Regulation of human endogenous retrovirus-K Gag expression in teratocarcinoma cell lines and human tumours

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Human endogenous retrovirus-K (HERV-K) Gag protein is produced by both Tera 1 and PA-1 (ovarian teratocarcinoma) cells, but only Tera 1 cells release the protein in the form of particles. It was unclear how Gag production was regulated in these cell types. Although both Tera 1 and PA-1 cells express Gag, demethylation upon treatment with 5-azacytidine (5-AZC) or exposure to the chromatin-modifying agent n-butyrate resulted in an increase in Gag protein levels only in Tera 1 cells. Consistent with this cell type-specific overexpression of Gag in response to demethylation, exposure to 5-AZC caused undermethylation of the gag gene and adjacent 5'LTR only in Tera 1 but not PA-1 or Raji cells. Similarly and importantly, undermethylation of gag sequences and expression of Gag were also correlated in primary human testicular tumours. These results therefore suggest that endogenous retroviral elements are subject to regulation through the methylation of CpG dinucleotides.

The human genome contains endogenous retroviral elements (HERVs) with homology to the retroviruses of humans and other vertebrates. Several families are distinguished on the basis of homology with exogenous retroviruses, primer binding sites or other characteristics. Whereas most of these HERVs are defective on account of multiple stop codons within essential viral genes, members of the HERV-K family often harbour open reading frames for the gag, pol and env genes (for review, see Wilkinson et al., 1994). Recently, expression of HERV-K Gag polyprotein and processed protein has been observed in different teratocarcinoma cell lines such as GH and Tera 1 as well as in testicular tumour biopsies (Löwer et al., 1993; Sauter et al., 1995). A high percentage of patients with such tumours possess elevated antibody titres against Gag (Sauter et al., 1995). Furthermore, gag and env gene mRNAs have been identified in a large number of undifferentiated germ line tumour biopsies but only rarely in other tumour types or normal tissue (Herbst et al., 1996). The biological relevance of the expression of these proteins is unclear; a direct involvement of HERV-K proteins in carcinogenesis has not been excluded. This prompted us to begin to study the nature of the induction of HERV-K related gag expression in Tera 1 and PA-1 teratocarcinoma cells and primary testicular tumours.

Previous studies had shown that the human ovarian teratocarcinoma cell line PA-1 expresses a defective mRNA from type C-related human endogenous retroviral proviruses upon exposure to retinoic acid (Kannan et al., 1991). It was therefore interesting to ask whether these same cells might produce HERV-K related Gag as was already known for teratocarcinoma Tera 1 cells. Cellular extracts were prepared from exponentially growing PA-1 cells by sonication and analysed by immunoblotting using polyclonal rabbit serum directed against the entire Gag polyprotein (serum no. 6897; Sauter et al., 1995). An 80 kDa protein was detected which corresponded to the full-length Gag polyprotein expressed in Tera 1 cells (Fig. 1a). We failed to detect the 80 kDa protein when the antiserum was preincubated with bacterially expressed Gag, confirming the identity of the 80 kDa signal. Preimmune serum did not detect HERV-K Gag protein (data not shown). Notably, the partially processed Gag proteins present in Tera 1 cell extracts could not be detected in the extracts from PA-1 cells. Furthermore, whereas Gag polyprotein and processed proteins were readily observable in the 100,000 g pellet obtained from Tera 1 cell culture supernatant and after clearing by low-speed centrifugation and filtration, such proteins were not present in the pellet from PA-1 cells (Fig. 1b). From these results we conclude that the human ovarian teratocarcinoma PA-1 cells, like Tera 1 cells, express HERV-K related Gag polyprotein, but, in contrast to Tera 1 cells, do not process or release the protein.

Budding retrovirus-like particles have been identified in electron micrographs of human teratocarcinoma cells (Boller et al., 1993; Löwer et al., 1993). Our data on the 100,000 g pellets

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suggest that the Gag proteins produced in Tera 1 cells are encoded by HERV-K sequences and demonstrate that they are released in the form of particles. To confirm the presence of free retroviral particles, we performed density gradient sedimentations in 20–70% sucrose. Identification of Gag protein at a density fraction of 1.16 g/ml would have established co-
sedimentation with intact particles of a retrovirus-like density (Beard, 1963). Immunoblot analysis of each fraction employing serum no. 6897 directed against Gag revealed proteins of the correct size in fractions 5–11 (Fig. 1c). Fractions 8 and 9 contained the 80 kDa polyprotein and processed proteins of 39, 30, 22, 19 and 17 kDa. Refractometric measurements indicated that the densities of the fractions ranged from 1.10–1.18 g/ml. Thus, the Gag proteins produced in Tera 1 cells were associated with a structure that sedimented at a density characteristic of retrovirus-like particles. Such particles could not be observed in PA-1 cell supernatant. Whether these particles are capable of reinfection is unknown. Since most of the Gag protein sedimented at a density of 1.14 g/ml, yet intact retroviral particles mostly sediment at 1.16–1.17 g/ml, it is possible that some Gag was associated with viral core particles that lacked an envelope.

