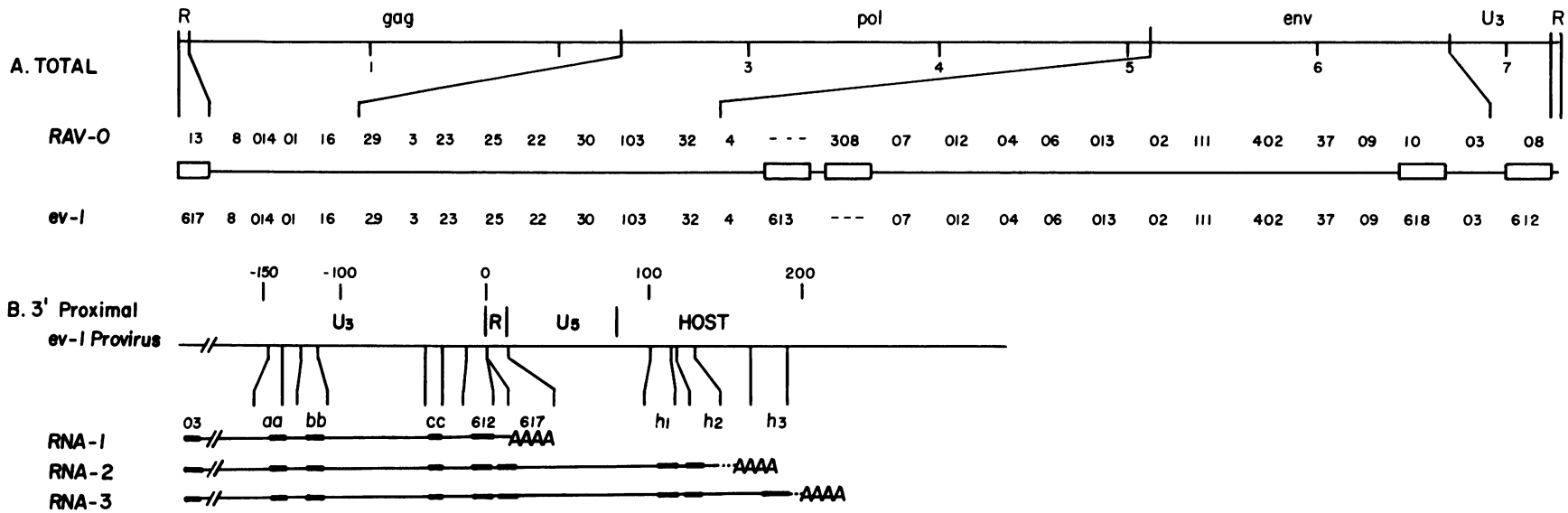

FIG. 5. (A) Oligonucleotide maps of RAV-0 and *ev*-1 RNA. Relative order of RAV-0 and *ev*-1 T_1 oligonucleotides are shown, along with gene designation. The top line indicates the approximate physical locations of the various regions, as determined by restriction and genetic mapping (33, 35, and our unpublished results). The scale is in kilobases. (B) Map of the right end of *ev*-1 proviral RNA and host flanking sequences. 3′ proximal oligonucleotides found in RAV-0 and *ev*-l-encoded virion RNA (see Fig. 6) are aligned with relevant DNA sequences, showing the inferred structure of the three *ev*-l transcripts. The positions of the oligonucleotides are inferred from the sequence obtained by Hishinuma et al. (16).

