Effects of 5-Azacytidine on Expression of Endogenous Retrovirus-Related Sequences in C3H 10T1/2 Cells

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In a previous study (22) we found that transient exposure of C3H 10T1/2 mouse embryo fibroblasts to 5-azacytidine (5-azaC) induced several changes in growth properties. The treated cells showed progressive changes in morphology, saturation density, growth rate, and serum dependence. By passage 5, the cells had acquired the ability to grow in 0.3% agarose, and by passage 30, they had given rise to fully transformed foci that grew in agarose, agar, and liquid suspension. This progression was rapidly accelerated if the cultures derived from 5-azaC-treated cells were exposed for 48 h to the carcinogen benzo[a]pyrene. The present studies demonstrate that both type C and type A, but not type B, retrovirus-related sequences were expressed in the 5-azaC-treated cells. There was negligible expression of these sequences in the control 10T1/2 cells. The level of expression of the related RNAs tended to correlate with loss of anchorage dependence and other markers of an increase in the transformed phenotype. These changes were associated with hypomethylation of the corresponding cellular DNA sequences, as revealed by differential digestion with the restriction enzymes *HpaII* and *MspI*. These studies provide evidence that aberrations in DNA methylation and induction of expression of certain endogenous retroviruses may be one of a series of critical events during the course of multistage carcinogenesis, thus enhancing the evolution of malignant tumor cells.

Recent advances in research on retroviruses, oncogenes, and recombinant DNA techniques provide new concepts and methods for understanding the molecular basis of multistage carcinogenesis. In addition, these and other types of studies have indicated the existence in eucaryotic cells of specific DNA sequences, termed promoters and enhancers, that control the transcription of flanking structural genes. Furthermore, there is evidence that the control of gene transcription is also modulated by DNA methylation, both in the case of endogenous retroviruslike sequences and in other cellular genes as well (18, 40). Multistage carcinogenesis may, therefore, involve not only mutationlike events in cellular oncogenes but also disturbances in gene transcription due to alterations in the function of specific promoter-enhancer sequences or aberrations in DNA methylation or both (50). Indeed, there is increasing evidence that carcinogens can impair DNA methylation (10, 23, 51) and that the DNA of tumors is often hypomethylated (13, 15). It is also of interest that mammalian cells normally contain abundant copies of endogenous retroviruslike sequences whose structures resemble known transposable elements in lower organisms, i.e., the copia elements of *Drosophila* and the Ty elements of yeast cells. Furthermore, rodent tumors and transformed cell lines, induced by a variety of agents, often display the expression of these endogenous retrovirus-related sequences, including those belonging to the C-type, intracisternal A-particle (IAP)-type, virus-like (VL30), and mouse mammary tumor virus (MMTV) families (9, 17, 25, 32, 36, 41, 52).

In a previous study (21), we extended the findings of Benedict et al. (1), indicating that 5-azacytidine (5-azaC) induces not only various differentiated phenotypes in C3H 10T1/2 cells but also cells that are morphologically transformed. We also found that a single exposure to 5-azaC led to hypomethylation of specific DNA sequences that persisted in the progeny cells for as long as 50 subsequent

passages. In a separate study (22), we demonstrated that transient exposure of C3H 10T1/2 cells to 5-azaC induced a process of stepwise cell transformation. This progression was rapidly accelerated if the cultures derived from 5-azaC-treated cells were exposed for 48 h to the carcinogen benzo[a]pyrene (BP). Thus, it appears that aberrations in DNA methylation enhance the process of cell transformation, but the precise mechanisms involved are not known.

In the present studies we have examined 5-azaC-treated cultures at different stages of neoplastic development for the expression of polyadenylated [poly(A)⁺] RNAs homologous to the murine type C Moloney murine leukemia virus (Mo-MLV), type B MMTV, and IAP sequences. The IAP proviral sequences are of particular interest because of recent evidence indicating that they can undergo rearrangements in murine plasmacytomas, thus becoming inserted into mouse κ light-chain genes (19) and into the oncogene *c-mos* (16, 26). In the latter case, the rearrangement results in transcriptional activation of this oncogene (16, 26). MMTV is the causative agent of mouse mammary tumors, presumably through viral infection and integration into the mouse genome (6). Since protection from exposure to milk-borne MMTV does not completely prevent mouse mammary tumors, it appears that endogenous proviral MMTV sequences may also be involved in tumorigenesis (8, 17). It is known that the expression of endogenous MMTV sequences is regulated by DNA methylation and glucocorticoid hormones (11, 35). It was of interest, therefore, also to examine the state of DNA methylation and expression of these sequences in our 5-azaC-treated cells.

