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TRANSCRIPTIONAL ACTIVATION OF ENDOGENOUS RAT RETROVIRUS WITH AND WITHOUT HYPOMETHYLATION OF PROVIRAL DNA

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SUMMARY: Induction of latent, endogenous retrovirus from normal vertebrate cells has been reported following exposure in-vitro to the thymidine analog 5-bromodeoxyuridine (BrdU), as well as to the cytidine analog 5-azacytidine (azaC). Although the mode of action of BrdU is still unclear, azaC is known to cause the hypomethylation of DNA in replicating cells. This work was performed in order to determine whether a common molecular mechanism existed. Although both drugs were equally effective in the induction of the same proviral DNA in normal rat embryo cells, only azaC-treated sequences demonstrated extensive hypomethylation of proviral genes. In contrast, viral-specific sequences from BrdU-treated DNA were indistinguishable from untreated samples with respect to digestion with Hpa II and Msp I. It is likely, therefore, that the drugs effect viral induction by different mechanisms, and that hypomethylation of structural genes may not be a requisite for transcriptional activation in general of proviral DNA.

Latent, endogenous retroviruses of certain eukaryotic species have been studied as representative model systems to determine inherent regulatory mechanisms for expression of structural genes (1). Although these particular nucleotide sequences are normally under strict transcriptional suppression, several experimental treatments have been successfully employed to specifically activate the proviral genes. Induction of retrovirus synthesis ard release have been accomplished most effectively as a result of exposure in-vitro to halogenated analogs of thymidine, especially 5-bromodeoxyuridine (ErdU) (2,3). More recently, similar proviral activation has been achieved by the deliberate hypomethylation of nuclear DNA following the administration of the cytidine analog 5-azacytidine (azaC) (4,5). This study was undertaken to determine whether a common molecular mechanism or pathway was involved in both instances.

A great deal of information has been reported during the last decade regarding the virogenic effect of BrdU (6-8). On the other hand, much interest

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has recently focused upon DNA methylation to be a putative means of transcriptional control for the co-ordinated regulation of structural genes (9, 10). Indeed, a number of workers have reported the deliberate induction of several finite gene products from a variety of cultured animal cells subsequent to experimental alterations in the extent and distribution of methylcytosine moieties in nuclear DNA (11). More specifically, Niwa and Gugahara (4), as well as Groudine and associates (5), described the successful induction of endogenous retrovirus from normal mouse and avian cells, respectively, following chemical suppression of DNA methylation by azaC. Inasmuch as our previous studies have focused upon the characterization of the molecular basis for endogenous rat retrovirus induction by BrdU (6-8, 12), we sought to determine whether a common mechanism of activation existed between the virogenic effects of the two different nucleoside analogs.

MATERIALS AND METHODS

Normal rat embryo fibroblasts were established periodically and maintained as described (6). Log-phase cultures were exposed for 48 hours to either 10 µg/ml of BrdU (Sigma) or to several changes of 1 µg/ml of azaC (Sigma). Cells were scraped from plastic flasks and high-molecular weight DNA was enzymatically extracted by the proteinase K (Worthington) method as before (12). Approximately 50 µg of each DNA type were digested with 100 units of either Hpa II or Msp I (Bethesda Research Labs) overnight at 37°C. Digests were subsequently loaded (20 µg/lane) onto horizontal slab gels of 1% agarose and electrophoresed until the tracking dye migrated 20 cm. Gels were stained with ethidium bromide (0.5 µg/ml), photographed under ultraviolet light, denatured and neutralized, and transferred to nitrocellulose membranes (Schleicher and Schuell, BA-85) all essentially according to Southern (13). Probe DNA was extracted from cloned phage according to Kirby (14), and radiolabeled with a nick translation kit (Bethesda Research Labs) containing [α^{32}P]-labeled dCTP and dTTP (Amersham). DNA blots were preincubated for 2 hours at 37°C in Denhardt's solution (15) containing micrococcal DNA. Approximately 10^6 cpm of radiolabeled probe were heat denatured, placed in Denhardt's solution, and hybridized at 37°C for 72 hours with gentle shaking. Membranes were then thoroughly washed for 5 hours at 37°C, dried, and placed against Kodak X-Omat film at -80°C from 2-7 days. For RNA dot blots, cultured cells were extracted according to Groudine and Weintraub (16) in the presence of vanadyl-ribonucleoside complex ribonuclease inhibitor (Bethesda Research Labs). Total RNA preparations were transferred to "Gene Screen" membranes (New England Nuclear) utilizing a "HybriDot" device from Bethesda Research Labs. Membranes were dried and prepared for hybridization to the same radiolabeled probes as before.