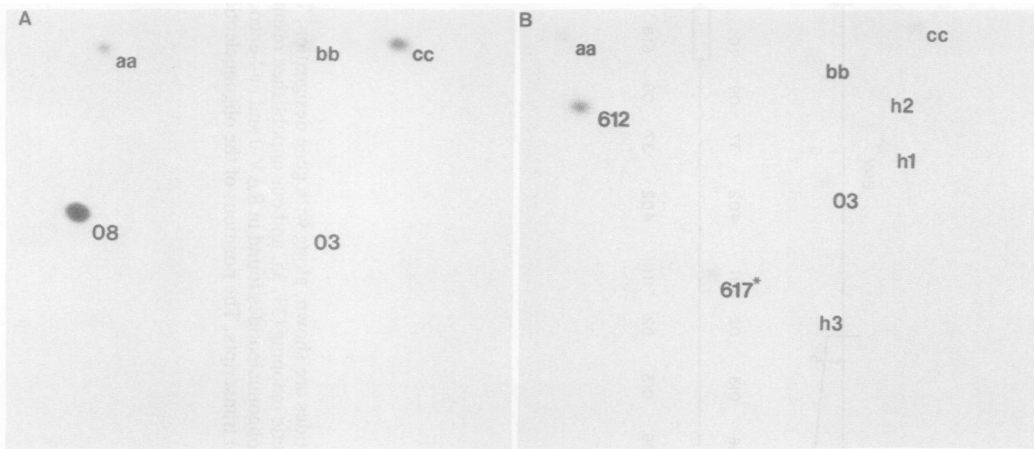

FIG. 6. 3'-Proximal regions of RAV-0 and *ev*-1 virion RNA. Short (about 300 nucleotides long) polyadenylated fragments were prepared from RAV-0 and 1836 particle RNA, digested with RNase T_1, and subjected to electrophoresis as in Fig. 4.

transcript of the *ev*-1 provirus was capable of coding for the protein detected in vivo, RNA isolated from 1836 particles was added to an in vitro translation system. Analysis of total reaction productions on SDS-polyacrylamide gels showed production of the ΔPr76 protein (data not shown).

Chromatin structure and methylation of Ev-1 DNA. Active chromatin may be distinguished from inactive chromatin in several ways. Expressed genes are more sensitive to digestion by DNase I, often displaying one or more "hypersensitive" sites (46). In addition, expressed genes are usually undermethylated at the dinucleotide CpG (24). These characteristics have been established for the endogenous provirus *ev*-3 and the *ev*-1 provirus in 5-azacytidine-induced cells (13, and unpublished results). The finding that 5-azacytidine induces *ev*-1 expression and results in concomitant undermethylation and generation of DNase I hypersensitivity in *ev*-1 DNA strongly suggests that undermethylation is important in transcriptional regulation. After 5-azacytidine induction, *ev*-1 proviral DNA is generally hypomethylated, with no apparent site specificity (13, and unpublished results). The identification of 1836 cells, which contained a spontaneously active *ev*-1 provirus, allowed determination if all or only a subset of the alterations seen after 5-azacytidine treatment was evident in these cells.

To analyze the relative DNase I sensitivity of *ev*-1 DNA in 1836 cells, nuclei were isolated and treated with increasing concentrations of DNase I, and the DNA was purified. For comparison, we also analyzed DNA from 1837 cells. After digestion with *Eco*RI, electrophoresis in 1% agarose gels, and transfer to nitrocellulose, the samples were hybridized with the *gag*-specific probe shown in the bottom of Fig. 7. Without DNase I digestion (0 DNase, Fig. 7A), both the 1836 and 1837 DNA yielded the 8.4-kbp, left-hand *ev*-1-specific *Eco*RI fragment (18, 35). As mentioned previously, both 1836 and 1837 DNA contain *ev*-1 and another endogenous provirus, *ev*-6. However, *ev*-6 is missing the 5' LTR and most or all of *gag* (15, 18). Thus, by using a *gag*-specific probe, emphasized in heavy black in the line drawing at the bottom of Fig. 7, we detected only the 8.4-kbp, left-hand *ev*-1-specific fragment and not the 7.0-kbp *ev*-6-specific 5' *Eco*RI fragment (18). After digestion with increasing concentrations of DNase I, two prominent subbands (2.15 and 1.25 kb) were evident in 1836 but not in the overexposed 1837 series. Assuming that one end of these subbands corresponds to the defined *Eco*RI restriction site in the *gag* gene and the other end to a double-stranded cut introduced by DNase I (49), the probable locations of the *ev*-1 hypersensitive sites in 1836 DNA were assigned to the 5' LTR and to a site in *gag*. Extensive mapping has confirmed these locations and also revealed another hypersensitive site in the 3' LTR of *ev*-1 in 1836 DNA (data not shown). The DNase I hypersensitive site in the *gag* gene of *ev*-1 from 1836 cells is not a unique feature of this provirus. Such sites have been detected in several active endogenous and exogenous proviruses, although their presence is more variable than those seen in the LTRs (unpublished observations). The relatively "low" intensity of the 1836 subbands and the continued presence of the parental 8.4-kbp *Eco*RI band at high concentrations of DNase I suggest that not all cells in the population contain a copy of this locus in an active conformation.