Demethylation has been described as a potential mechanism in the development of tumours (for review, see Jones & Buckley, 1990; Jost & Saluz, 1993). In the past, we have shown that the expression of HERV-K Gag is associated specifically with germ cell tumours in humans (Sauter et al., 1995). Moreover, it has been documented that antibodies directed against the entire Gag, cORF and Env proteins can be found in a large number of teratocarcinoma patients (Denner et al., 1995); however, these studies did not address the molecular mechanisms that resulted in the activation of these genes. It was therefore interesting to ask whether 5-azacytidine (5-AZC) or n-butyrate might affect the expression of HERV-K Gag in the human teratocarcinoma cell lines Tera 1 and PA-1.

Exposure of Tera 1 cells to 10 μM-5-AZC for up to 168 h and subsequent immunoblot analysis of cell extracts revealed that Gag full-length protein (60 kDa) and the processed 39 kDa protein were expressed substantially above the uninduced level only at 120 h post-induction (Fig. 2a). However, since 0.1% ethanol (the 5-AZC solvent) already profoundly repressed the basal level of Gag expression, the induced levels should also be compared to these controls. It then becomes evident that 5-AZC induces Gag expression (or counteracts the ethanol effect) as early as 96 h after the beginning of exposure. Furthermore, when in a separate set of experiments cells were induced for up to 168 h with 5-AZC and were compared with cells not exposed to the solvent, induction of Gag expression was clearly observable (data not shown). Together, these data suggest that demethylation as a result of 5-AZC treatment further stimulated Gag expression in Tera 1 cells. Significantly, and in accord with our interpretation that 5-AZC stimulates Gag production, the increased level of cellular Gag correlated with a concomitant enhanced production of retrovirus-like particles. This was evidenced by a stronger Gag protein signal in the 100,000 g pellet from the culture supernatant (Fig. 2a).

The induction of Gag expression was fully reversible after removal of the agent (data not shown). Treatment of Tera 1 cells with 3 mM-n-butyrate resulted in an increased expression of Gag polyprotein and processed protein after 72 h in comparison to untreated control cells (Fig. 2b). Again, the enhanced intracellular concentration of Gag translated into an increase in immunodetectable Gag in the 100,000 g pellet, indicative of its enhanced incorporation into particles. As with 5-AZC, the effect of n-butyrate on the expression of Gag was reversible.

Remarkably, and in contrast to Tera 1 cells, PA-1 teratocarcinoma cells did not show an increase in the production of Gag upon 5-AZC (Fig. 2a) or n-butyrate (Fig. 2b) treatment. Rather, n-butyrate caused abolition of Gag production. Similarly, Raji Burkitt’s lymphoma cells silent for Gag expression could not be activated by the two agents (data not shown). These findings are in accord with the notion that demethylation through 5-AZC and n-butyrate treatment can result in a selective activation of genes independent of cell type (see, for instance, Jüttermann et al., 1994). n-Butyrate is known to inhibit the histone deacetylase in a non-competitive manner. The resulting state of histone hyperacetylation causes chromatin decondensation and thereby affects gene transcription (Kruh, 1982). However, we cannot exclude the possibility that other effects of n-butyrate contribute to the described cellular phenotype. Other reports have documented that n-butyrate can induce Epstein–Barr virus in the producer cell line P3HR-1 (Szyf et al., 1985) as well as in latently infected cells (Luka et al., 1979; Boos et al., 1987). Notably, in our system, 5-AZC treatment seems to result in an increased production of free particles. Along the same lines, previous studies have shown that 5-AZC can induce the production of intracisternal A particles (IAPs), and the corresponding IAP genes have been demonstrated to be hypomethylated in response to 5-AZC in established and primary mouse plasmacytomas (Hojman-Montes de Oca et al., 1984; Lueders & Kuff, 1995). Furthermore, it is known that mouse and avian endogenous retroviruses as well as simian foamy virus can be regulated through methylation/demethylation (Breznik & Cohen, 1982; Groudine et al., 1981; Schweizer et al., 1993). Our data showing that Gag expression can be activated in Tera 1 cells by demethylation or chromatin structure modification are thus entirely consistent with these processes making sequences accessible to transcription.

