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Carcinogen- and radiation-transformed C3H 10T½ cells contain RNAs homologous to the long terminal repeat sequence of a murine leukemia virus

(chemical carcinogenesis/endogenous retrovirus)

PAUL KIRSCHMEIER*, SEBASTIANO GATTONI-CELLI*, DINO DINA†, AND I. BERNARD WEINSTEIN*

*Cancer Center/Institute of Cancer Research and Division of Environmental Science, Columbia University, New York, New York 10032; and †Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT **Carcinogen- or radiation-transformed C3H 10T½ murine fibroblasts transcribe a set of poly(A)⁺RNAs that contain sequences homologous to the long terminal repeat (LTR) sequence of Moloney murine sarcoma virus. These LTR-containing RNAs consist of a series of discrete bands ranging in size from about 38 to 18 S. The higher molecular weight molecules (30–38 S) in this set of RNAs also contain sequences homologous to the *gag*, *pol*, and *env* genes of a murine leukemia virus. A 24S RNA contains sequences homologous to the *env* gene of murine leukemia virus. A 20S and an 18S RNA also share homology with the LTR probe but fail to hybridize to the *gag*, *pol*, or *env* probes or to a probe for the U3 region of the LTR sequence. Thus, the latter transcripts do not appear to arise from a known endogenous murine leukemia virus genome. Although this entire set of RNAs is absent from normal C3H 10T½ cells (or is present at an extremely low level), these RNAs are induced by BrdUrd or 5-azacytidine. The presence of these RNAs may provide highly sensitive molecular markers of transformation of murine cells.**

Chemical carcinogens and radiation can induce the malignant transformation of cells *in vitro* (for review, see ref. 1). The simplest explanation is that these agents induce point mutations in genes that regulate normal growth. Other hypotheses suggest that more complex mechanisms are involved, such as DNA rearrangements or stable alterations in gene expression via mechanisms that govern normal differentiation (2, 3). Any of these hypotheses must account for the fact that, in contrast to oncogenic viruses, chemical carcinogens and radiation cannot introduce new genetic information. Therefore, they must act through genes normally present in the target cell. A major challenge in carcinogenesis research is the identification of these host gene(s).

Recent studies on the avian and mammalian RNA sarcoma viruses may provide important clues as well as specific probes for identifying these genes. These studies have revealed that many vertebrate species, including humans, normally contain a set of single-copy genes that are homologous to the transforming genes (also designated *src* or *onc* genes) of the RNA tumor viruses. At least a dozen of these types of genes have now been identified in normal vertebrate cells through the use of probes obtained from different RNA sarcoma viruses. The viral gene usually lacks the intervening sequences found in its cellular homologue, but the coding regions of the two genes are otherwise nearly identical (for reviews on *onc* genes, see refs 4–9).

The major difference between the cellular *onc* genes and their viral homologues is their level of physiological expression (6–9). Cells transformed by a RNA sarcoma virus can contain 10,000–20,000 copies of RNA homologous to the viral *onc* gene. However, in normal cells, the cellular *onc* genes show negligible or a very low level of expression—i.e., about 5–10 copies per cell (6). The c-*mos* gene, which is homologous to the *onc* gene of Moloney murine sarcoma virus (Mo-MuSV), is expressed at less than 0.5 copy per cell in various cell types (4). Although the endogenous *onc* genes are not usually expressed at high levels, there is evidence that they do contain genetic information capable of transforming cells. When the c-*mos* gene is spliced to a long terminal repeat (LTR) sequence (see below) and normal cells are transfected with this DNA, transformation occurs (10). This and other evidence (11, 12) indicates that cellular *onc* genes can lead to cell transformation if they acquire signals that cause a high level of expression.

Several lines of evidence indicate that the LTR regions of retrovirus genomes play a crucial role in controlling transcription (11, 12). The specific sequences involved in promotion of transcription are present in the U3 portion of the LTR sequence (13). The R region of the LTR is coincident with the site of initiation of viral RNA synthesis, and the U5 region represents the portion of the LTR sequence that is present at the 5′ terminus of the mature viral RNA (13). The LTR sequence also has structural features similar to those of transposable elements of bacteria, suggesting that the LTR sequences might also be involved in gene transposition (12). They could act, therefore, as mobile promoters capable of initiating the transcription of sequences adjacent to sites into which they might become integrated (12). Consistent with this possibility are recent studies on an avian leukosis virus (RAV-2) which lacks its own transforming gene. It appears that insertion of the LTR sequence of this virus into the host cell DNA activates transcription of a flanking host *onc* sequence designated c-*myc* and that the expression of the latter sequence results in cell transformation (11).