To determine if the *ev*-1 provirus from 1836

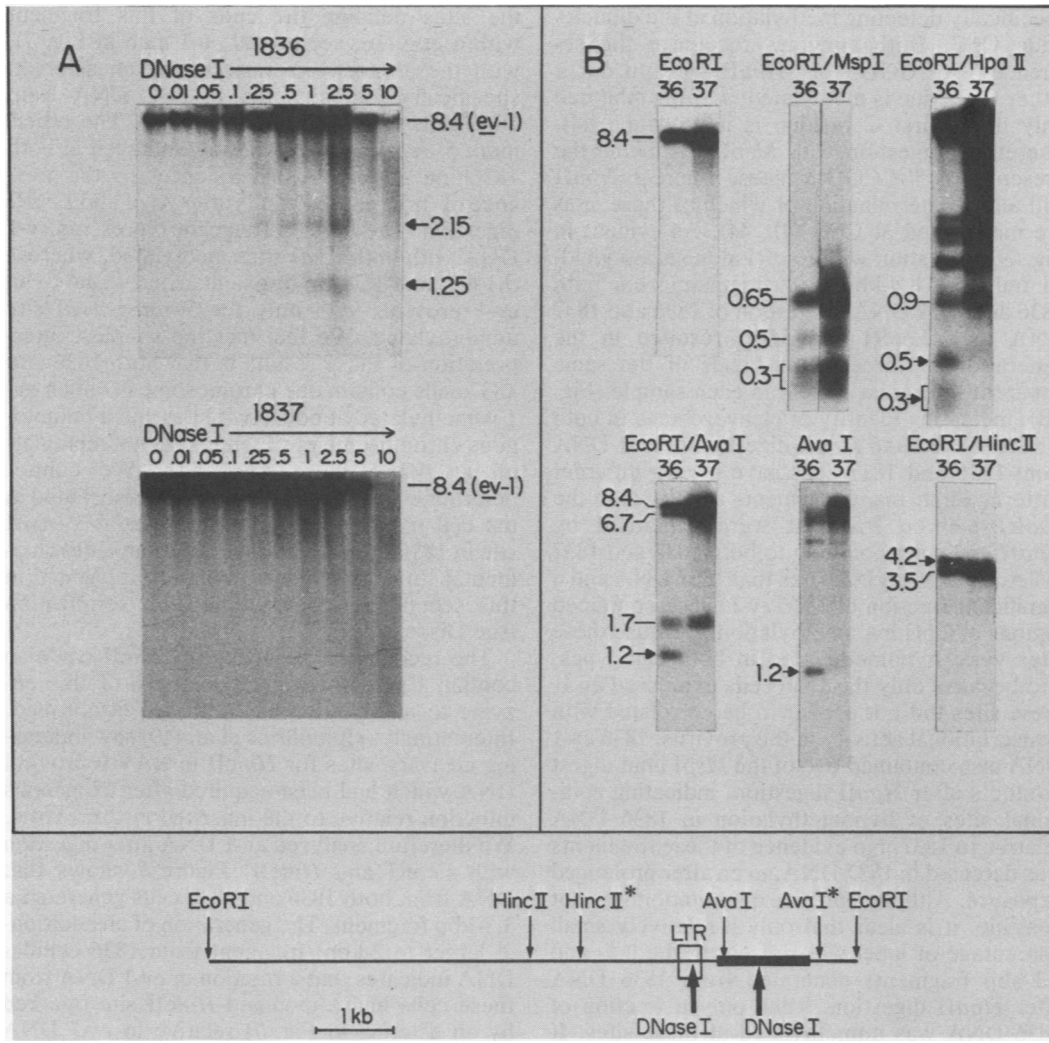

FIG. 7. Chromatin structure and methylation of the *ev*-1 provirus. (A) DNase I generated hypersensitive sites in 1836, but not in sibling 1837 cells. Nuclei from 1836 and 1837 cells were treated with increasing concentrations of DNase I at a DNA concentration of 1 mg/ml for 10 min at 37°C. DNA was isolated, digested with *Eco*RI, subjected to electrophoresis in neutral 1% agarose horizontal gels, transferred onto nitrocellulose filters, and hybridized to a [^{32}P]*gag* probe subcloned from a clone of *ev*-1 DNA provided by A. Skalka (35) and containing sequences indicated in heavy black on the map at the bottom of this figure. (The starting clone contained the 5′ end of *ev*-1 plus host flanking sequences; the provirus therefore extends to the right of the LTR as shown.) Numbers at the top of each lane refer to concentrations of DNase I (in micrograms per milliliter). The locations of the 1836 hypersensitive sites inferred from this experiment are indicated on the *ev*-1 map. (B) Methylation at CpG dinucleotides in 1836 and 1837 DNA. DNA from 1836 and 1837 cells was isolated, digested sequentially with *Eco*RI and then with either *Msp*I, *Hpa*II, *Ava*I, or *Hinc*II. In addition, one sample was digested with only *Ava*I. After electrophoresis in horizontal 1.4 agarose gels, the DNA was transferred and hybridized as above. In most cases, the 1837 lane is overexposed to demonstrate the lack of detectable limit digests. The map presented at the bottom of this figure shows *Ava*I and *Hinc*II sites within the parental 5′ *ev*-1 8.4-kbp *Eco*RI fragment, and was derived from our own work as well as that of Hishinuma et al. (16). The *Ava*I* site is undermethylated in one variant provirus of some 1836 cells, and the *Hinc*II* site is either absent or undermethylated in one 1836 variant.

cells contained a different pattern of methylation relative to the inactive provirus in 1837 cells, DNA was isolated from each cell type, digested with the restriction endonucleases *Msp*I and *Hpa*II in combination with *Eco*RI, run in neutral agarose gels, and analyzed by hybridization with the same probe.

