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EXPRESSION OF LONG TERMINAL REPEAT (LTR) SEQUENCES IN CARCINOGEN-INDUCED MURINE SKIN CARCINOMAS

Gerard M. Housey[1], Paul Kirschmeier[1], Seymour J. Garte[2], Fred Burns[2], Walter Troll[2] and I. Bernard Weinstein[2]

Cancer Center/Institute of Cancer Research[1], Columbia University, New York, N.Y. 10032

Department of Environmental Medicine[2], New York University, New York, N.Y. 10016

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RNA sequences homologous to the Long Terminal Repeat (LTR) sequence of Moloney Murine Leukemia Virus proviral DNA are expressed in murine squamous cell carcinomas of the skin induced by chemical carcinogens. These transcripts range in size from 8.2 to less than 2.4 kb but their size profile varies between individual tumors. These RNAs are not detected in the poly A^+ RNA fraction obtained from the epidermis of control mice or carcinogen induced skin papillomas. The poly A^+ RNAs from the livers and spleens of some of the mice with skin carcinomas also revealed LTR related sequences, whereas these RNAs were not detected in the livers and spleens of control mice or of carcinogen-treated mice that did not develop carcinomas. Thus, chemical carcinogenesis in mouse skin is associated with constitutive expression of endogenous retrovirus related sequences in the carcinomas as well as in certain apparently normal host tissues.

Malignancies induced by chemical carcinogens are associated with complex changes in gene expression in the target cell (1,2). These changes may be qualitative; e.g. new alleles of host genes may be generated by mutation or gross rearrangements in DNA sequences, or they may be quantitative, due to gene amplification or stable alterations in gene expression via mechanisms that are not well defined (for review see 1-3). The mouse genome contains multiple DNA sequences that are homologous to the Long Terminal Repeat (LTR) sequence of Moloney Murine Leukemia Virus (MoMuLV) proviral DNA (4). These sequences are often found juxtaposed to the structural genes of endogenous retroviruses, but they can also occur in the absence of flanking retroviral information (5,6). The LTR sequences are of particular interest because they contain signals related to the control of gene expression including a TATAAA box, the CCAATC sequence and

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enhancer sequences (7). They can also potentiate the activity of other promoters even when the LTR itself is located 3' to an adjacent gene (8). In addition, the LTRs have structural similarities to the transposable elements of bacteria, yeast, and Drosophila (9). We have previously demonstrated that C3H 10T1/2 mouse fibroblasts transformed by either chemical carcinogens or radiation express a set of polyadenylated RNAs that contain sequences homologous to the LTR sequences of MoMuLV (10). These RNAs were not detected, or were present at very low levels, in the corresponding untransformed cells. The LTR positive RNAs that were detected in the transformed cells consisted of 3 major size classes: an 8.2kb class that probably represents transcripts of a complete endogenous retrovirus proviral genome (containing gag, pol and env genes), RNA species of about 3.8kb that contain env homologous sequences, and sequences less than 3.0kb that did not share homology to our gag, pol, or env probes, or to the U3 portion of our LTR probe (10). Since these findings were obtained in a cell culture system it was not clear whether they were relevant to carcinogenesis in the intact animal. The present study was undertaken, therefore, to determine whether primary skin carcinomas induced in mice by chemical carcinogens also express similar species of poly A^+ RNAs.

Materials and Methods

Tumor Induction - Tumors were induced in HA/ICR mice as follows. The hair was clipped from the dorsal skin and the chemicals dimethylbenz(a)-anthracene (DMBA) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were applied topically by pipette in 0.2ml acetone. One group received a single application of DMBA (25μg) followed by TPA (5μg) thrice weekly. A second group received weekly applications of DMBA (5μg) alone. Tumors were excised 208 days after the start of the treatments and a sample of each tumor was taken for histological classification. At the same time livers and spleens of the treated animals that had either carcinomas, papillomas, or no detectable skin lesions were also collected. Control tissues (epidermis, livers and spleens) were obtained from age-matched untreated HA/ICR mice. The epidermis was isolated as previously described (11).

RNA Isolation - RNA was extracted from the above murine tissues by the guanidinium thiocyanate method and the poly A^+ fraction isolated by oligo(dT) cellulose chromatography (12). PolyA^+ RNA was denatured, gel electrophoresed, blotted and hybridized to ^{32}P nick-translated probes as previously described (12, 13-15).