Spent tissue culture fluids were clarified by low-speed centrifugation. Shed retroviruses were pelleted following ultracentrifugation in a Type 50.2 Beckman rotor subjected to 200,000 x g for 30 minutes at 5°C, resuspended in a small volume of cold buffer (100 µl, 0.01M Tris-HCl, pH 7.3, 0.1M NaCl, 1mM EDTA) and layered onto the surface of a preformed 30-60% linear sucrose gradient and sedimented as before (6). Gradient fractions corresponding to the buoyant density of retroviruses (1.15 g/cc) were pooled, diluted in 5 volumes of buffer, and sedimented at 250,000 x g as before. The pellets were resuspended in 50 µl of DNA polymerase buffer (6) made 40 µg/ml of poly r(A): oligo (dT) and 0.2 mM [^{3}H]dTTP (20 Ci/mmole, New England Nuclear Corp.), and incubated at 37°C for 1 hour. All reaction mixtures were made 20% in TCA and 100 µg/ml in bovine serum albumin on ice. Precipitates were isolated, washed thoroughly, and quantitated for acid-insoluble radioactivity as before (6,7). Purified avian myeloblastosis virus reverse transcriptase (Bethesda

Research Labs) was used for control purposes. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Normal strains of diploid rat fibroblasts were periodically established in tissue culture from 14-16 day old rat embryos as described (6-8). Monolayers of early passages were exposed to each of the nucleoside analogs during log-phase of growth. Inasmuch as these cells have a doubling time of approximately 12 hours (7), cultures were exposed to each drug for 48 hours to assure that incorporation had occurred in both strands of chromosomal DNA. Culture fluids were collected for an additional 24 hours for subsequent bioassays for reverse transcriptase activity. Cells were then scraped from culture vessels, high-molecular weight DNA was extracted and purified by the proteinase K method, and digested with either the restriction endonuclease Hpa II or its isoschizomer Msp I as before (12). DNA blots were hybridized with a total, nick-translated probe composed of lambda gtWESlambda B containing the complete circle of Moloney murine leukemia virus (MoMuLV) inserted at the Sal I site; a cloned retrovirus known to possess extensive homology with the endogenous rat proviral nucleotide sequences (17).

In confirmation of previous reports (4,5), it was apparent that azaC was indeed capable of stimulating the synthesis of latent retroviruses as reflected by the levels of reverse transcriptase activity in spent culture media (Table 1). In comparison to untreated embryo cells, BrdU and azaC were equally competent in the chemical activation of the latent endogenous virus. The specificity of the assays was established whenever purified viral reverse transcriptase was added to the reaction solutions. In order to confirm that the same nuclear proviral DNA sequences were activated by both drugs from the rat cells, RNA "dot blots" were performed. It became apparent that the radio-

Table 1
Reverse Transcriptase Activity of Spent Culture Fluids

Source of Fluids	Acid-Insoluble Radioactivity-[^{3}H]cpm
Fresh culture media alone	23
Spent media from untreated cells	25
Spent media from BrdU-treated cells	4,000
Spent media from azacytidine-treated cells	3,300
Fresh culture media + purified reverse transcriptase	7,600

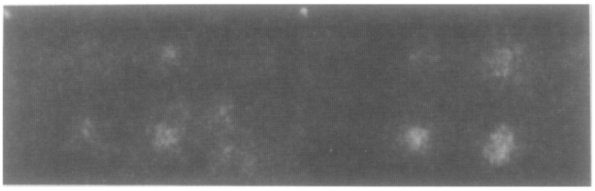

Figure 1. RNA "dot blots" of total cellular RNA isolated from rat embryo cell cultures after 48 hours of exposure to either BrdU or azaC. RNA was quantitatively extracted, blotted onto membranes at various concentrations, and hybridized as described against the [^{32}P]-labelled cDNA probe representing the complete, endogenous proviral sequences. Approximately 5, 10, 25, and 50 µg of each RNA were blotted. The left 4 RNA dots were from azaC-treated cells; the right 4 RNA dots were from BrdU-treated cells. RNA from untreated cells failed to hybridize.

labeled probe effectively hybridized to blots of total cellular RNA (Figure 1) irrespective of whether cells were exposed to either BrdU or azaC. Furthermore, the intensities of the dots were nearly identical, suggesting that equal amounts of viral messenger RNA were temporally transcribed.

Because the majority of eukaryotic DNA methylation is in the form of methylcytosine (9-11), the isoschizomers Hpa II and Msp I were selected for these studies in that while both endonucleases recognize the same nucleotide sequence (CCGG), only the former enzyme will not cleave if the internal cytosine is methylated (5,9,11). Msp I will cleave at the recognition site irrespective of methylation (5,9,11). Therefore, differences in digestion patterns reflect any corresponding differences with respect to inherent methylation patterns at the common (CCGG) site in both DNA samples. Following exhaustive hybridization of the radiolabeled viral probe to the restriction-digested DNA blots, autoradiograms were prepared (Figure 2). It was immediately apparent that proviral DNA sequences were present as two major bands following Hpa II digestion of control, untreated rat embryo DNA (Bands 1 and 2). Additionally, these native sequences were extensively methylated inasmuch as Msp I digestion reproducibly generated several lower molecular weight fragments (Bands 3-5). DNA obtained from BrdU-treated cells exhibited essentially identical hybridization profiles when compared to the respective control counterparts. On the other hand, the corresponding DNA hybridization profiles from azaC-treated cells were similar to each other, regardless of restriction enzymes. Hpa II and Msp I digestion profiles were nearly indistinguishable; an indication that endogenous, post-replicative methylation had indeed been extensively, if not quantitatively, inhibited as expected. Furthermore, these profiles were essentially identical to the Msp I digests of control and BrdU-treated DNA.