MATERIALS AND METHODS

Cell cultures. Stock cultures of the C3H 10T1/2 mouse embryo fibroblast cell line (at passages 11 through 14) were grown in 90-mm petri dishes in Dulbecco modified Eagle medium supplemented with 10% calf serum (Flow Laboratories, Inc., McLean, Va.). Cultures were grown in a humidified incubator at 37°C with 5% CO₂ in air and were fed

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TABLE 1. Progression of 5-azaC-treated 10T1/2 cells^a

Treatment and stage	Passage no.	Morphology	Growth in:		
			Agarose	Agar	Liquid
Control	0-30	Flat	0	0	0
5-azaC					
1	0-4 ^b	High saturation density	1+	0	0
2	5-30 ^c	Type III foci	3+	0	0
3	30 up	Type IV foci	4+	4+	4+

^a Cells were treated with 3 μ M 5-azaC for 24 h, serially passaged, and scored for parameters. Some of these cultures were subsequently treated with BP (1 μ g/ml) for 48 h and then assayed when confluent.

^b Cells treated with BP at passage 0 rapidly yielded stage 2-type cells.

^c Cells treated with BP at passage 8 rapidly yielded stage 3-type cells.

fresh medium twice a week. Cultures not treated with 5-azaC were designated C, cultures derived from 5-azaC-treated cells were designated A, and cultures derived from cells treated with BP were designated BP. P designates passage, and the superscript designates the passage number after 5-azaC treatment (or the passage number of the corresponding control). The derivation and growth characteristics of these cell lines are described elsewhere (22).

Probes. To prepare the Mo-MLV long-terminal-repeat (LTR) fragment, plasmid p600, which contains the LTR sequence of Moloney murine sarcoma virus (2), was digested with *Pst*I, and a 580-base-pair fragment was isolated by electrophoresis and electroeluted from the gel. The *env* fragment comprised the *Xba*I-*Xba*I 2.2-kilobase (kb) region of plasmid p8.2 (44). Plasmids pMIA6 and pMIA1 (5) were used to prepare the probes for the LTR and internal viral sequences of the IAP sequence, respectively. An *Eco*RI-*Hind*III fragment, which includes most of the IAP proviral sequences between the 5' and 3' LTR of this provirus, was isolated from pMIA1. A *Hind*III fragment was isolated from pMIA6, which included the 3'-terminal IAP proviral sequence, the entire 3' LTR, and the associated mouse flanking sequence. A *Pst*I-digested 1.45-kb MMTV LTR-specific fragment was obtained from plasmid p201 (33) (American Type Culture Collection, Rockville, Md.).

Nucleic acid hybridizations. ³²P-labeled probes were prepared from these fragments by nick translation, as previously described (49). For Southern blot hybridizations, cellular DNA was isolated, and Southern blots were prepared and hybridized to the specific probes, as previously described (46). For Northern blot hybridizations, poly(A)⁺ RNAs were extracted by the cesium chloride cushion method (7), electrophoresed on 1% agarose gels in the presence of 6% formaldehyde, and hybridized to the specific probes, as previously described (39, 48).

RESULTS

Summary of the effects of 5-azaC on the progressive nature of cell transformation in C3H 10T1/2 cells. Our previous studies (22) indicated that transient exposure of C3H 10T1/2 cells to 5-azaC initiated a gradual and progressive series of changes in the morphology and growth properties of the subsequent progeny cells. We have divided this progression into three stages (Table 1). During stage 1 (from passage 0 to passage 4 after 5-azaC treatment), the monolayer cultures displayed morphologic changes (including the appearance of myoblasts and adipocytes), but no distinct foci of transformed cells were seen. In contrast to the control cultures, the treated cultures were able to form colonies in agarose, but these were microcolonies, and the treated cells did not form colonies in agar (Table 2) (22). Cells from stage 2