Preparation of Probes - DNA fragments containing either the LTR sequence or env sequence of murine C type retroviruses were purified and nick-translated as previously described (10).

Results

We examined polyA$^+$ RNAs from various tissues of HA/ICR mice whose skin had been treated repeatedly with DMBA alone or with DMBA once followed by repeated applications of TPA, as well as tissues from control mice, for species that are homologous to the LTR of MoMuLV proviral DNA. These RNA samples were electrophoresed, blotted onto nitrocellulose and hybridized to the LTR probe as described in Materials and Methods.

Representative blot hybridizations are shown in Figure 1 and the results of all of these studies are summarized in Table 1. PolyA$^+$ RNAs from all of the 6 squamous cell carcinomas contained species that hybridized to the LTR probe, with variations in abundance of individual classes of RNAs among the tumors. Five of these tumors were induced by the DMBA plus TPA protocol and one by repeated doses of DMBA alone. The LTR probe hybridized to an 8.2kb species present in 3 of the carcinomas. All of the carcinomas contained a 3.8 kb species as well as a heterogeneous group of RNAs less than 3.0 kb that hybridized to the LTR probe.

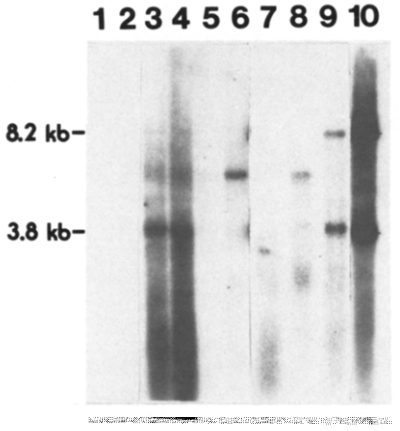

Figure 1: PolyA$^+$ RNA was isolated from various tissues (see Table 1), separated in 0.8% agarose gels containing 6% formaldehyde, transferred to nitrocellulose filters and hybridized to the nick-translated LTR probe, as described in Materials and Methods. Lane 1: RNA from normal epidermis of an untreated HA/ICR mouse. Lane 2: RNA from normal liver of an untreated HA/ICR mouse. Lane 3: RNA from the liver of animal #1. Lane 4: RNA from the skin carcinoma of animal #1. Lane 5: RNA from the skin carcinoma of animal #2. Lane 6: RNA from the skin carcinoma of animal #3. Lane 7: RNA from the spleen of animal #26. Lane 8: RNA from the liver of animal #26. Lane 9: RNA from the skin carcinoma of animal #26. Lane 10: RNA from a methylcholanthrene transformed C3H 10T1/2 cell line (positive control). Lane 5 displayed faint bands at 6.0, 3.8 and <3.0 kb but these are not well visualized in this figure.

TABLE 1

TISSUE	TREATMENT			LTR-HOMOLOGOUS POLY A^+ RNAs (kb) DETECTED:			
	ANIMAL NUMBER	GROUP NUMBER	METHOD	8.2	6.0	3.8	>3.0
Liver	C1	Control	Age-Matched	-	-	-	-
Spleen	C1	Control	Controls:	-	-	-	-
Liver	C2	Control	No Treatment	-	-	-	-
Spleen	C2	Control		-	-	-	-
Epidermis	C3	Control		-	-	-	-
Liver	9	1	DMBA once,	-	-	-	-
Spleen	9	1	then TPA	-	-	-	-
Papilloma	9	1	three times	-	-	-	-
Liver	15	1	per week	-	-	-	-
Spleen	15	1		-	-	-	-
Papilloma	15	1		-	-	-	-
Liver	22	1		-	-	-	-
Spleen	22	1		-	-	-	-
Liver	1	2		+	+	+	+
Carcinoma	1	2		+	+	+	+
Carcinoma	2	2		-	+	+	+
Carcinoma	3	2	DMBA once,	-	+	+	+
Liver	26	2	then TPA	-	+	+	+
Spleen	26	2	three times	-	-	-	+
Carcinoma	26	2	per week	+	-	+	+
Liver	39	2		-	-	-	-
Spleen	39	2		-	-	-	-
Papilloma	39	2		-	-	-	-
Carcinoma	39	2		+	-	+	+
Liver	48	3	DMBA three	-	-	-	-
Spleen	48	3	times per	-	+	+	+
Carcinoma	48	3	week	-	+	+	+