Taken together, the above findings suggest a model for cell transformation by chemical carcinogens or radiation. If damage to cellular DNA by these agents were to induce gene rearrangements (2), then certain endogenous *onc* genes or LTR sequences might be inserted into aberrant positions in the host genome and thus cause the expression of genes that induce cell transformation. In theory, these events could occur in the absence of a replicating leukemia virus (12). In a previous study designed to test this hypothesis we used a probe to the c-*mos* sequence

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Abbreviations: *onc* gene, oncogene or transforming gene; Mo-MuSV, Moloney murine sarcoma virus; MuLV, murine leukemia virus; c-*mos*, c-*rasH*, and c-*myc* are the cellular homologues of the *onc* genes of Mo-MuSV, Harvey sarcoma virus, and MC-29 virus, respectively; *gag*, *pol*, and *env*, genes encoding viral group-specific antigen, reverse transcriptase, and envelope glycoprotein, respectively; LTR, long terminal repeat of Mo-MuSV; bp, base pair(s).

Table 1. Cell lines used in the present study

Designation	Transforming agent	Source
C3H 10T½	None	C. Heidelberger
C3H 10T½ CB-1	X-rays	C. Borek
C3H 10T½ CB-2	Benzo[*a*]pyrene	C. Borek
C3H 10T½ JL-1	X-rays	J. Little
C3H 10T½ JL-2	UV light	J. Little
C3H 10T½ JL-3	Methylcholanthrene	J. Little
C3H 10T½ MCA	Methylcholanthrene	I. B. Weinstein
C3H 10T½ UR-1	Methylcholanthrene	U. Rapp
C3H 10T½ UR-1	Methylcholanthrene	U. Rapp

All cell lines were of transformed phenotype except C3H 10T½ which was of normal phenotype.

(4). We found, however, that this sequence had not undergone rearrangement and that it remained hypermethylated and transcriptionally silent in several radiation- and carcinogen-transformed murine cell lines. We also obtained evidence that another *onc* gene, c-*rasH*, had not undergone rearrangement in these transformed cells (unpublished studies).

In the present study we have taken a different approach by examining whether there are differences between normal murine cells and murine cells transformed by radiation and chemical carcinogens in terms of the expression of RNA species containing sequences homologous to a probe prepared to the LTR sequence of a murine retrovirus.

MATERIALS AND METHODS

Cell Cultures. The cell lines used in this study are listed in Table 1. C3H 10T½ c18 and its transformed derivatives were grown in Dulbecco's modified Eagle's medium containing 10% calf serum on plastic tissue culture plates (Nunc) at 37°C in an atmosphere of 5% CO_2 in room air.

Preparation of Probes. Specific DNA fragments were prepared with the appropriate restriction enzymes (New England BioLabs) from the previously described recombinant plasmid p600 (13) which contains the LTR sequence of Mo-MuSV and from plasmid p101 (Fig. 1) which contains the entire Mo-MuSV genome. To prepare the LTR fragment, plasmid p600 was digested with *Pst* I and the 580-base-pair (bp) fragment was purified by gel electrophoresis and then extracted from the gel. The U3 DNA fragment of the LTR sequence was isolated by digesting plasmid p600 with *Sac* I and *Pst* I. This yielded a 350-bp fragment that lacked the U5 and R sequences of the LTR (see introduction). To prepare the *gag–pol* fragment, plasmid p101 was digested with *Pst* I and the 3.1-kilobase fragment was isolated by gel electrophoresis. This fragment was then digested with *Bgl* I and the larger of the two fragments (2.4 kilobases) was isolated by gel electrophoresis. The *env* fragment comprised the *Hin*dIII–*Hin*cII region of cloned Friend leukemia virus DNA (14). This fragment was subcloned into the equivalent sites of pBR322 by J. Chinsky and kindly provided by him. The entire plasmid was used to prepare the *env* probes. Labeling of DNAs with nucleoside [α-^{32}P]triphosphates was performed *in vitro* by nick-translation (15).