(passage 5 up to about passage 30) gave larger colonies in agarose, including some giant colonies, but still did not grow in agar (Table 2). Cells at stage 3 (passage 30 or later after 5-azaC treatment) grew well in both agarose and agar, and in monolayer culture they formed distinct type IV transformed foci (Table 2). We found that the carcinogen BP was able to accelerate this progression, since when 5-azaC-treated cells at passage 0 were exposed to BP, they rapidly acquired the characteristics of stage 2 cells, and when 5-azaC-treated cells at passage 8 were exposed to BP, they rapidly yielded stage 3-type cells (Tables 1 and 2) (22). Clones isolated from stage 1, 2, or 3 cultures demonstrate these altered growth properties even more dramatically than the mass cultures, although we have not yet studied the serial progression of individual clones taken from early-stage cultures. We should emphasize that the control untreated C3H 10T1/2 cultures did not show the type of serial progression in phenotype seen in the progeny of the 5-azaC-treated cells.

Altered expression of endogenous sequences homologous to Mo-MLV. We have previously reported that several C3H 10T1/2 cell lines transformed by chemical carcinogens or radiation constitutively express a series of poly(A)⁺ RNAs that contain sequences homologous to the LTR sequence of Mo-MLV proviral DNA (Mo-LTR) (25). These transcripts could not be detected in early-passage normal C3H 10T1/2 cultures, but they were detected when the poly(A)⁺ RNA fraction was examined 48 h after the cells were exposed to 5-azaC (3 μ M for 24 h). Since the studies described above indicated that with serial passage the 5-azaC-treated 10T1/2 cells underwent progressive changes and that this process was accelerated by exposure to BP, it was of interest to correlate these phenotypic changes with the expression of LTR-related transcripts. The poly(A)⁺ RNA fraction was isolated from cells at various stages (Table 1), fractionated by gel electrophoresis, and blot hybridized to a ³²P-labeled

TABLE 2. Cloning efficiency in soft agarose and agar of control and 5-azaC-treated C3H 10T1/2 cells^a

Cell type and clone ^b	Cloning efficiency (%) ^c in:				
	Small	Medium	Large	Giant	Agar (diam, >0.5 mm)
C-P ⁰	0	0	0	0	0
C-P ¹	0	0	0	0	0
C-P ⁵	0	0.01	0	0	0
A-P ⁰	0	0	0	0	0
A-P ¹	0.14	0	0	0	0
A-P ⁵	0	0	0.75	0.07	0
3	0	0	50	0	0
4	0	20	0	0	0
7	0	20	0	0	0
10	0	0	30-50	0	0
15	20	0	0	0	0
23	0	50	0	0	0
54	0	0	20-30	0	0
60	0	0	30-50	0	0
A-P ³⁰	0	0	>80	0.01	0.02
A-P ³⁰ , 4a	0	0	>40	40	40
BP-A-P ⁸	0	0	>80	0.05	0.02
BP-A-P ⁸ , 4a	0	0	>40	40	40
MCA-5	0	0	0	30	18

^a For additional details, see reference 22.

^b For a description of cell types, see reference 22.

^c Expressed as percentage of initial number of cells plated. Small, medium, large, and giant designate the diameters of the colonies, i.e., <0.05, 0.05 to 0.15, 0.15 to 0.5, and >0.5 mm, respectively.

FIG. 1. Expression of RNAs homologous to a Mo-LTR DNA probe in control and 5-azaC-treated cells at various passages. (A) Uncloned cells; (B) various clones derived from 5-azaC-treated cells (see text for details). Poly(A)⁺ (10 μ g) was applied in each lane, electrophoresed, and blot hybridized to the Mo-LTR probe, as described in Materials and Methods. rRNAs were used as markers.