The enzymes *Msp*I and *Hpa*II are useful for

specifically detecting methylation at the dinucleotide CpG. Both enzymes recognize the sequence 5'-CCGG-3', but *Hpa*II will not cut if either C residue is methylated; *Msp*I is inhibited only if the first C residue is methylated (44). Therefore, digestion with *Msp*I will define the presence of 5'-CCGG-3' sites, whereas *Hpa*II will allow determination of which of these sites are methylated at CpG (41, 44). As evident in Fig. 7B, digestion with *Eco*RI alone again yielded only the 8.4-kbp *ev*-1 fragment from both 1836 and 1837 DNA. Digestion of 1836 and 1837 DNA with *Eco*RI and *Msp*I resulted in the generation of three major bands of the same apparent molecular weight in each sample (Fig. 7B), indicating identity of cleavage sites in both DNAs. *Eco*RI and *Hpa*II digestion of *ev*-1 DNA from 1836 and 1837 cells gave a very different pattern. First, many fragments smaller than the *Eco*RI 8.4-kbp fragment were generated by *Hpa*II and were common to both 1836 and 1837 DNA. This result indicates that 1837 DNA and a significant fraction of 1836 *ev*-1 DNA contained regions of CpG hypomethylation. Because these sites were hypomethylated in both cell types, and because only the 1836 cells expressed *ev*-1, these sites did not appear to be correlated with transcriptional activity of the provirus. 1836 *ev*-1 DNA also contained two of the *Msp*I limit digest products after *Hpa*II digestion, indicating additional sites of hypomethylation in 1836 DNA relative to 1837. No evidence of these fragments was detected in 1837 DNA, even after prolonged exposure. Although precise quantitation was not possible, it is clear that only a relatively small percentage of label was present in the 0.5- and 0.3-kbp fragments generated from 1836 DNA after *Hpa*II digestion. Thus only a fraction of 1836 DNA was unmethylated at these sites. If differences in methylation patterns do correlate with transcriptional activity, the low yield of these fragments is consistent with the idea, suggested by DNase I sensitivity data, that only a fraction of 1836 cells contain an active *ev*-1 provirus.

