Variation in p30-Related Proteins in Gross Virus-Induced Tumor Cell Lines Derived from *H-2* Congenic Mice

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p30-related proteins were examined in three in vitro-passaged Gross virus-induced cell lines derived from mice congenic at the *H-2* complex: BALB.K-gv-1 (*H-2^k*) (BkGV1), which produces infectious, oncogenic virus, and BALB.B-gv-1 (*H-2^b*) (BbGV1) and BALB/c-gv-1 (*H-2^d*) (BdGV1), which stopped producing infectious virus early in their passage history. The producer BkGV1 line made p30 and its expected precursors. BbGV1 and BdGV1 cells had multiple changes in p30-related protein expression. BbGV1 cells (i) synthesized a truncated *gag* precursor polypeptide of ca. 47 kilodaltons, (ii) expressed fewer p30-related molecules per cell on their surface than BkGV1 cells, (iii) produced functional reverse transcriptase, and (iv) budded morphologically mature virions which were neither infectious nor oncogenic. BdGV1 cells (i) produced no p30 but expressed some high-molecular-weight proteins with p30 determinants, (ii) expressed fewer p30-related determinants on their surface than BbGV1 cells, and (iii) harbored immature intracisternal viral particles (type A) but did not exhibit budding virions.

Several observations in mice suggest that the expression of retroviruses, the genotype of the major histocompatibility complex (*H-2*), and the pathogenesis of virus-induced leukemias may be interrelated. The *H-2^k* haplotype is associated with susceptibility to the development of leukemia induced by endogenous and certain exogenous (e.g., Gross and Friend) retroviruses, whereas mice expressing the *H-2^b* haplotype are relatively resistant (reviewed in references 12 and 14). Available evidence suggests that this phenomenon may involve both control of immune responses to virus-induced antigens and cellular control of virus expression (reviewed in references 12, 14, 19, and 20). To examine possible effects of an *H-2*-linked gene(s) on virion production in the absence of any interaction with the host, cell lines derived from tumors induced by either Gross virus (GV) or Friend virus in *H-2*-congenic mice have been studied (8, 19). The data from these studies suggest that a gene(s) linked to *H-2* may be exerting intracellular control over infectious virus production (19). Too few cell lines have been studied to determine whether an *H-2*-linked gene(s) actually mediates the change to noninfectious status in vitro or to ascertain the molecular processes involved in the cessation of infectious virus production in these cell lines. However, the nonproducer Friend virus-induced cultured tumor cell lines which have been studied contain multiple changes in virus protein processing (1, 5, 15). Because both GV- and Friend virus-induced cell lines which have become nonproducers of infectious virus may nonetheless continue to express murine leukemia virus (MuLV)-encoded proteins (8, 19), it is important to determine whether the molecular lesions related to virus production in the GV-induced nonproducer cell lines are similar to those observed in Friend virus-induced cell lines.

To investigate the continued expression of viral proteins in the absence of infectious virus production in GV-induced cells, we analyzed processing and expression of the *gag*-encoded virus core protein p30 and its related precursor polypeptides in three cell lines. The BALB.K-gv-1 (BkGV1) line, derived from mice expressing the *H-2^k* haplotype, continues to produce infectious, oncogenic virus even after

several years in culture. The BALB.B-gv-1 (BbGV1) and the BALB/c-gv-1 (BdGV1) lines, derived from mice expressing the *H-2^b* and *H-2^d* haplotypes, respectively, have ceased to produce infectious virus detectable in the XC and Mink S⁺L⁻ assays. Cells from all three lines continue to express molecules with antigenic specificities associated with viral proteins (19).