Mo-LTR-specific probe. A single treatment with 5-azaC induced the appearance of a series of RNAs homologous to the LTR probe (Fig. 1A). The level of these transcripts increased considerably by passage 5 after 5-azaC treatment (Fig. 1A, A-P⁵), by which time the cells also displayed morphologically transformed foci (type III) and had acquired the ability to grow in agarose but not agar (Table 2) (22). At early passage, the control 10T1/2 cells were negative or produced only trace amounts of these transcripts (Fig. 1A, C-P⁰), whereas five passages later they produced clearly detectable amounts of these transcripts (Fig. 1A, C-P⁵), but at much lower levels than the 5-azaC-treated cells at a comparable passage (Fig. 1A, A-P⁵). As previously reported for carcinogen-transformed cells (25), the profile of LTR-positive bands included two prominent bands at about 8.2 and 3.5 kb and two weaker bands at about 2.0 and 1.2 kb. The former two bands, but not the latter two, also hybridized to an Mo-MLV *env*-specific (Mo-*env*) probe (data not shown). They probably represent, therefore, full-length and spliced transcripts, respectively, of endogenous proviral Mo-MLV-related sequences. The genomic sequences that give rise to the two lower-molecular-weight RNAs (2.0 and 1.2 kb) are not known (see Discussion in reference 25). Eight randomly picked clones obtained from the A-P⁵ culture were also examined for the presence of these RNAs. Except for clone 15, which grew very poorly in 0.3% agarose (Table 2), all of these clones displayed LTR-homologous RNAs. A representative blot is shown in Fig. 1B.

Thus, a single exposure of 10T1/2 cells to 5-azaC caused rapid induction (within 4 or 5 days) of the expression of a series of poly(A)⁺ RNAs that were apparently derived from endogenous Mo-MLV-related proviral DNA sequences. These transcripts were constitutively expressed (i.e., in the absence of further exposure to 5-azaC) and increased in amount with subsequent serial passage. Their increased expression paralleled the ability of the cells to grow in 0.3% agarose (Table 2), although it is not clear that the two events are causally related.

In view of the above results, it was of interest to analyze the methylation pattern of endogenous Mo-LTR and *env*

DNA sequences. DNAs were extracted from 10T1/2 control cultures, 10T1/2 A-P¹ and A-P⁵ cells, and also from four independently isolated clones derived from A-P⁵ cells (clones 4, 15, 23, and 54). The samples were digested with *Hpa*II or *Msp*I and then analyzed by Southern blot analysis with a ³²P-labeled *env*-specific probe. The patterns obtained with *Msp*I digestion of the various DNA samples were very similar (Fig. 2a). On the other hand, with *Hpa*II digestion, all of the DNA samples obtained from 5-azaC-treated cells revealed two or three low-molecular-weight bands not seen (or only faintly detected) in the control 10T1/2 cells. These additional bands were most prominent in the A-P⁵-derived clones. Since these new bands correspond to low-molecular-weight bands seen with *Msp*I-digested DNA (Fig. 2a), we presume that they reflect *env* DNA sequences that are hypomethylated. When the same blot shown in Fig. 2a was hybridized with a Mo-LTR probe, the results also provided evidence for hypomethylation of these DNA sequences in the 5-azaC DNA samples (data not shown). However, the results were less clear because of the large number of endogenous LTR sequences in the mouse genome (25). To improve the resolution of the low-molecular-weight bands, a 2% agarose gel was placed at the bottom of a 1% gel. Figure 2b shows the Southern blot analysis obtained with this composite gel and the Mo-LTR probe. Although the profile is quite complex, the *Hpa*II digest of A-P⁰ and A-P⁵ DNA samples revealed more prominent low-molecular-weight bands than the control 10T1/2 DNA sample. These bands were especially prominent in the DNA samples from the A-P⁵-derived clones. Similar results were obtained in a repeat study (data not shown).

Altered expression of endogenous IAP DNA sequences. IAP DNA sequences are highly reiterated in the murine genome (28). They occur as heterogeneous types that have different discrete sizes, although they all share certain conserved sequences (28, 45). They are expressed during embryonic development and in certain murine tumors (5). They contain LTR sequences that enhance gene transcription (30), and they can undergo rearrangements within the murine genome, thus causing insertion mutations and oncogene activation

FIG. 2. Methylation patterns of cellular Mo-MLV *env* and Mo-LTR homologous DNA sequences in control and 5-azaC-treated C3H 10T1/2 cells. Cellular DNAs were extracted from control 10T1/2 (lanes 1 and 8), A-P⁰ (lanes 2 and 9), A-P⁵ (lanes 3 and 10), and A-P⁵ clone 23 (lanes 4 and 11), 54 (lanes 5 and 12), 4 (lanes 6 and 13), and 15 (lanes 7 and 14) cells and digested with *HpaII* or *MspI*. DNA (40 µg) was applied to each lane, electrophoresed in 1% agarose gels, and blot hybridized to either the Mo-MLV *env* probe (a) or the Mo-LTR probe (b). In panel b, a composite gel was used to increase the resolution. The size markers were *HindIII*-digested λ DNA.