Additional digestion of 1836 and 1837 DNA with *Eco*RI and either *Ava*I (5'-CPyCGPuG-3') or *Hin*cII (5'GTPyPuAC-3') also generated different cleavage products from these two DNAs. Based on the known restriction map of cloned *ev*-1 DNA (16; Fig. 7), the observed differences are most likely due to differential methylation at some of these sites (see Discussion). *Ava*I will not cleave DNA if the C of CpG in its recognition sequence is methylated (25). Digestion of cellular DNA with *Eco*RI and *Ava*I showed that *ev*-1 DNA from 1837 cells was missing a 1.2-kbp fragment which was generated from 1836 *ev*-1 DNA either with *Eco*RI and *Ava*I or with *Ava*I alone (Fig. 7B). Mapping studies have located the sites defining the ends of this fragment within *gag* (16; see partial *ev*-1 map in Fig. 7), with the 3'-most site (marked by an asterisk) specifically unmethylated in *ev*-1 DNA from 1836 cells (Fig. 7; see Discussion). The other, more 5' *Ava*I site was variably modified in both 1836 and 1837 DNA, as evidenced by the presence of three shared bands after *Ava*I and *Eco*RI digestion. The 8.4-kbp fragment represents *ev*-1 DNA with both *Ava*I sites methylated, whereas the 6.7- and 1.7-kbp fragments correspond to an *ev*-1 provirus with only the 5'-most *Ava*I site unmethylated. We feel that the simplest interpretation of these results is that both 1836 and 1837 cells contain one chromosome in which *ev*-1 is methylated at both *Ava*I sites and a homologous chromosome in which *ev*-1 is not methylated at the 5'-most *Ava*I site. We cannot determine how these homologs are distributed in the cell population. The unmethylated 3' *Ava*I site in 1836 cells might then represent a developmental "mistake" generated and propagated in this second allele sometime after fertilization (see Discussion).

The recognition sequence of *Hin*cII can also contain CpG; however, sensitivity of this enzyme to methylation has not been established. Interestingly, Humphries et al. (19) saw increasing cleavage sites for *Hin*cII in RAV-0 proviral DNA which had been acquired after exogenous infection relative to the inherited *ev*-2 provirus. We therefore analyzed *ev*-1 DNA after digestion with *Eco*RI and *Hin*cII. Figure 7 shows that DNA from both 1836 and 1837 cells generated a 3.5-kbp fragment. The generation of an additional larger (4.2-kbp) fragment from 1836 cellular DNA indicates that a fraction of *ev*-1 DNA from these cells had a modified *Hin*cII site (marked by an asterisk in Fig. 7) relative to *ev*-1 DNA from 1837 cells. These results suggest that some of the 1836 proviruses have lost the more 3' *Hin*cII site or that this site contains a CpG in which the C is methylated in one 1836 allele.

These data show that a significant fraction of *ev*-1 proviral DNA from 1836 cells was distinguishable from that of 1837 by the presence of DNase I hypersensitive sites, by decreased sites of methylation, and by at least two DNA modifications, which may also be methylation differences. The observation that only a fraction of the *ev*-1 DNA in 1836 cells contained these characteristics associated with active DNA suggests that not all cells in the population contain a copy of the *ev*-1 locus in an active conformation.

DISCUSSION

A variant *ev*-1 provirus with a novel phenotype. The *ev*-1 provirus, found in virtually all white leghorn chickens (38), is usually relatively inactive, coding for barely detectable levels of RNA