Our findings on the response of Tera 1 cells to 5-AZC treatment hinted at a role of methylation in the regulation of HERV-K gag. It was unclear, however, whether the demethylation affects the gag sequences directly or via the activation of cellular effectors of the HERV-K LTRs. To address this issue, we analysed genomic DNA from Tera 1, PA-1 and Raji cells, in the presence or absence of 5-AZC. DNA was isolated by standard procedures and subsequently subjected to complete digestion with the restriction endonuclease AfII. This resulted in the presence of the HERV-K gag sequences within a 3.9 kb fragment. Next, this fragment was digested with either the methylation-sensitive enzymes HpaII or Smal, or their methylation-insensitive isoschizomers MspI or XmaI following established methods. The results are summarized in Fig. 3(a, b).
Fig. 2. For legend see facing page.
is evident that the relevant \textit{gag} DNA sequences from Tera 1, PA-1 and Raji cells are methylated and thus inaccessible to \textit{HpaII} cleavage (Fig. 3\(a\), lanes 2, 6 and 10). In contrast, all DNAs are readily cut by the methylation-insensitive \textit{MspI}, as expected. Since Tera 1 cells but not PA-1 or Raji cells were conditionally overexpressing Gag in response to 5-AZC, and if this effect is the result of demethylation of the \textit{gag} sequences, one would expect that exposure to 5-AZC leads to the appearance of DNA fragments upon \textit{HpaII} cleavage similar to those found upon \textit{MspI} cleavage. This is indeed shown in Fig. 3\(a\), lane 4. Significantly, \textit{gag} DNA from the two cell types that failed to express Gag expression after 5-AZC treatment (PA-1, Raji) also failed to become demethylated. Similar results were obtained upon cleavage with the isoschizomers \textit{SmaI} and \textit{Xmal} (Fig. 3\(b\)). Here, too, only Tera 1 cells responded to 5-AZC with demethylation of \textit{gag} genes. From these combined data it follows that a correlation exists between Gag protein expression and \textit{gag} gene demethylation in response to 5-AZC. It was truly remarkable that, although the Raji cell genome has previously been shown to be demethylated to almost 90\% upon 5-AZC exposure (Krawisz & Lieberman, 1984), HERV-K sequences failed to be demethylated and activated in these cells. In contrast, in Tera 1 cells which like Raji contain 30–50 copies of the HERV-K elements per haploid genome, most of
these elements became demethylated under 5-AZC irrespective of the location in the genome, as judged from the restriction digest pattern. These findings underscore the conclusion that HERV-K is regulated cell type-specifically.

One critical question was whether the correlation between Gag expression and demethylation observed in tissue culture can be established for human primary germ line tumour cells. To begin to address this issue we performed Southern blot analyses on DNA from testicular tumour biopsies. The results of the study are summarized in Fig. 3 (c). As in the cell lines, we observed demethylated and methylated gag sequences in tumour tissue. Demethylation was evident in five out of the eight tumour samples tested (tumours 4–8 in Fig. 3c). In contrast, DNA isolated from spleen and placenta control tissues as well as from tumours 1–3, harboured fully methylated gag sequences (Fig. 3c). Although there were some differences in the ratios of methylated to demethylated tumour DNA as judged from the variations in the hybridization signal patterns, these patterns were highly reproducible for each individual DNA sample. The differences most likely stem from the fact that the extent of demethylation varies between tumour samples and that demethylation may not affect all copies of HERV-K. To correlate the status of methylation with the expression of Gag protein, immunoblot analysis on protein extracts from the tumours was performed in parallel (Fig. 3c). Seven of eight primary tumours (except tumour 1) showed a correlation between the demethylation of gag sequences (as evidenced by HpaII/SmaI digests) and expression of Gag protein. This suggests that in primary testicular tumours as well as in cell lines, methylation/demethylation of DNA sequences is involved in the regulation of HERV-K gag expression. Further tumour samples will have to be analysed to finally establish the correlation between demethylation and HERV-K Gag expression.

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Fig. 3. Southern blot analysis demonstrating the methylation pattern of HERV-K gag sequences in cell lines and primary tumour cells. (a) DNA from the indicated cell types, with or without 5-AZC treatment as indicated, was digested with the methylation-sensitive restriction enzyme HpaII (H) or the methylation-insensitive MspI (M). In vitro methylated Tera 1 DNA (Tera 1m) served as a positive control and HERV-K negative B95-8 cell DNA was used as a negative control. (b) Similar to (a), cells were digested with the methylation-sensitive SmaI or -insensitive XmaI. (c) Genomic DNA isolated from spleen (Spl.) and placenta (Pla.) (controls) and from testicular tumours (T1–T8) was subjected to MspI/HpaII or to SmaI/XmaI digestion. Ten lag of genomic DNA was digested with AfII and the methylation-sensitive (S) restriction enzymes HpaII or Smal, or insensitive (I) enzymes MspI or XmaI. Fragments separated on a 1% agarose gel and transferred to a positively charged nylon membrane (Hybond-N+, Amersham) were probed with the 2.5 kb KpnI-ClaI fragment (Mueller-Lantzsch et al., 1993) using the ECL-random prime labelling kit from Amersham. The washing conditions were of intermediate stringency; 1% SSC and 0.5% SSC were used at 62°C for 1 h. Protein (15 µg) extracted from the indicated tissues was subjected to SDS-PAGE and probed by Western blot with the anti HERV-K Gag polyclonal serum no. 6897 (Sauter et al., 1995). ND, Not done. The correlation between demethylation and Gag expression is summarized.

References


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