Summary of LTR-homologous poly A^+ RNA species detected by blot-hybridization in various tissues of HA/ICR mice. Mice were exposed to DMBA plus TPA or DMBA alone, tissues were isolated at 15-18 months, the poly A^+ RNA fraction was isolated and analyzed by blot hybridization using a probe to the LTR sequence of MoMuLV, as described in Materials and Methods. Mice were divided into 4 groups: Control, age-matched controls that received no treatment; Group 1, animals (#9, 15 and 22) that received DMBA plus TPA and developed papillomas and carcinomas, Group 3, animal (#48) that received only multiple doses of DMBA, which induced a carcinoma in one animal (#48). RNA species homologous to the LTR probe detected by blot hybridization (see Figure 1) were classified according to size in kb. The symbol (-) indicates absence of this size class and (+) designates its presence in the indicated tissue.

Carcinomas from mice numbered 1,2 and 3 also contained a 6.0kb RNA recognized by the LTR probe, which was particularly prominent in the carcinoma from animal number 3 (Figure 1). The 6.0 and less than 3.0 kb

RNAs did not hybridize to the env probe (data not shown here). In contrast to the positive findings with the carcinomas, we could not detect LTR positive transcripts with equivalent amounts of polyA$^+$ RNA extracted from the untreated epidermis of age-matched control mice.

We were also interested in determining whether LTR related polyA$^+$ RNAs were present in other tissues of mice that developed skin carcinomas, or in tissues of mice that received the carcinogen and promoter treatment but did not develop skin carcinomas. We were surprised to find that in 2 of 4 instances examined the livers of mice with skin carcinomas also contained polyA$^+$ RNAs recognized by our LTR probe. Furthermore, two of the three spleens from mice with carcinomas which we examined also contained these RNAs. As in the carcinomas, these RNAs ranged in size from 8.2 to less than 3.0 kb. In the case of animal #26 the major species detected in the liver was 6.0 kb, although this species was not detected in the carcinoma or the spleen of the same animal. We did not detect LTR related polyA$^+$ RNAs in livers or spleens of mice that did not have skin carcinomas. The latter mice were age-matched and exposed to the identical carcinogen-promotor regimen in the same experiment as the mice that developed skin carcinomas. None of the papillomas that we tested contained LTR related polyA$^+$ RNAs. The papillomas were isolated from two sources: treated mice that developed skin papillomas but not carcinomas (#9 and #15) and a mouse that developed both skin carcinomas and papillomas (#39). Age matched control untreated mice from the HA/ICR strain contained no LTR related polyA$^+$ RNA species in their livers, spleens, or epidermis. We also examined polyA$^+$ RNAs from livers and spleens of age-matched untreated Balb/c, C3H/HeJ and SENCAR mice and found no LTR related species (data not shown). Despite the positive findings obtained with the livers and/or spleens of animals 1, 26 and 48 none of these tissues displayed gross or histologic evidence of primary or metastatic tumor.

Discussion

Previously, we showed that a group of LTR related transcripts are present in radiation or carcinogen transformed C3H/10T 1/2 cells but not in the parental untransformed cell line (10). The results of the present study indicate that similar transcripts can appear in primary skin carcinomas induced in mice by chemical agents. Thus, our earlier findings are not confined to malignant transformation induced in cell culture. The LTRs of endogenous murine leukemia proviruses contain regulatory elements that control transcription (4). Many of these elements are linked to structural genes of retroviruses and form a transcriptional unit capable of generating infectious ecotropic, xenotropic or mink-cell focus forming virus (4-7). Other endogenous murine LTRs are linked to viral structural genes that have not been recovered as infectious viruses (5). These LTRs, which have weak promoting activity, can contain a 190bp insert 48bps upstream from the CCAATC box and a 14bp duplication near the 5' end of the direct repeat enhancer sequence. Still other endogenous murine LTRs are not flanked by virally related sequences (5). The LTR probe used in the present studies does not distinguish transcripts promoted by these different classes of endogenous LTRs. Based on previous studies (10), we assume that the 8.2kb transcripts detected in the skin carcinomas represent full length transcripts of either an ecotropic or xenotropic endogenous provirus, and the 3.8kb species represents the corresponding spliced *env* transcripts. The RNAs less than 3.0 kb may include the low molecular weight *env* transcript described by Boccara et al (16) and found in radiation induced T-cell leukemias of Balb/C mice, and RNA transcripts from LTR sequences that flank various cellular genes. The 6.0kb species found in four of the carcinomas, in the liver in animal #26 and in the spleen of animal #48 might be transcribed from an unusual endogenous proviral DNA sequence. Indeed Khan et al. (5-6) have cloned several endogeneous proviral DNA sequences that could generate 6.0kb transcripts. In addition to the RNAs that are homologous to the MoMuLV LTR, we have

recently obtained evidence that poly A^+ RNAs homologous to the LTR sequence of intracisternal A particle proviral DNA are also expressed at high levels in murine skin carcinomas and papillomas (17) (unpublished studies).