Nucleic Acid Separation and Hybridization. Total RNA was extracted from subconfluent cells and the poly(A)$^+$RNA fraction was isolated on an oligo(dT)-cellulose column (Collaborative Research, Waltham, MA) as described (16). This RNA was then denatured, gel electrophoresed, blotted, and hybridized to ^{32}P-labeled probes by published methods (17–19). High molecular weight DNA, isolated as described (4), was cleaved with specific restriction enzymes (New England BioLabs) and analyzed by the blotting technique described by Southern (20).

RESULTS

Transformed Cell Lines Contain RNAs Homologous to LTR Sequences. The normal murine cells used in these studies were C3H 10T½ c18, originally isolated by Reznikoff *et al.* (21). Although they are an aneuploid cell line, they have a very low saturation density, display anchorage-dependent growth, and are not tumorigenic in syngeneic mice (21). They can be reproducibly transformed *in vitro* by several chemical carcinogens, UV light, and x-ray irradiation (22), thus providing a series of comparable cell lines for analysis of the mechanism of cell transformation.

To analyze possible differences between normal and transformed C3H 10T½ cells in terms of expression of endogenous

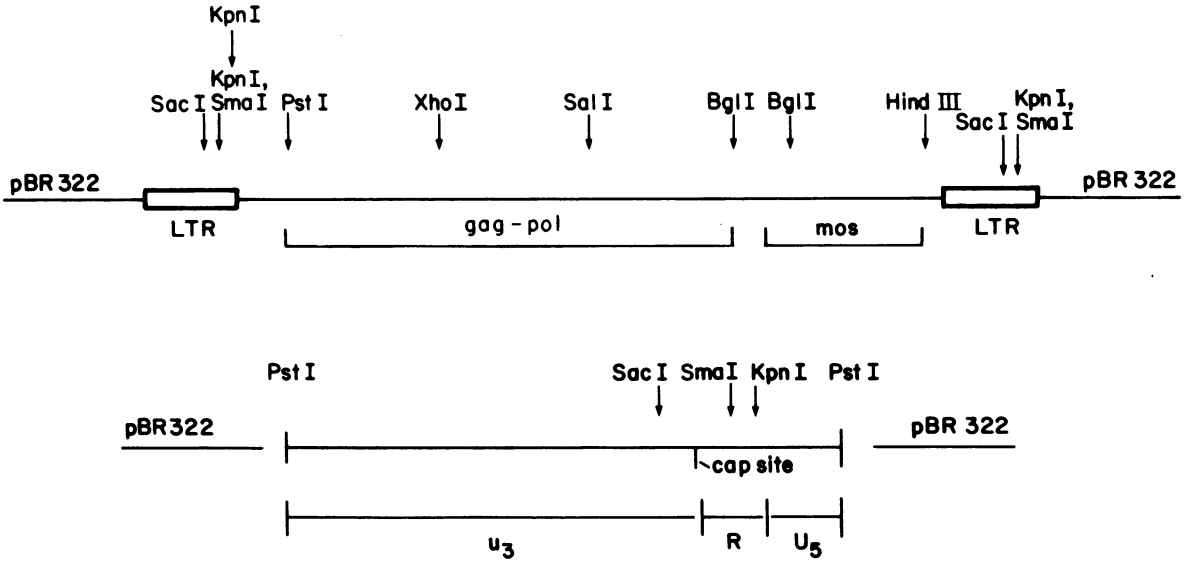

FIG. 1. Restriction endonuclease map of the cloned DNAs used in the present study. (*Upper*) The entire genome of Mo-MuSV was cloned into plasmid pBR322; the resultant plasmid is called p101. (*Lower*) The LTR region of Mo-MuSV was cloned into pBR322 and the resultant plasmid is called p600 (13).