(15, 19, 26). It was of interest, therefore, to examine possible changes in integration, methylation, and expression of IAP DNA sequences in 10T1/2 cells after treatment with 5-azaC.

Two different probes were prepared with DNA fragments obtained from the recombinant plasmids pMIA1 and pMIA6 (5). We isolated from pMIA1 an *EcoRI*-*HindIII* fragment which included most of the IAP proviral sequences that are present between the 5' and 3' LTRs of this provirus. From pMIA6, a *HindIII* fragment was isolated which included the 3'-terminal IAP proviral sequence, the entire 3' LTR (338 base pairs), and the associated murine flanking sequence, for a total length of 1.2 kb (5). These fragments were used to prepare IAP-internal and IAP-LTR ³²P-labeled probes, respectively. When the *PstI*-digested DNAs extracted from 10T1/2 control and various 5-azaC-treated cells were subjected to Southern blot analysis with ³²P-labeled IAP-LTR probe, several distinct bands of 7.0, 6.5, 4.0, 2.6, 2.2, and 1.3 kb and a few bands smaller than 1 kb were observed in all of the DNA samples (data not shown). Similar patterns have been reported in other strains of mice and in other murine

cell lines (28). Thus, the 5-azaC treatment did not lead to gross rearrangements or amplification of specific IAP sequences. To compare the methylation patterns of IAP-related DNA sequences, the DNAs obtained from control cells and from cells at passages 0 to 4 after 5-azaC treatment were cleaved with *PstI* followed by either *HpaII* or *MspI*, electrophoresed on 1% slant gels (3), and then blot hybridized to the ³²P-labeled IAP-internal probe. The *MspI*-digested DNA contained two prominent bands at 1.8 and 1.2 kb (Fig. 3, A-P⁵). These bands were also present in the *HpaII*-digested DNAs from all of the 5-azaC-treated cells (A-P⁰ through A-P⁵), but these two bands were not present in the *HpaII* digest of the control DNA. Thus, cellular IAP genes or their immediately adjacent DNA sequences, or both, underwent demethylation in 5-azaC-treated cells. This demethylation occurred rapidly after drug treatment, since the change was seen in the A-P⁰ cells, and persisted in all subsequent passages.

The size of IAP transcripts found in various murine tumors and tumor cell lines (20, 27, 31, 36, 52) varied from 7.0 (35S) to 3.5 kb. Recently, Piko et al. (37) reported a predominant and discrete IAP RNA of 5.4 kb in murine embryos. The level of expression of this RNA correlated with embryonic development. In view of these findings and the results related to DNA methylation (Fig. 3), it was of interest to determine whether 5-azaC treatment led to the production of IAP RNA in C3H 10T1/2 cells. Poly(A)⁺ RNAs were prepared from the control C-P⁰ and C-P⁵ cells and from A-P⁰, A-P¹, and A-P⁵ cells, electrophoresed in 6% formaldehyde agarose gels, and blot hybridized with the ³²P-labeled IAP-LTR probes. The RNA from C-P⁰ cells revealed only two very faint bands at 6.5 and 5.0 kb, which were increased in amount in the C-P⁵ cells (Fig. 4a). RNAs from all of the 5-azaC-treated cells showed a marked increase in these two bands and also revealed two additional

FIG. 3. Methylation patterns of cellular IAP DNA sequences in control 10T1/2 and 5-azaC-treated cells at passages 1 through 5. Of each DNA sample, 40 µg was digested with *HpaII* or *MspI*, as indicated, electrophoresed in 1% slant agarose gels (3), and blot hybridized to the IAP LTR probe. The size markers were *HindIII*-digested λ DNA and *HaeII*-digested ΦX174 replicative-form DNA.

