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Expression of Xenotropic-Like *env* RNA Sequences in Normal DBA/2 and NZB Mouse Tissues

ELSBETH J. LEE,†* JOSEPH KAMINCHIK,‡ AND W. DAVID HANKINS‡

Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20205

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Using a DNA probe prepared from cloned *env* gene sequences of Friend spleen focus-forming viruses, we detected the differential expression of multiple RNA species in uninfected DBA/2 fibroblasts and in various tissues from adult DBA/2 and NZB mice. The size of the major RNA species detected was estimated to be 24S. The 24S RNA species was enriched in polyadenylate-selected preparations and thus may represent a message for endogenous viral envelope glycoproteins. The viral origin of the 24S RNA was further characterized by its hybridization to DNA probes containing the long terminal repeats of Harvey murine sarcoma virus, mouse mammary tumor virus, or the U_3 region of an endogenous xenotropic virus. Although the *env*-related 24S RNA failed to react with either Harvey murine sarcoma virus or mouse mammary tumor virus long terminal repeat probes, it hybridized well with the xenotropic virus long terminal repeat probe. Therefore, it is likely that the RNA detected with the Friend spleen focus-forming virus *env* probe reflects transcription of xenotropic envelope sequences in uninfected tissues. Our finding that the level of 24S RNA varied in different organs indicated some tissue specificity in the expression of these xenotropic-like *env* proteins.

The envelope (*env*) glycoproteins, gp70s, are the primary determinants of the host ranges of murine type C viruses and have been classified as ecotropic (originating in and infecting mice), xenotropic (originating in mice but only infecting other species), and amphotropic (infecting mice and other species). In addition, free gp70 has been found in murine serum (10) and in the male genital tract of certain strains of mice (6). Comparing tryptic digests of serum, seminal fluid, and viral gp70s, Elder et al. (8) devised a classification for gp70 on the basis of immunological and structural properties and demonstrated that gp70 in serum is more closely related to the *env* protein of NZB xenotropic virus than to that of ecotropic or amphotrophic viruses.

This relationship may be of significance, since various studies have associated gp70 with such poorly understood maladies as autoimmune and graft-versus-host diseases (1, 13, 14, 24). In addition, the leukemia-inducing potential of both the polycythemia- and anemia-inducing strains of Friend spleen focus-forming virus (SFFV) has been shown to reside in or near the envelope-coding region of the viral genome (16–18).

The precise origin of these pathogenic *env* sequences is not known. However, the available evidence (4, 9, 12, 20, 23) suggests that the erythroleukemia-inducing SFFV may be a modified member of the mink cell focus-forming (MCF) viruses which arise by recombination between exogenous ecotropic viruses and endogenous (nonecotropic) virus-like sequences. Molecular probes prepared from cloned SFFV have greatly facilitated attempts to identify sequences in normal cells that are similar to those in SFFV and MCF virus. For example, a subcloned fragment between the *Bam*HI and *Eco*RI restriction sites of the SFFV *env* region (B-E probe) did not hybridize with Friend helper virus but did hybridize well with SFFV DNA, several MCF viral DNAs, and, with somewhat lower affinity, xenotropic viral DNAs. Using the B-E probe to analyze uninfected, endonuclease-restricted murine DNA, Chattopadhyay et al. (4) identified two families of endogenous xenotropic-like sequences from which the SFFV envelope could have been derived. Thus, these authors were able to distinguish between endogenous MCF and endogenous xenotropic sequences. For the purpose of this report, we will refer to these sequences collectively as "xenotropic-related" *env* sequences, since these putative families cannot be readily distinguished by hybridization techniques alone.

Since the xenotropic-related gp70s have been implicated in normal and abnormal physiology, we and others have sought to identify, in normal, uninfected mouse tissues, protein and RNA components that might be related to these endogenous envelope sequences (2, 6, 10). In the present study, we used the SFFV B-E probe to analyze RNAs from a variety of uninfected cell types from DBA/2 and NZB mice.