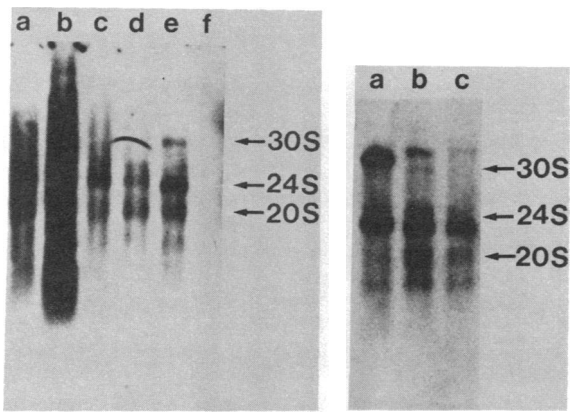

FIG. 2. RNA blot analysis showing hybridization of the LTR probe to poly(A)$^+$RNA from normal, transformed, and drug-induced C3H 10T½ cells. Size markers are given in the margins. (*Left*) The gels contained poly(A)$^+$RNAs from the following cell lines: a, C3H 10T½ JL-2; b, C3H 10T½ CB-2; c, C3H 10T½ JL-1; d, C3H 10T½ CB-1; e, C3H 10T½ JL-3; and f, normal C3H 10T½. For a description of these cell lines see Table 1. (*Right*) The gels contained poly(A)$^+$RNAs from the following cell lines: a, C3H 10T½ UR-1; b, C3H 10T½ cells treated with 5-azacytidine; and c, C3H 10T½ cells treated with BrdUrd. For the studies involving drug treatment, C3H 10T½ cells were seeded at 5×10^5/10-cm plate and 24 hr later they were exposed to 3 μM 5-azacytidine or BrdUrd at 50 μg/ml for 48 hr prior to extraction of the RNA.

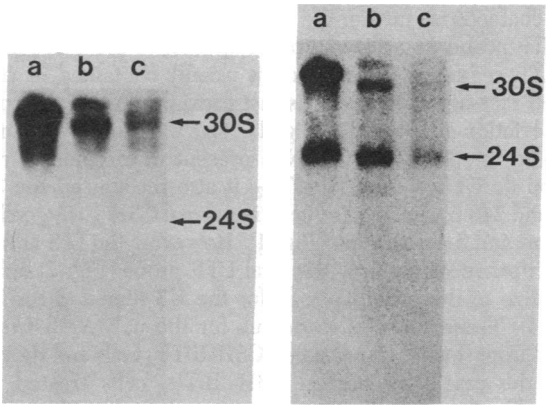

FIG. 3. Hybridization of the *gag–pol* probe (*Left*) and the *env* probe (*Right*) to poly(A)$^+$RNA from transformed and drug-treated murine cells. The sources of the poly(A)$^+$RNAs were: a, C3H 10T½ UR-1; b, C3H 10T½ treated with 5-azacytidine; and c, C3H 10T½ treated with BrdUrd. Cells were exposed to 5-azacytidine and BrdUrd and the poly(A)$^+$RNA fraction was prepared and analyzed as in Fig. 2.

LTR sequences, we extracted the poly(A)$^+$RNA from these cells, separated it by gel electrophoresis, and then, after blotting to nitrocellulose, hybridized the RNA with a ^{32}P-labeled DNA probe for LTR sequences. With the poly(A)$^+$RNA from normal C3H 10T½ cells there was negligible hybridization to the LTR probe (Fig. 2 *Left*, lane f). On the other hand, the poly(A)$^+$RNA from five different transformed C3H 10T½ cell lines (lanes a–e) showed appreciable hybridization to this probe. At least five distinct poly(A)$^+$RNA species, ranging from about 38 S to 18 S, were detected in the transformed lines. We have analyzed a total of eight transformed cell lines that were originally derived from normal C3H 10T½ after exposure to chemical carcinogens, UV light, or x-rays and all of these displayed RNAs homologous to the LTR probe, yielding profiles similar to those shown in Fig. 2 and Table 1. On the other hand with both an early- and a late-passage clone of normal C3H 10T½ cells there was always undetectable or only slight hybridization to this probe.