We do not know whether any of the above described RNAs contribute to the transformed phenotype. Thus far, we have not detected RNAs homologous to the LTR of MoMuLV in skin papillomas but this requires further study. If the expression of these RNAs is specifically associated with carcinomas then they may be a marker of tumor progression rather than initiation or promotion. The presence of LTR homologous RNAs in the livers or spleens of some of the mice bearing skin carcinomas could be due to: 1) metastases from the skin carcinoma, 2) a direct effect of the carcinogen and/or promoter on these tissues following absorption from the skin, 3) the spread of retrovirus particles from the tumor to these tissues, or 4) the production of a factor(s) by the skin carcinoma that induces the expression of these RNAs at distant sites. Our histologic studies rule out the first possibility. Further studies are required to evaluate the other possible mechanisms. Although the HA/ICR mice used in this study exhibit approximately a 10% incidence of leukemia at age 18 to 24 months (18 and unpublished studies), the animals in this study were 6 to 7 months old at sacrifice and displayed no evidence of leukemia.

Regardless of their significance, the expression of these LTR homologous RNAs provides further evidence that carcinogenesis is often associated not only with oncogene activation but also with gross disturbances in the expression of endogenous retrovirus related sequences (1).

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References

1. Weinstein, I.B., Gattoni-Celli, S., Kirschmeier, P., Lambert, M., Hsiao, W.-L., Backer, J. and Jeffrey, A. (1984) In: CANCER CELLS 1/The Transformed Phenotype, Levine, A., Vande Woude, G., Watson, J.D. and Topp, W.C. (eds.) Cold Spring Harbor Laboratory, 229-237.
2. Cairns, J. (1981) Nature 289, 353-357.
3. Bishop, J.M. (1983) Cell 32: 1018-1020.
4. Coffin, J. (1982) RNA Tumor Viruses Weiss, R., Teich, N., Varmus, H. and Coffin, J. (eds.) Cold Spring Harbor Laboratory, 1109-1204.
5. Khan, A.S., Wallace P. Rowe and Malcolm A. Martin. (1982) J. Virol. 44 625-636.
6. Khan, A.S. and Martin, M.A. (1983) Proc. Natl. Acad. Sci., USA 80, 2699-2703.
7. Dhar, R., Mc.Clements, W., Enquist, L.W. and Vande Woude, G.F. (1980) Proc. Natl. Acad. Sci. USA 77 3937-3941.
8. Blair, D.G., Oskarson, M., Wood, T.G., McClements, W.L., Fischinger, P.J. and Vande Woude, G.F. (1981) Science 212, 911-943.
9. Finnegan, D.J. (1983) Nature 302, 105-108.
10. Kirschmeier, P., Gattoni-Celli, S., Dina, D. and Weinstein, I.B. (1982) Proc. Natl. Acad. Sci. USA 79 2773-2777.
11. Belman, S., Troll, W. and Garte, S.J. (1978) Cancer Res. 38, 2978-2982.
12. Chirgwin, J.M. Przybyla, A.C. MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18 5294-5299.
13. Rave, W., Crkuenjakov, R. and Boedtker, H. (1979) Nucleic Acids Res. 6 3559-3567.
14. Thomas, P.J. (1980) Proc. Natl. Acad. Aci. USA 77 5201-5205.
15. Wahl, G.M., Stern, M. and Stark. G.R. (1974) Proc. Nat. Acad. Sci. USA 76 3683-3687.
16. Boccara, M., Souyri, M., Magarian, C., Stavnezer, E. and Fleissner, E. (1983) J. Virol., 48, 102-109.
17. Augenlicht, L.H., Kobrin, D., Pavlouee, A. and Roystom, M.L. (1984) J. Biol. Chem. 259: 1842-1847.
18. Prejear, J.D.,Peckham, J.L., Kasey, A.E., Griswold, D.P., Weisburger, E.K. and Weisburger, J.H. (1973) Can. Res. 33 2768-2773.