Retrovirus protein synthesis (reviewed in reference 6) results from the transcription of integrated proviral DNA to viral RNA which subsequently is translated into large precursor polypeptides. These large polypeptides are cleaved to form several mature virus proteins. The MuLV core proteins are formed from the *gag*-encoded precursor polypeptide Pr65^{gag}, which is cleaved into p15, p12, p30, and p10 molecules. A second primary translation product of the *gag* mRNA is gPr80^{gag} which contains Pr65^{gag} and which is glycosylated to form gP95^{gag} and gP85^{gag} found on the surface of infected cells. A third *gag*-related polypeptide is Pr180^{gag-pol} which is the precursor of reverse transcriptase.

In this study, we report that (i) nonproducer GV-induced cell lines had distinct alterations in *gag* gene product processing and production, (ii) cell surface expression of *gag* protein-related antigens was higher in producer than it was in nonproducer cell lines, and (iii) the nonproducer BbGV1 line made budding virus particles which were neither detectable by XC assay nor oncogenic, and BdGV1 cells harbored intracisternal particles which did not appear to bud.

MATERIALS AND METHODS

Cell lines. The cultured tumor cell lines BkGV1, BbGV1, and BdGV1 have been described previously (19). All were derived from thymic lymphomas induced by inoculating BALB-adapted Gross passage A virus into neonatal mice. All three cell lines initially produced infectious ecotropic virus detectable by XC assay, but the BbGV1 and BdGV1 lines became nonproducers early in their passage history. Cells from each line express Thy 1.2 and appropriate *H-2* antigens; however, the BkGV1 cells, although continuing to express *H-2D^k* molecules on their cell surface, have stopped expressing *H-2K^k* antigens (16; T. V. Rajan, personal communication). All cells were grown in RPMI 1640 medium

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FIG. 1. Proteins precipitated by anti-AKR-p30 serum from cell lines metabolically labeled with [35 S]methionine for 2 h (5-day exposure).

supplemented with glutamine, penicillin, streptomycin, and 15% horse serum.

Labeling and disruption of cells. Cells growing at logarithm phase were washed three times in Hanks balanced salt solution and incubated in methionine-free medium for 30 min at 37°C. Cells were suspended at 2×10^6 /ml in methionine-free medium containing 250 μ Ci of [35 S]methionine (Amersham Corp.; 1,300 Ci/mmol) per ml. Cells were incubated for 2 h at 37°C for one-dimensional (1-D), two-dimensional (2-D), and peptide mapping studies. Cells for pulse-chase experiments were incubated with [35 S]methionine for 15 min, washed twice, and incubated for various times in growth medium containing excess unlabeled methionine. After incubation, all cells were washed three times in Hanks balanced salt solution and suspended at 10^6 cells per ml in lysing buffer containing 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in 0.01 M Tris (pH 7.2)–0.15 M sodium chloride (4). After 30 min at 4°C, the lysate was used or stored at -70°C .

Antiserum and immunoprecipitation. Immunoprecipitations were performed with monospecific goat-anti-AKR-leukemia-virus-p30 serum, lot 79S-142, obtained from the Viral Oncology Program, National Cancer Institute, Bethesda, Md. Cell lysates were precleared with Formalin-fixed *Staphylococcus aureus* Cowan 1 at 4°C for 30 min. *S. aureus* Cowan 1 was removed by centrifugation, antiserum was added, and samples were incubated overnight at 4°C. *S.*

FIG. 2. Pulse-chase analysis of proteins precipitated by anti-AKR-p30 serum from cell lines metabolically labeled with [35 S]methionine for 15 min and incubated in medium containing cold methionine for 0 to 120 min. Each lane contained the precipitates from 5×10^5 cells (10-day exposure).

aureus Cowan 1 was added, samples were incubated for 30 min at 4°C, and complexes were removed by centrifugation and washed twice in lysing buffer. Samples for 1-D, pulse-chase, and peptide analysis were resuspended in eluting buffer of 2% SDS–5% 2-mercaptoethanol–62.5 mM Tris-hydrochloride (pH 7.4)–10% glycerol–0.001% Coomassie brilliant blue dye and heated to 100°C for 2 min. Samples for 2-D gels were washed in lysing buffer without SDS and resuspended in a lysis buffer of 9.5 M urea–2% Nonidet P-40–1.6% (pH 5 to 7) Ampholines (LKB Instruments, Inc.)–0.4% (pH 3.5 to 10) Ampholines (LKB)–5% 2-mercaptoethanol (13).