Induction of LTR-Containing Transcripts by BrdUrd or 5-Azacytidine. We next asked whether similar transcripts could be induced in normal C3H 10T½ by two compounds that have the capacity to modulate cellular differentiation and can also induce the production of endogenous murine leukemia viruses—i.e., BrdUrd, a halogenated pyrimidine (23), and 5-azacytidine, a drug that inhibits DNA methylation (24). We found that exposure of normal C3H 10T½ cells to either of these drugs for 48 hr induced the expression of a series of poly(A)$^+$RNA that were homologous to the LTR probe (Fig. 2 *Right*). The induced RNAs were similar in size to those found in the transformed C3H 10T½ cell lines, except that the normal cells exposed to either BrdUrd or 5-azacytidine contained an additional RNA species of 38 S. Because exposure of normal C3H 10T½ cells to BrdUrd or 5-azacytidine led to a rapid induction at the population level of these LTR-containing transcripts, it seems likely that what is unusual in the transformed cells is the constitutive expression of these transcripts rather than the presence of LTR-containing DNA sequences that are unique to the transformed cells. Because this conclusion is based only on sizing techniques, a more detailed analysis will be required to establish the exact relationship between these transcripts.

Mapping of LTR-Containing Transcripts. The poly(A)$^+$RNA transcripts detected with the LTR probe in the above studies could originate from endogenous murine retrovirus genomes or from the expression of host genes unrelated to the retroviruses but flanked by LTR sequences. Therefore, we performed a set of experiments to determine whether these transcripts contained, in addition to LTR-like sequences, sequences homologous to the known retrovirus genes *gag*, *pol*, and *env* and the U3 region of the LTR sequence (see Fig. 1).

Fig. 3 *Left* shows the DNA·RNA hybridization profile obtained when a probe for the *gag–pol* region of the murine retroviral genome was hybridized to the poly(A)$^+$RNA of carcinogen-transformed C3H 10T½ cell lines. Although this probe did hybridize to high molecular weight RNAs (30–38 S), there was no detectable hybridization to the lower molecular weight RNAs (18–24 S) detected with the LTR probe. The probe to the *env* region of the murine retroviral genome also hybridized to RNAs of about 30–38 S and, in addition, to an RNA of about 24 S, but it did not hybridize to the 18–20S RNAs detected with the LTR probe in transformed C3H 10T½ cells (Fig. 3 *Right*). Similar findings were obtained with the other transformed cell lines (Table 2). No significant hybridization was detected with the *gag*, *pol*, or *env* probe and the poly(A)$^+$RNA from normal C3H 10T½ cells. These results suggest that the higher molecular weight RNAs (30–38 S) present in transformed C3H 10T½ cells are transcribed from an endogenous retroviral genome(s). The 24S RNA may represent a mRNA for the viral envelope glycoprotein because it hybridized to the LTR and *env* probes but not to the *gag–pol* probe.

Table 2. Summary of DNA·RNA hybridization analysis

	LTR			*gag–pol*			*env*			3		
Source of RNA	a	b	c	a	b	c	a	b	c	a	b	c
C3H 10T½ normal	−	−	−	−	−	−	−	−	−	−	−	−
C3H 10T½ JL-2	+	+	+	+	−	−	+	+	−	+	+	−
C3H 10T½ CB-1	+	+	+	+	−	−	+	+	−	+	+	−
C3H 10T½ UR-1	+	+	+	+	−	−	+	+	−	+	+	−
C3H 10T½ UR-2	+	+	+	+	−	−	+	+	−	+	+	−

Letters indicate sizes of RNA that hybridize with the respective probe: "a," about 30–38 S; "b," about 24 S; "c," about 18–20 S. For typical profiles, see Fig. 2.

To characterize further the spectrum of RNAs detected with the LTR probe we utilized a probe specific to the U3 region of the LTR sequence. This region is usually contained in virus-related messages because it is just proximal to the viral polyadenylylation site (ref. 13; Fig. 1). The U3 probe was homologous to the 30S and 24S RNAs present in carcinogen-transformed derivative of C3H 10T½; it also recognized the 38S, 30S, and 24S transcripts induced in normal C3H 10T½ cells by BrdUrd and 5-azacytidine (Fig. 4). However, the 20S and 18S transcripts recognized by the total LTR probe (Fig. 2) did not hybridize to the probe specific for the U3 region of the LTR (Fig. 4). These findings were true for the poly(A)$^+$RNAs obtained from several transformed C3H 10T½ cells and they also were the case with normal C3H 10T½ cells treated with BrdUrd or 5-azacytidine.