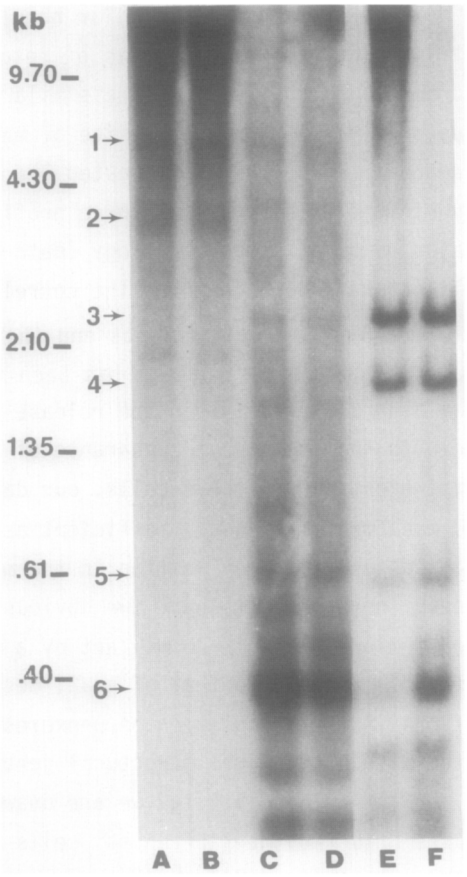

Figure 2. Southern blot analysis of restriction-digested DNA from normal and nucleoside analog-treated rat embryo cells. Digestion, blotting, and hybridization to [32P]-labeled proviral cDNA were as described in Methods. A, control DNA + Hpa II; B, BrdU-DNA + Hpa II; C, control DNA + Msp I, D, BrdU-DNA + MsP I; E, azaC-DNA + Hpa II; F, azaC-DNA + Msp I.

Interestingly, a sharp band of highly methylated, possibly repetitive DNA was consistently visible during ultraviolet examination of the Msp I digests of all DNA samples in ethidium bromide-stained gels. These sequences migrated with an average length of 0.400 kb pairs, and shared some homology with radiolabeled, viral specific probes. These methylated, repetitive families have been shown to be present in approximately 18,000 copies per haploid genome (18). Inasmuch as azaC treatment most likely does not cause total demethylation of the eukaryotic genome in living cells, it is not surprising that Band 6 in Lane E is not as intense as that in lane F. It is also possible that many small fragments of proviral DNA co-electrophorese to this broad area, and react with the radiolabeled probe. Preliminary data from another laboratory have confirmed that labeled cDNA from the

Kirsten murine sarcoma virus (KiMuSV) shares similar homology to these same sequences following hybridization to Msp I digests of fetal and adult rat DNA (18). Finally, in order to further confirm the similar organizational status of the endogenous proviral genes irrespective of methylation in the three types of cells, control, BrdU, and azaC-treated DNA were digested with Eco RI, blotted, and hybridized as before. All three profiles were identical, and consisted of two major bands of strong homology (data not shown).

Several recent publications have documented the correlation between the extent of methylation of endogenous proviral genes and their subsequent experimental expression (19,20). A common observation has been that hypomethylation of latent retroviral DNA generally results in the release from strict transcriptional suppression with the subsequent appearance of shed viral particles. With respect to azaC-treated rat embryo cells, our data are in agreement. On the other hand, BrdU treatment was equally efficient as a virogenic agent, but in the absence of any detectable modification in the methylation status of viral structural genes. Therefore, at least two obvious explanations for these observations come to mind. First, BrdU may act by a molecular mechanism entirely independent of and unrelated to that of azaC. Second, both agents may act to effect modifications in the methylation of unexpressed, regulatory DNA sequences not represented within the viral structural genes of the cloned cDNA probe. With respect to the latter possibility, we are unaware of any published report which associates BrdU treatment of animal cells with resultant alterations in enzymatic DNA methylation. Nonetheless, we are currently evaluating the role of the long terminal repeat (LTR) nucleotide sequences in a similar fashion as was done for the proviral DNA probe.

Because other experimental treatments unrelated directly to DNA methylation have been shown as well to be effective inducers of endogenous provirus expression (21-24), perhaps the presence of a methylcytosine or bromouracil moiety in a critical regulatory site in chromosomal DNA perturbs the physiologic interaction between the substituted nucleotide sequences and associated nuclear proteins of potentially regulatory significance. Such a situation could be the basis of a common mechanism for chemically-induced relaxation of transcriptional suppression.Therefore, it seems likely that the ultimate regulation of virus expression may transpire at the level of DNA and regulatory proteins interacting at finite chromosomal regions. Inductive drugs of several types may exert their influence at these sites. However, these studies demonstrate that proviral DNA can indeed be activated and transcribed in living cells in a fully methylated state. Consequently, caution should be used in the formation of universal hypotheses which attribute activation of a particular gene with concurrent demethylation of the associated chromosomal DNA.

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