We first examined RNAs extracted from uninfected DBA/2 and NIH 3T3 fibroblasts. Cultured DBA/2 and NIH 3T3 fibroblasts were grown under standard tissue culture conditions. Methods for extraction and analysis of RNA were similar to those described by Maniatis et al. (19) with some modifications. Cell pellets were lysed with a solution containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; pH 8), 3 mM $MgCl_2$, 0.5% Triton X-100, 0.25 M sucrose, and 0.15 M NaCl. Supernatants containing total cytoplasmic RNA were recovered, adjusted to a final concentration containing 0.1 M Tris (pH 9), 0.5% sodium dodecyl sulfate (SDS), and 0.1 mM EDTA, extracted with phenolchloroform (1:1), and ethanol precipitated. For subsequent studies in this report, total RNA was extracted from freshly frozen mouse tissues (−70°C) by homogenizing tissues in a Waring blender containing 0.1 M Tris (pH 9), 0.5% SDS, and 10 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories), an RNase inhibitor. The homogenates were extracted and precipitated as described above; they were then treated with 3 M sodium acetate to eliminate contaminating DNA. Polyadenylate-enriched DBA/2 fibroblast RNA, after purification by passage through oligodeoxythymidylate-cellulose (P. L. Biochemicals) (19), represented ca. 1 to 5% of the total cytoplasmic RNA. RNA samples were

* Corresponding author.

† Present address: Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

‡ Present address: Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD 20205.

electrophoresed through 1% agarose and transferred onto nitrocellulose as described earlier (19). Blots were prehybridized for 2 h at 42°C in buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt solution, 0.1% sodium dodecyl sulfate and 0.1 mg of boiled calf thymus DNA per ml of buffer; the blots were then hybridized to nick-translated DNA probes for 20 h in a solution containing 50% formamide, 5× SSC, 1× Denhardt solution, 0.01% sodium dodecyl sulfate, and 0.5 mg of boiled DNA per ml of buffer. After hybridization, blots were washed and exposed to Kodak X-Omat AR film at −70°C (19).

The SFFV *env*-specific DNA probe used in this study was a *Bam*HI-*Eco*RI-restricted fragment, designated the B-E probe, that was purified from a plasmid containing a molecularly cloned subgenomic *env* fragment of the polycythemia-inducing strain of SFFV (18). The Harvey murine sarcoma virus long terminal repeat (Ha-LTR) probe, a 0.6-kilobase permuted LTR fragment purified from an infectious clone of Harvey murine sarcoma virus and inserted into pBR322 (3), was provided by Esther Chang. The Harvey murine sarcoma virus was originally isolated from a rat infected with ecotropic Moloney murine leukemia virus, and it possessed LTRs identical to those of that virus (11, 22). Another probe was prepared from xenotropic LTR DNA. The *Pst*I-*Sma*I-cleaved DNA fragment, which contained the U_3 region and was ca. 30 base pairs into the R region of the LTR, was inserted into M13 (15) and was a gift of Arifa Kahn. The 1.3-kilobase permuted mouse mammary tumor virus long terminal repeat (MMTV-LTR) DNA fragment that was cloned into pBR322 (7), provided by Gordon Hager, was prepared as the MMTV-LTR probe.

When Northern analysis was performed with the B-E probe, a single RNA band of ca. 24S was detected in DBA/2 fibroblast RNA (Fig. 1A, lane c) but not in NIH 3T3 fibroblast RNA (data not shown). In this report, the single RNA species of ca. 24S will be referred to as 24S RNA.

Since the B-E probe was derived from the envelope region of SFFV, we considered it likely that the 24S RNA species represented endogenous envelope mRNA. To test this hypothesis, we selected for polyadenylate-containing RNA from DBA/2 cell extracts and hybridized this RNA to the B-E probe. The 24S RNA species that hybridized to the B-E probe was enriched in polyadenylate-selected DBA/2 fibroblast preparations, compared with the unselected RNA preparations (Fig. 1A, lanes h and c, respectively). Non-polyadenylated RNA was not capable of hybridizing to the B-E probe (Fig. 1A, lane i).

These results are consistent with the supposition that the 24S RNA species represents an envelope message of an endogenous provirus. However, both genomic bands (35S) and *env* bands (24S) were detected by the B-E probe in RNA from the infected cells, which served as positive controls in this experiment (Fig. 1A, lanes b and j). The absence or paucity of similar genomic bands (35S) in total or polyadenylate-selected RNA from uninfected DBA/2 cells (Fig. 1A, lanes c and h, respectively) led us to further investigate the viral origin of the 24S RNA species.

Toward this end, we sought other evidence that the RNA detected by the B-E probe was virus associated. Since an envelope message in virus-infected cells is spliced and remains associated with the LTR region, we hybridized the putative endogenous viral RNA to LTR probes prepared from exogenous (Harvey murine sarcoma virus), endogenous (xenotropic) viruses, and mouse mammary tumor viruses. Ha-LTR (Fig. 1B) and MMTV-LTR (data not shown) did not hybridize with RNA from uninfected DBA/2 tissues. However, the xenotropic LTR (xeno-LTR) probe detected RNA species which were indistinguishable from those recognized by the B-E probe. Thus, a major species was observed at 24S (Fig. 1C, lanes c and h). As with the B-E probe, a very small amount of hybridizable RNA was detected in the genomic region with the xeno-LTR probe, and this RNA required polyadenylate selection. Although the significance of the high ratio of envelope RNA to genomic RNA remains unclear, these LTR hybridization results suggest that the RNA species detected in uninfected tissues by the B-E probe may be of xenotropic viral origin. This conclusion is consistent with previous DNA hybridization studies (4, 23), which indicate that B-E sequences cross-hybridize with xenotropic viral sequences.