Taken together, these results suggest that, although the 24–38S RNAs detected in transformed C3H 10T½ cells and in normal 10T½ cells exposed to BrdUrd or 5-azacytidine appear to be transcribed from an endogenous retrovirus genome, this may not be the case for the lower molecular weight (18–20S) RNAs detected in these cells with the LTR probe.

Endogenous LTR Sequences in the Genome of C3H 10T½ Cells. Normal mouse DNA is known to contain several endogenous murine leukemia virus (MuLV) genomes which can cross-hybridize to probes obtained from various strains of murine retroviruses. To determine whether it was feasible to assign a putative DNA template for the transcription of the 18S and 20S RNAs, we hybridized LTR probes to restriction endonuclease-cleaved mouse DNA.

Fig. 5 shows the results obtained by Southern blot analysis when the DNA from normal C3H 10T½ cells or from the transformed cell line C3H 10T½ JL-3 was digested with the restriction enzymes *Bgl* I and *Bgl* II, separated by gel electrophoresis, and hybridized to the ^{32}P-labeled LTR probe. There were at least 30 (and probably more) DNA fragments present in both the normal and transformed cells that were homologous to this probe. It was not possible to demonstrate reproducible differences between the normal and transformed cell lines, but the profiles are extremely complex and this requires further study. Because it was possible that the LTR sequences expressed in the transformed cells might be less methylated than those that were not expressed, we performed double-restriction digestion with *Bgl* I or *Bgl* II and then *Hpa* II or *Msp* I as isoschizomers capable of detecting differences in DNA methylation (25). If the transcribed LTR DNA sequence(s) were hypomethylated, they should be cleaved more extensively by *Hpa* II and this would be reflected in the gel profiles with the LTR probe. However, the restriction enzyme profiles obtained in this analysis revealed such a complex profile that it was not possible to resolve a fragment showing unique sensitivity to *Hpa* II (data not shown here).

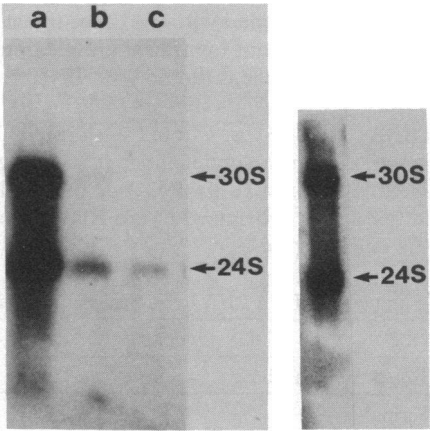

FIG. 4. (*Left*) Hybridization of the U3 probe to poly(A)$^+$RNA from transformed and drug-treated murine cells. The sources of the poly(A)$^+$RNAs were: a, C3H 10T½ UR-1; b, C3H 10T½ treated with 5-azacytidine; and c, C3H 10T½ treated with BrdUrd. (*Right*) C3H 10T½ JL-2 treated with BrdUrd. Cells were exposed to 5-azacytidine and BrdUrd and the poly(A)$^+$RNA was prepared and analyzed as in Fig. 2.

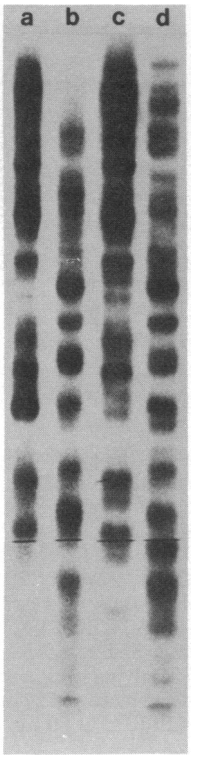

FIG. 5. Southern blot analysis of restriction enzyme-digested chromosomal DNA from C3H 10T½ and C3H 10T½ JL-1. Lanes: a, C3H 10T½ DNA digested with *Bgl* I; b, C3H 10T½ DNA digested with *Bgl* II; c, C3H 10T½ JL-1 DNA digested with *Bgl* I; d, C3H 10T½ JL-1 DNA digested with *Bgl* II.