FIG. 4. Expression of RNAs homologous to IAP DNA probes in control 10T1/2 and 5-azaC-treated cell populations (a), in clones derived from A-P⁵ cells (b), and in various transformed cells (see text for a description of these cell types) (c). Poly(A)⁺ RNA (10 μ g) was applied to each lane, electrophoresed in 1% agarose, and blot hybridized to either the IAP-LTR probe (a and c) or the IAP-internal probe (b). Sizes were calculated on the basis of 28S and 18S rRNA markers. The original autoradiogram displayed faint bands at 6.5 and 5.0 kb in the C-P⁰ sample and distinct 8.4- and 7.6-kb bands in the A-P⁵ sample, which are not clearly seen in this photograph.

discrete bands of 8.4 and 7.6 kb (Fig. 4a). These RNAs were particularly prominent in the later-passage A-P⁵ cells. Eight clones derived from A-P⁵ cells were also studied for IAP RNA expression with the IAP internal probe (Fig. 4b). They were all strongly positive. However, in contrast to the expression of Mo-LTR-related sequences (Fig. 1B), the individual clones differed both quantitatively and qualitatively in terms of the profile of specific IAP RNAs present (Fig. 4b). There was no simple correlation between these profiles and the relative abilities of these individual clones to grow in 0.3% agarose (compare Fig. 4b and Table 2). It is of interest that among the eight clones examined, clone 15 had the lowest level of IAP transcription (Fig. 4b) and also had a low level of expression of RNA homologous to the Mo-LTR probe.

We also examined the poly(A)⁺ RNAs obtained from two clones derived from type IV foci, one isolated from A-P³⁰ cells (A-P³⁰ clone 4a) and the other isolated from a type IV focus obtained when A-P⁸ cells were treated with BP (BP-A-P⁸ clone 4a). For comparison, poly(A)⁺ RNAs from two tumor-derived murine cell lines, the P3 plasmacytoma (24, 38) and L5178Y lymphoma (14), were included in this study. With the IAP-LTR probe, the control 10T1/2 cells (Fig. 4c, C-P⁵) again showed only trace amounts of IAP RNA, while the RNAs from A-P³⁰ clone 4a, BP-A-P⁸ clone 4a, P3, and L5178Y cells showed large amounts of IAP-related RNAs. The profiles of bands were rather similar in all of the transformed cells, although the P3 cells contained much more total IAP-related RNAs than the other three cell lines. Two distinct low-molecular-weight bands were present for all of the transformed cells. These two bands were more prominent when we used the IAP-LTR-specific probe rather than the IAP-internal probe (compare Fig. 4c and b).

Endogenous type B MMTV-related sequences remain transcriptionally silent even though the proviral sequences are hypomethylated in the 5-azaC-treated C3H 10T1/2 cells. Endogenous MMTV proviral sequences are generally transcriptionally silent and hypermethylated in the normal mammary

and nonmammary tissues of most strains of mice. These endogenous sequences are expressed, however, both in the normal mammary gland and in mammary tumors in C3H/Sm mice that have a low spontaneous incidence of mammary tumors (4, 17). MMTV sequences are also expressed in certain murine thymomas (29, 35). When the Northern blots containing the poly(A)⁺ RNA samples shown in Fig. 4a, b, and c were hybridized to the MMTV-LTR-specific probe, none of the normal or 5-azaC-treated C3H 10T1/2 cells showed expression of MMTV-related sequences. On the other hand, the P3 myeloma and L5178Y lymphoma cell lines revealed expression of MMTV-LTR-specific transcripts of 4.5 and 2.2 kb (Fig. 5). Graham and his co-workers have also demonstrated a 2.2-kb LTR-specific sequence in

FIG. 5. Expression of RNAs homologous to MMTV-LTR probes in control 10T1/2 and transformed cells. Poly(A)⁺ RNA (10 μ g) was electrophoresed in 1% agarose and blot hybridized to MMTV-LTR-specific probe.

FIG. 6. Methylation patterns of cellular MMTV DNA sequences in control 10T1/2 and 5-azaC-treated cells at passages 1 through 5. Of each DNA sample, 40 μ g was digested with *Hpa*II and *Msp*I, as indicated, electrophoresed in 1 to 2% composite agarose gel, and blot hybridized to MMTV-LTR probe. The size markers were *Hind*III-digested λ DNA.