SDS-PAGE. Slab gel electrophoresis was performed as previously described (9) with 5% stacking gels and 11% running gels. For 2-D gels, the first dimension was performed in nonequilibrium pH gradients, and the second dimension was performed on 11% SDS-polyacrylamide gel electrophoresis (PAGE) slab gels as previously described (13). Gels were stained with Coomassie brilliant blue in a solution of glacial acetic acid–methanol–water (1:4:5), destained, treated with En^3Hance (New England Nuclear Corp.), rehydrated, and dried. Dried gels were exposed to X-ray film (Kodak XAR-5) at –70°C.

Peptide mapping. Immunoprecipitated cell lysates were resolved on 11% SDS-PAGE tube gels. Gels were cut into 2-mm slices which were eluted in 0.3 ml of 0.1% SDS overnight at room temperature, and samples were counted in a scintillation counter. Peaks corresponding to protein bands found in 1-D gels were pooled, lyophilized, and stored at –70°C. For analysis, samples were resuspended in deionized water and subjected to digestion with various concentrations of enzyme (3). Enzymes used were *S. aureus*-V8 protease and α -chymotrypsin (Miles Laboratories, Inc.). Digested peptides were resolved on 15% SDS-PAGE slab gels.

RESULTS

Alteration of gag-gene protein synthesis in nonproducer cell lines. Proteins precipitated by anti-p30 serum from tumor cells metabolically labeled for 2 h are shown in Fig. 1. Pulse-chase analyses of p30-related protein production in equicellular amounts of each of the cell lines are shown in Fig. 2. Lysates of producer BkGV1 cells showed that gPr80^{gag} and Pr65^{gag} were labeled within the pulse period (0-min chase), reflecting the fact that these molecules are primary translation products (6). Pr180^{gag-pol} was not apparent in the pulse-chase gel. As the levels of these precursor molecules decreased with time, gP95^{gag}, gP85^{gag}, a 58-kilodalton (kd) protein, and p30 levels increased. The nonproducer BbGV1 and BdGV1 cells, however, showed very different profiles of proteins precipitated by anti-p30 serum. In BbGV1 cells, Pr180^{gag-pol} was barely detectable in the cells labeled for 2 h which are shown in Fig. 1, but in all other preparations it was not detectable even on overexposed films or when another amino acid was used to label the cells (data not shown). gPr80^{gag} and gP95^{gag} were not detectable, although an 85-kd molecule was produced. A 65-kd protein was seen in 2-h labelings but was only faintly visible in pulse-chase gels. p30 did not appear until 30 to 60 min after the pulse. The 58-kd protein was visible in cells labeled for 2 h but was only faintly visible in the pulse-chase gel. Proteins of ca. 36 and 38 kd, which appeared in 2-h labelings in both BkGV1 and BbGV1 cells, were also faintly visible in the pulse-chase-labeled cells. The most prominent feature of the BbGV1 cells was a constant heavy band of ca. 47 kd which was also seen in 1-D gel analysis of BbGV1 cells labeled for 2 h. This

FIG. 3. 2-D gels of proteins prepared as described in the legend to Fig. 1 (7-day exposure).

protein is not present in BkGV1 cells. Densitometry tracings of this band showed a slight increase after 15 min of chase, followed by slight steady decreases for the remainder of the chase period (data not shown); however, background variation may have been great enough to account for these differences. Nonproducer BdGV1 cells had a p30-related protein profile different from each of the other lines. Pr180^{gag-pol} was present, but no gPr80^{gag}, gP95^{gag}, or Pr65^{gag} proteins were detectable. A band of ca. 90 kd was present, and the 58-kd band (also seen in BkGV1 and BbGV1 cells) appeared 15 min after the pulse. This finding may indicate that the 58-kd protein was cleaved from the 90-kd protein. No p30 was detectable.