Although we did not expect the MMTV-LTR to cross-hybridize with xenotropic proviral sequences, the complete lack of hybridization of the Ha-LTR probe to these sequences was an unexpected result. The adequacy of the Ha-LTR probe was established by a control experiment (Fig. 1B, lane b) in which this probe clearly hybridized to both genomic and envelope RNAs of ecotropic virus. Our results, as well as sequence data from previous studies (5, 7, 15), indicate that cloned LTRs from ecotropic and xenotropic viruses appear to differ significantly in both their primary structure and the classes of viral RNA which they are capable of detecting. One practical implication of this finding is that multiple classes of LTR probes should be used to analyze tumor RNA for LTR-activated transcripts.

We next determined whether the expression of xenotropic-related RNA species was restricted to cultured DBA/2 fibroblasts. RNA was extracted from several DBA/2 mouse tissues and hybridized to the four probes previously used (B-E, Ha-LTR, xeno-LTR, and MMTV-LTR). As with the fibroblasts, no hybridizable RNA was detected in DBA/2 kidney, liver, muscle, or splenic tissues when RNA was hybridized to either the Ha-LTR (Fig. 1B, lanes d through g) or the MMTV-LTR probe (data not shown). The B-E and xeno-LTR probes both detected variable levels of 24S RNA in DBA/2 tissues. The highest level of 24S RNA was detected in the kidney, less was detected in the liver and spleen, and no xenotropic-related RNA was detected in femoral muscle tissue (Fig. 1A and C, lanes d through g). Therefore, these data suggest that xenotropic-related RNA is not unique to fibroblasts but also that there is some tissue-specific regulation of the expression of such sequences. The quantitative differences were not due to unequal RNA degradation, as each tissue sample was estimated to have equal quantities of rRNA (28S and 18S) upon visual examination under UV illumination of agarose gels stained with ethidium bromide. This visual examination also revealed a slight shift in the migration of the 28S and 18S rRNAs from tissue preparations extracted in the presence of the RNase inhibitor, vanadyl ribonucleoside complex. The shift in the 28S and 18S patterns consistently correlated with and probably explains the slight variation in the migration of the 24S RNA.

To further verify the specificity of the hybridization probes, we reacted the B-E, Ha-LTR, and xeno-LTR probes with RNA from various tissues of NZB mice (a strain known to release endogenous xenotropic virus). As in the DBA/2 tissues described above, no hybridization was observed with the Ha-LTR probe (Fig. 1B, lanes k through n), and an RNA species of ca. 24S was detected in all NZB tissues examined with the B-E probe (Fig. 1A, lanes k through n) and the xeno-LTR probe (Fig. 1C, lanes k through n). The xeno-LTR probe also detected several larger RNA species (ca. 30S, 33S, and 35S) in addition to the 24S RNA species. Since NZB mice release xenotropic viruses, it is likely that the