C3H/Sm mouse mammary tumors (17). When the DNA methylation patterns of endogenous MMTV sequences were examined in C3H 10T1/2 and 5-azaC-treated cells during serial passages with *Hpa*II-*Msp*I differential digestion, we found extensive loss of methylation in the 5-azaC-treated cell populations compared with normal C3H 10T1/2 cells (Fig. 6). Yet, as mentioned above, MMTV-homologous transcripts could not be detected in these 5-azaC-treated cells. Thus, the expression of MMTV-related sequences may depend on additional factors (such as glucocorticoids and other hormones) or on the state of cell differentiation, in addition to the loss of DNA methylation.

DISCUSSION

The present study indicates that the progeny of C3H 10T1/2 cells that have been transiently exposed to 5-azaC display constitutive expression of transcripts related to at least two distinct families of endogenous retroviruslike DNA sequences, i.e., sequences related to Mo-MLV (C type) and sequences related to the IAP family (A type). We presume that this reflects hypomethylation of the related DNA sequences induced by the original exposure to 5-azaC. Indeed, we obtained evidence, by restriction enzyme analysis, for hypomethylation of the endogenous Mo-LTR, Mo-*env*, and IAP DNA sequences in these cells (Fig. 1 and 3). However, the extent of expression of these RNAs did not precisely correlate with the apparent extent of hypomethylation of the related DNA sequence. For example, clone 15 showed extensive hypomethylation of the *env* DNA sequence, but little or no expression of the related poly(A)⁺ RNA was detected (Fig. 1A and B). There is evidence that the critical sites for the control of transcription by DNA methylation are confined to the 5' end of genes. Thus a precise correlation might not be expected unless these regions are specifically examined. Such examination was not possible in the present study because of the multiplicity and complexity of the endogenous Mo-LTR, Mo-*env*, and IAP DNA sequences. Nevertheless, it would appear that endogenous type C and

type A retrovirus-related sequences are more readily susceptible to transcriptional activation by 5-azaC and certain other agents (43, 47) than are other cellular genes. Thus, in a previous study (21) and in this study we could not detect transcription of the β -globin sequence, the *c-mos* locus, or MMTV proviral sequences in 5-azaC-treated cells, even though we found that these sequences had undergone extensive demethylation, and yet the same cells display transcription of the Mo-LTR, Mo-*env*, and IAP sequences (this study). The reasons for this specificity are not now apparent, but this phenomenon may be a clue to why carcinogen-transformed cells also tend to switch on the transcription of certain endogenous retrovirus-related sequences. It is known that in certain avian and rodent systems, various chemical carcinogens (including BP) can also induce the transient expression of retroviruses (47).

In this study the cells that showed anchorage independence (i.e., growth in soft agarose and agar) tended to have elevated levels of retrovirus-related RNAs, and even higher levels of these RNAs were seen, in general, in the fully transformed clones. We have no direct evidence, however, that the expression of these transcripts actually contributes to the evolution of the transformed phenotype of the 5-azaC-treated cells. As a matter of fact, in 5-azaC-treated cells, the expression of the retrovirus-related sequences precedes the acquisition of the fully transformed phenotype. Presumably, further genomic changes are needed for cell transformation.

Rodent tumors and transformed cell lines often display the expression of various endogenous retrovirus-related sequences. In addition, RNAs homologous to the retrovirus-like element copia present in *Drosophila* are expressed in transformed *Drosophila* cells but not in normal adult *Drosophila* tissues (42). Since both copia (12) and IAP (16, 19, 26) elements are known to undergo gene transposition, and since all of these elements share structural features of transposable elements (34), it is possible that their expression in tumors might lead to gene transposition, via reverse transcription, and thus contribute to genomic changes that occur during the process of tumor progression. Due to the high copy number (from 30 to over 1,000) (25, 31), it may be difficult to detect the transposition of an endogenous type A- or type C-related DNA sequence by Southern blot analysis. The LTR-specific transcripts of both type A and type C viral sequences that we have found in 5-azaC-treated cells are worthy of further study, since some of them may be linked to cellular sequences that influence cell growth. Regardless of its functional significance, it is clear that the expression of endogenous retrovirus-related DNA sequences frequently accompanies the process of cell transformation. Thus, this phenomenon provides a useful marker of alterations in gene transcription associated with the carcinogenic process.

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