Anti-p30 precipitates of two of the cell lines resolved on 2-D gels are shown in Fig. 3. Pr65^{gag} and p30 from BkGV1 cells migrated as at least three spots each. In BbGV1 cells, a 65-kd band migrated as a smear, and p30 migrated as two spots. When BkGV1 and BbGV1 precipitates were analyzed on the same gel (data not shown), the two p30 species of BbGV1 exactly overlapped the most basic and most acidic species of BkGV1 p30. In other preparations of BbGV1 cells, p30 contained three spots identical to those precipitated from BkGV1 cells. In contrast, the 65-kd smear of BbGV1 appeared at the basic end of the Pr65^{gag} of BkGV1. The 47-kd protein appeared as multiple spots in the same charge

FIG. 4. Isolation and peptide mapping of proteins prepared as described in the legend to Fig. 1. (a) Proteins were resolved on tube gels, and peaks of activity corresponding to the major bands seen in Fig. 1 were isolated as described in the text. (b) Peptides generated by incubation of 25,000 cpm of each isolated protein with 3 μ g of *S. aureus*-V8 protease for 2 h at 37°C (7-day exposure).

range as Pr65^{gag} of BkGV1. BbGV1 cells displayed only a doublet of spots of ca. 22-kd which was not analyzed further (data not shown).

Homology of the 47-kd protein of BbGV1 cells and Pr65^{gag} of BkGV1 cells. Pr65^{gag} and p30 from BkGV1 cells and the 65-, 47-, and 38-kd and p30 proteins from BbGV1 cells were isolated by tube gel separation (Fig. 4a) and analyzed for homology by peptide mapping with *S. aureus*-V8 protease (Fig. 4b). The p30 molecules of both cell lines were very closely related, having shared the same pattern of bands, which in some cases varied in intensity. The 65-kd protein isolated from BbGV1 cells shared almost no fragments with Pr65^{gag} of BkGV1 cells. The 38- and 47-kd proteins from BbGV1 cells shared an extensive number of bands with each other and with Pr65^{gag} of BkGV1 cells. Particularly noticeable was the high-molecular-weight triplet in the 47-kd protein digested by *S. aureus*-V8 protease which was found in the Pr65^{gag} of BkGV1 cells. Digestion with α -chymotrypsin demonstrated similar homology (Fig. 5).

Cell surface expression of p30-related proteins. p30 antigenic determinants were expressed on 99% of cells from all three cell lines when analyzed in the fluorescence-activated cell sorter (data not shown). The BkGV1 line expressed higher fluorescence intensity than BbGV1 cells, and BbGV1 cells expressed higher fluorescence intensity than BbGV1 cells. Thus, a hierarchy exists with respect to the number of p30 determinants per cell expressed on the surface.

Production of virions by cell lines. Although BbGV1 cells and their cell-free supernatants were negative for infectious virus as determined in the XC assay, electron micrographs showed viral particles in the extracellular spaces and budding from the cell surface (data not shown). In contrast, the BbGV1 cells exhibited no budding from the cell surface, nor were particles seen extracellularly; however, immature (A-type) particles could be observed in the intracisternal area (data not shown). BALB.B mice inoculated as neonates with cell-free supernatants from cultures of BkGV1 tumor cells developed thymic lymphomas after an 8- to 12-week latent period, whereas those inoculated with cell-free supernatants from cultures of BbGV1 cells failed to develop any disease after 10 months of observation (data not shown), indicating that the virus being shed by BbGV1 cells was nononcogenic or noninfectious or both. Additionally, supernatants from BkGV1 and BbGV1 but not from BbGV1 cells had functional reverse transcriptase (RT) (data not shown).