DISCUSSION

The major finding in this study is that eight of eight C3H 10T½ cell lines transformed by chemical carcinogens, UV light, or x-rays contain a series of poly(A)$^+$RNAs that range in size from about 18 S to 38 S and that contain sequences homologous to the LTR region of a murine retrovirus, Mo-MuSV. We have also found that, although these RNAs are absent from (or barely detectable in) the normal parental C3H 10T½ cell line, a similar set of poly(A)$^+$RNAs is induced when the normal cells are exposed to BrdUrd or 5-azacytidine.

Previous studies have indicated that normal C3H 10T½ cells do not produce leukemia virus particles spontaneously; nor do C3H 10T½ cell lines that have been transformed by chemicals or radiation (26, 27). The transformed cell lines are readily induced to produce such particles when exposed to IdUrd and they may also produce virus particles after prolonged serial passage (23, 26). Two of the transformed cell lines that we found to be positive for the presence of the above-described RNAs, C3H 10T½ UR-1 and UR-2 (Table 1), have been specifically assayed for spontaneous production of leukemia virus particles and found to be negative (Ulf Rapp, personal communication). Thus, the presence of these RNAs does not simply reflect overt

production of leukemia virus. Nevertheless, the 30–38S and the 24S RNAs that we detected in the transformed C3H 10T½ cell lines and in normal C3H 10T½ exposed to BrdUrd or 5-azacytidine are related to the structural genes of murine retroviruses because they hybridize to probes for the *gag–pol* or *env* sequences of MuLVs.

On the other hand, the 20S and 18S RNAs that we have detected with the LTR probe have no homology with our *gag–pol* or *env* probes or with the U3 region of the LTR sequence. It seems likely that transcripts arising from endogenous MuLV genomes would be recognized by either the *gag–pol* or *env* probes used in the present studies. There is evidence that the *gag* and *pol* genes of the various MuLVs are well conserved (28–30). Because the alleles of the *env* genes are more heterogenous, this aspect requires further study. Some of the transcripts that we have detected may reflect the expression of non-virus-related host sequences that utilize endogenous LTR sequences as promoters. Southern blot analyses indicate that the genome of normal C3H 10T½ cells contains more than 30 copies of LTR sequences (Fig. 5). The transformation of C3H 10T½ cells by chemicals or radiation need not cause rearrangement of LTR sequences with respect to host genes, because we found that similar 20S and 18S transcripts were rapidly induced at the population level when normal cells were treated with BrdUrd or 5-azacytidine. What appears to be unusual in the transformed cells is the constitutive expression of these sequences.

Halogenated pyrimidines are well-known inducers of endogenous MuLVs (23, 31). Recent experiments suggest that these compounds do not have to be incorporated into proviral DNA sequences to induce virus production (32). By contrast, it appears that 5-azacytidine induces expression of endogenous leukemia virus by direct incorporation into the DNA of the promoter region of the provirus (33). We assume that the induction of synthesis of RNAs containing LTR sequences by BrdUrd and 5-azacytidine in normal C3H 10T½ cells, described here, occurs by analogous mechanisms. It has been suggested that the organization or "phasing" of nucleosomes in the chromatin structure may influence gene expression (34, 35). If this is the case, then altered nucleosome phasing in transformed cells might favor the constitutive transcription of regions of the genome that contain LTR sequences.

Finally, we must stress that our results do not indicate whether or not the constitutive expression of RNAs containing LTR sequences in C3H 10T½ cells transformed by chemical carcinogens or radiation plays a critical role in the establishment or maintenance of the transformed state in these cells. The facts that we have observed the expression of these RNAs in all of the eight transformed cell lines that we have examined and that LTR sequences play a key role in the mechanism of cell transformation by an avian leukosis virus (11) suggest that our findings are relevant to the process of cell transformation. Nevertheless, our data do not exclude the possibility that the expression of these RNAs in transformed C3H 10T½ cells reflects events secondary to the transformation process. In any case, these highly specific markers of constitutive gene activation in transformed murine cells may provide sensitive molecular probes for further studies on the mechanism of neoplastic transformation.

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