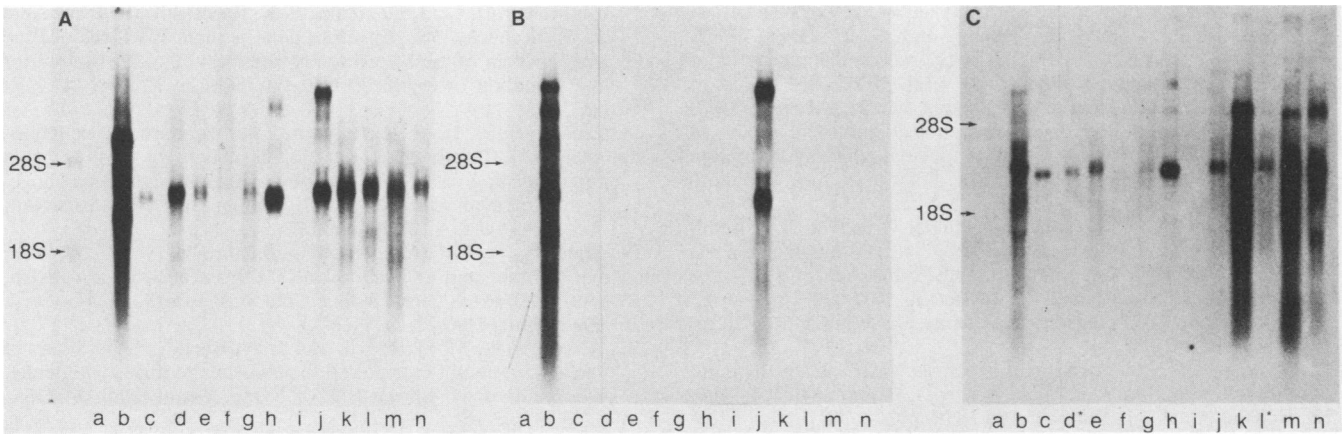

FIG. 1. Northern analysis of different DBA/2 and NZB cell RNAs hybridized to each of the following ^{32}P-labeled DNA probes; (A) B-E, (B) Ha-LTR, and (C) xeno-LTR. Lanes in each of the three panels represent 30 μg of total RNA extracted from the following: (a) Fischer rat embryo fibroblasts, (b) Fischer rat embryo cells infected with $SFFV_A$-Friend murine leukemia virus complex, (c) DBA/2 fibroblasts, (d) DBA/2 kidney tissue, (e) DBA/2 liver tissue, (f) DBA/2 muscle tissue, (g) DBA/2 spleen tissue, (h) polyadenylate-selected DBA/2 fibroblast RNA (1 μg), (i) non-polyadenylated DBA fibroblast RNA, (j) NIH 3T3 fibroblasts infected with Moloney MCF virus, (k) NZB kidney tissue, (l) NZB liver tissue, (m) NZB spleen tissue, and (n) NZB thymus tissue. In Fig. 1C, lanes d and l represent less than 15 μg of RNA.

larger RNA species reflect the viral genomes of defective as well as nondefective endogenous (xenotropic) proviruses. The lack of detection of these genomes and smaller RNA species in NZB tissues by the B-E probe (Fig. 1A) might be explained by the finding that the B-E probe has a somewhat lower affinity for xenotropic *env* sequences than does the xeno-LTR probe (4). Alternatively, the xeno-LTR probe may detect a more diverse population of xenotropic sequences, whereas the BE probe may detect only a subset of this population.

In summary, we hybridized RNA from numerous uninfected mouse tissues to a probe prepared from an erythroleukemia-inducing RNA tumor virus and detected RNA transcripts of envelope and genomic sizes. Since similar sequences were recognized by LTR probes from xenotropic but not ecotropic viruses, we suggest that the transcripts arose from xenotropic proviruses.

Further analysis of the RNA sequences detected by the B-E probe will be necessary to determine which cellular proteins are encoded by these putative messages. However, on the basis of protein studies, two possibilities can be suggested. First, Elder et al. (8) have proposed that the endogenous gp70s are derived from a family of genes. Thus, 24S RNA may carry information from some or all of these genes. It is noteworthy that Hara et al. (10) correlated increased levels of gp70 in serum with increased reactivity of kidney and liver tissues with antiserum prepared against xenotropic viruses. Since we observed high levels of 24S RNA in kidney and liver, it is possible that gp70 in serum may result from tissue-specific expression of the *env* portion of xenotropic proviral genomes and may be encoded by the 24S mRNA detected by the B-E probe. Second, it is possible that 24S RNA encodes a xenotropic-related protein that has previously been shown to be expressed on the surfaces of cells from DBA/2 mice and certain other strains of mice (21). It has been postulated (1a, 21) that the expression of these xenotropic-related envelope proteins on the cell surface confers resistance against MCF virus infection. It is interesting that NIH 3T3 fibroblasts or normal splenic tissues from NIH mice, which do not express any xenotropic virus-related envelope proteins (21), do not express any 24S RNA that hybridizes to the B-E probe (data not shown).

In addition to the 24S species, several higher-molecular-weight bands were detected in the polyadenylate-selected RNA from DBA/2 fibroblasts and in preparations of total cytoplasmic RNA from NZB tissues. It is possible that these RNAs were transcribed from genomic proviral sequences. However, the RNAs were heterogeneous in size, and many of the bands were somewhat smaller than the genomes of xenotropic viruses described in the literature (15). It is suggested, therefore, that these genomic RNAs (and perhaps the 24S species as well) may be transcriptional products of incomplete proviruses. If this were the case, it is unlikely that infectious virions could be isolated from tissues, especially if the defect resided in regions of the viral genome necessary for replication or maturation of the virus. This may explain the appearance in the serum of NZB mice of virion-free gp70 (10).

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