DISCUSSION

These studies have demonstrated alterations of *gag* protein processing in BbGV1 and BbGV1 tumor cell lines each of which stopped producing infectious, XC-positive virus early in its passage history. The most prominent alteration was the presence of the 47-kd primary translation product in BbGV1 cells. The pattern of peptide fragments generated

from this protein was similar to the pattern of peptides from Pr65^{gag} of BkGV1 cells. The peptide fragments of the p30 proteins from both cell lines were identical with respect to size and appeared to be a subset of the peptides from BkGV1 Pr65^{gag} and the 47-kd protein of BbGV1. The 65-kd protein isolated from BbGV1 cells did not share fragments with any of the other proteins. We concluded that BbGV1 cells produced a truncated *gag* precursor polyprotein instead of Pr65^{gag} and that the p30 produced by BbGV1 cells was probably a product of the 47-kd protein. This conclusion is also supported by the fact that 47-kd was the only protein which appeared in a large enough quantity to account for the amount of p30 observed.

The slower rate of appearance of p30 in BbGV1 compared to BkGV1 cells may indicate that the 47-kd protein was cleaved to yield mature viral core proteins less efficiently than normal Pr65^{gag} isolated from BkGV1 cells. An alternative explanation could be that p30 was simply not detectable early in the pulse period because of the smaller amount made compared with BkGV1 cells. However, densitometry tracings of the gels shown in Fig. 1, which were from equal numbers of cells, demonstrated that p30 was made in approximately equal amounts, but twice as much 47-kd protein was made in BbGV1 cells as Pr65^{gag} in BkGV1 cells. This data is consistent with a hypothesis of inefficient processing of the 47-kd protein to p30. This could also explain the failure to detect a significant decrease in the 47-kd protein as the chase period increased. This finding also suggests that the missing portion of the polyprotein may be involved in cleavage of the precursor to its constituents. The *gag*-encoded p15 of avian retrovirus has viral protease activity (7, 17, 18), however, this protein has no obvious analog in MuLV (6). A MuLV-associated proteolytic protein of ca. 20 kd has been described, but it is apparently not *gag* encoded (21). The 47-kd protein was also precipitated by anti-p15 and anti-p12 sera, suggesting that it may possess at least portions of each of these molecules (data not shown); however, the anti-p15 serum appeared to have some cross-reactivity with p30. Because the 47-kd protein appeared to contain an intact p30, it probably was missing some segment of p15, p12, or p10 to account for its size.

The 47-kd protein appears to be a primary translation product. Defects which could account for this truncated *gag* precursor polyprotein are an alteration in mRNA, resulting in only part of the polyprotein being translated, or a shortened mRNA, resulting either from a defect in transcription of proviral DNA or a modified provirus. Decreased amounts of viral mRNA without loss of proviral DNA have been observed in Friend virus-derived nonproducer cell lines (2).

The proteins which appeared as distinct bands of 36 and 38 kd on slab gels appeared as one peak in tube gels (Fig. 4a). Peptide mapping demonstrated extensive homology between them and the 47-kd protein. That they only appear faintly in pulse-chase gels and that similar bands are made in BkGV1 cells suggest that they are breakdown by-products rather than intermediates.

In studies of the gPr80^{gag} pathway, pulse-chase experiments have shown that gP95^{gag} is labeled during the pulse, and in most experiments gP85^{gag} appears later (11). Precipitation with anti-p10 antiserum has indicated that gPr80^{gag} and gP95^{gag} contain p10, whereas gP85^{gag} does not; however, tryptic peptide analysis has indicated that gP85^{gag} contains some p10-related peptides (11). From these data, it was concluded that, after glycosylation of gPr80^{gag} to form gP95^{gag}, most of p10 is cleaved from gP95^{gag} to form gP85^{gag}. The pulse-chase profile of our cells indicates that

FIG. 5. Peptides generated by incubation of 25,000 cpm of each of the proteins isolated in Fig. 4a with 0.5 μ g of α -chymotrypsin for 2 h at 37°C (7-day exposure).

this relationship may not always occur. gPr80^{gag} was seen only in BkGV1 cells, but BbGV1 and BdGV1 cells produced 85- and 90-kd proteins, respectively. In contrast to the previous findings, gP85^{gag} of BkGV1 appeared before gP95^{gag}. Thus, it is difficult to determine the precursor-product relationship of these proteins.