and proteins (15), and has not previously been associated with endogenous virus expression. We report here a novel phenotype in chicken cells, conferred by a variant *ev*-1 provirus. The cells that contain this provirus, fibroblasts from embryo 1836, produced high levels of the *gag* gene product, ΔPr76, which was cleaved into virion structural proteins and assembled into virions. Analysis of DNA from 1836 cells revealed no additional avian leukosis virus-related DNA relative to nonexpressing siblings and that no gross alteration of proviral DNA had occurred. Although our analyses would not have revealed additional proviruses in a small minority of cells, a number of lines of evidence exclude this possibility for the 1836 phenotype. First, we were unable to detect the presence of infectious virus from any of the cultures characterized as positive for *ev*-1 expression, eliminating the possibility of accidental infection with a replication-competent virus as the basis for the 1836 phenotype. Had a replication-defective virus been introduced alone, it would have been unable to spread to a sufficient number of cells to be detectable. Second, analysis of cellular DNAs after digestion with *Eco*RI and *Bam*HI, which generate fragments from within the provirus, did not reveal additional virus information relative to the analysis with *Sac*I (data not shown). Furthermore, no trace of a provirus with an *Eco*RI cleavage site characteristic of exogenous virus LTRs (17, 18) could be detected. Third, the identical phenotype could be induced with 5-azacytidine from two sibling cultures, one of which contained only *ev*-1, as well as from chicken cells from a completely different source (13). Fourth, fingerprint analysis of the RNA released from 1836 and induced 1831 cells revealed an identical composition which was distinct from all other endogenous and exogenous viruses that we have studied (Coffin et al., manuscript in preparation). This genome contained many markers (such as oligonucleotides 01, 02, 03, etc.) common to most endogenous, but not exogenous, viruses. Furthermore, some characteristic oligonucleotides such as 613 and 617 have been previously found only in genomes of recombinants generated by exogenous virus infection of noninduced cells containing only *ev*-1 (K. Conklin, Ph.D. thesis, Tufts University, Boston, Mass., 1982). Analysis of two other characteristic oligonucleotides, 617 and 612, showed that they had the composition and mobility predicted from the nucleotide sequence of the LTR of *ev*-1 (16). Again, these characteristic oligonucleotides have been found in no other virus genome, save known recombinants with *ev*-1. We therefore conclude that the phenotype we are observing is a consequence of induction of the provirus at the *ev*-1 locus.

By restriction enzyme analysis, the *ev*-1 *pol* gene appears intact (35). It was somewhat surprising, then, that no protein related to the *pol* gene product was detectable in cells or virions. Recent results indicate that the *gag* and *pol* genes are in different reading frames in the virus genome (47; D. Schwartz, personal communication) and suggest that a spliced message is generated which allows production of the *gag-pol* fusion protein. *ev*-1, then, may be defective in production of a functional *pol* mRNA. Alternatively, the *pol* gene may contain a mutation near its 5′ end which leads to premature termination, generating a protein indistinguishable from ΔPr76 under our conditions. Resolution of the *pol* gene defect will apparently depend on sequencing of this region.

Analysis of 5-azacytidine-induced cells that contained only *ev*-1 allowed determination of the *env* gene expression from this provirus. After treatment with 5-azacytidine, 1831 cells did not produce the envelope glycoprotein precursor protein (gPr90), yet we were unable to detect the processed forms of this protein in either cells or virions. No significant size difference was detected between gPr90 from *ev*-1 cells and that from cells containing *ev*-6, suggesting comparable levels of glycosylation. The presence of envelope glycoproteins in virions from *ev*-6-containing cells implies that the defect in *ev*-1 is within *env* and not in the ability of *ev*-1-encoded virions to incorporate a functional envelope glycoprotein.

The RNA packaged in virions from 1836 and induced *ev*-1, *ev*-6 cells was identical to that isolated from virions of induced *ev*-1 cells. The fact that *ev*-6 RNA was not packaged is not surprising, since sequences located near the 5′ end of virion RNA appear to be required for efficient incorporation into virions (33), and the *ev*-6 provirus carries a deletion which includes this region (15, 18). The *ev*-1-encoded virion RNA contained heterogeneous 3′ ends that were apparently generated from readthrough of proviral sequences into host-flanking sequences and polyadenylation at sites downstream of the normal site. This heterogeneity is similar to that described for transcripts of some other eucaryotic genes (30), and may represent the existence of multiple polyadenylation sites which all give rise to stable, functional mRNA's.

ev-1 is widespread in white leghorn chickens and could have contributed by complementation or recombination to a number of previously described genetic phenomena of avian leukosis and sarcoma viruses. For example, we have found that a virus with variant host range (BO1-Pr-RSV) originally attributed to mutation (40) contained oligonucleotide 618, and was therefore likely a recombinant with *ev*-1 (data not