The 58-kd protein found in all three cell lines (Fig. 1) was similar to P58 found in E δ G2 cells (10), having appeared during the chase period and migrated near the basic end of Pr65^{gag} in 2-D gels. P58 may be a product unique to GV-infected cells as E δ G2 cells are also GV derived, and this protein has not been observed in cells infected with other retroviruses. The origin of P58 could not be determined from these experiments, although in BdGV1 cells the presence of only a 90-kd protein and the increased amount of P58 as the chase period lengthens suggests a precursor-product relationship.

The finding that Pr180^{gag-pol} was not detectable in most preparations of BbGV1 cells was unexpected because it has been identified as the precursor of RT (11), and this cell line produced functional RT (data not shown). This finding could be explained by a very rapid turnover rate or it may indicate that although Pr180^{gag-pol} is the usual precursor of RT, it may not be required for translation of the *pol* RNA into a functional protein.

Although we have previously classified the BbGV1 line as a nonproducer because it fails to produce plaques in an XC assay and foci in Mink S⁺L⁻ cells (19), electron micrographs demonstrated that the cells made budding virions (data not shown). The particles produced were not oncogenic in vivo,

indicating that the alterations in viral proteins changed the biological behavior of the virions but not their ability to replicate. Cultured tumor cells of Friend virus origin which are negative in the XC assay and have *gag* protein defects have also been shown to produce viral particles (5). In other nonproducer Friend virus-induced cell lines, the absence of detectable Friend MuLV *gag*-gene products (1) accompanying decreased Friend MuLV-specific RNA expression (2) has been interpreted to mean that the 5'-end of the MuLV genome is deficient (1). In the BbGV1 cells, only part of the *gag* gene product was affected, and the *pol* gene was apparently intact. BdGV1 cells evidenced nascent virus assembly, but neither budding virions nor particles in the supernatants were observed. Concurrently, these cells expressed few virus-encoded proteins, either in the cytoplasm or on their surface, and BdGV1 cell supernatant did not contain detectable RT (data not shown).

Both GV- and Friend virus-induced tumor cell lines have been observed to cease production of infectious virus after a few months in tissue culture (4, 8, 15, 19). Even though indirect evidence has been obtained which suggests that nonproducer cells may outgrow producers (4), it appears unlikely that a nonproducer culture results from the emergence of a single rare cell variant which overgrows the culture. A sufficient number of these cell lines have stopped producing infectious virus spontaneously *in vitro* to indicate that this is not a rare event. Furthermore, cessation of infectious virus production may be an inherent property of most cells of certain cell lines. Strong evidence on this point has been obtained from examination of clones derived at early passages from a cell line which became a nonproducer: of the sister clones which were producing infectious virus, nearly half (7 out of 16) became nonproducers upon further passage (4). Among tumor cell lines differing genetically only at the *H-2* complex, specific haplotypes (*H-2^b* and *H-2^d*) appear to be associated with the cessation of infectious virus production, which also suggests that host cell genes may regulate virus production (19). It is notable that other Friend virus-induced tumor cell lines which have been reported to have stopped producing infectious virus express the same two *H-2* haplotypes, albeit on different genetic backgrounds (1, 4, 15), but too few cell lines have been studied to determine whether *H-2*-linked genes actually mediate this change. The data presented in this report show that the lesions in the nonproducer cell lines derived from GV-induced tumors involve *gag* protein processing and in that respect are similar to lesions found in nonproducer Friend virus-induced cell lines (1, 5, 15). Thus, these findings suggest that in these cell lines the mechanisms of conversion to the nonproducer phenotype may be related, which is consistent with the hypothesis that virus production may be regulated by the host cell.

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