shown). We have also found that RAV-0, after repeated passage on *ev*-1-containing cells, acquires a significant amount of genomes which are recombinants with *ev*-1, as judged by the appearance of *ev*-1-specific oligonucleotides in its RNA (Coffin et al., manuscript in preparation). Thus, *ev*-1 is capable of contributing by recombination to both exogenous and endogenous viruses grown on cells which contain it. Such recombination could also lead to viruses which acquire specific defects. For example, two recently described variants of Rous sarcoma virus, SE521 (22) and PN3/2SR-D (37), have defects in glycoprotein processing similar to those in *ev*-1. We have found that the SE521 genome contains some *ev*-1-specific markers (K. Conklin, unpublished results), although we cannot conclusively state that the defect specifically derives from *ev*-1. Similarly, the low-molecular-weight p19 proteins seen in many recombinant viruses (31) may in fact derive from recombination with the *ev*-1 *gag* gene which is characterized by a low-molecular-weight p19. Finally, it should be noted that mutants whose functions can be complemented or repaired by recombination with *ev*-1 might be expected to be very difficult to isolate and maintain on *ev*-1-containing cells. Interestingly, very few temperature-sensitive mutants of RSV with lesions in *gag* have been isolated compared to the numbers of *src* and *pol*. Clearly, the use of *ev*-negative birds (1) is to be recommended for future attempts to isolate such mutants.

Chromatin structure and methylation of *ev*-1 DNA. In attempting to understand the molecular basis of *ev*-1 expression in the 1836 variant, we have examined the chromatin structure and DNA methylation patterns of *ev*-1-specific DNA in these cells. Our results show that expression of *ev*-1 in this variant is correlated with the acquisition of DNase I hypersensitive sites in both LTRs as well as in *gag*. We also observed hypomethylation at the dinucleotide CpG as detected with restriction endonuclease *Hpa*II. Specific differences in cleavage patterns of *ev*-1 DNA from 1836 and 1837 cells were also detected with *Ava*I and *Hin*cII.

Given the methyl sensitivity of *Ava*I cleavage, it is likely that the apparent absence of this site in 1837 and a fraction of 1836 *ev*-1 DNA is actually due to methylation. This notion is supported by the presence of this site in other *ev*-1-containing cells after exposure to 5-azacytidine (unpublished observations). Cloned *ev*-1 DNA also contains two *Hin*cII sites in the 5′ host flanking DNA. The apparent loss of one of these sites in a fraction of 1836 cells represents either methylation at this site or primary sequence alteration. If the basis is methylation, this result indicates that it may be the pattern of methylation rather than methylation per se that influences transcription. We are currently cloning and sequencing *ev*-1 DNA from 1836 cells to determine if the observed difference in *Hin*cII digestion is due to primary sequence alteration.

Heterogeneity was observed in the products obtained from *ev*-1 DNA after digestion with *Hpa*II, *Ava*I, and *Hin*cII. 1836 *ev*-1 DNA contained the two "normal" *ev*-1 proviruses seen in DNA from sibling embryos as well as a "variant" one. The most likely explanation for this result is that 1836 and 1837 cells contain two distinct *ev*-1 proviruses, one of which is marked by methylation at both *Ava*I sites and the other by methylation only at the 5′ proximal site. The novel *Ava*I fragment generated from 1836 DNA suggests the presence of an additional provirus, present in a fraction of the 1836 population, which is unmethylated at both of these *Ava*I sites. If this provirus represents the active *ev*-1, it could account for the hypomethylated fraction seen with *Hpa*II digestion and the component which is hypersensitive to DNase I digestion. The obvious prediction from this model is that 1836 cells consist of a mixed population of *ev*-1-silent and *ev*-1-expressing cells. This possibility is currently under investigation. The event(s) which gave rise to the "active" *ev*-1 provirus appears to be unique to embryo 1836. Analysis of eight sibling embryos of 1836 has failed to reveal another *ev*-1-expressing individual (data not shown), indicating that this phenotype was not inherited from the parents. It is possible that a novel pattern of methylation could arise during development. The recent observation that methylation of CpG dinucleotides in transfected DNA is not replicated with 100% fidelity (48) may support this notion.

The finding that 5-azacytidine induces *ev*-1 gene expression is strong evidence that methylation of DNA plays a role in endogenous retrovirus gene expression. Although we have not proven that undermethylation of *ev*-1 DNA itself effects transcriptional activation, the fact that the spontaneously active *ev*-1 provirus in 1836 cells contains an altered methylation pattern provides strong support for this hypothesis.

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