PREFERENTIAL NUCLEAR COMPARTMENTALIZATION OF ENDOGENOUS MINK CELL FOCUS-FORMING–RELATED RETROVIRAL TRANSCRIPTS

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Infectious type C retroviral RNA serves as a template for translation of a *gag-pol* polypeptide without undergoing splicing. However, the 8.4-kb retroviral transcript does not give rise to the *env* protein (1), perhaps because the *env* initiation site overlaps slightly with the 3' end of the *pol* gene (1). The full-length 8.4-kb transcript is processed into a 3.0-kb transcript by splicing the upstream long terminal repeat (LTR)[1] (donor site) with a splice acceptor site found just 5' to the *env* initiation site. This spliced 3.0-kb transcript is the template for the *env* fusion protein, which gives rise to SU and TM (previously called gp70 and p15E, respectively) (2).

Unspliced cellular transcripts tend to be retained in the nucleus; in contrast, spliced transcripts are rapidly transported to the cytoplasm and, therefore, are predominant there (3). However, unspliced infectious retroviral transcripts must be transported from the nucleus to the cytoplasm to be incorporated into virions (3). In contrast to infectious retroviruses, there are numerous inherited retroviral-related sequences in the genome, the great majority of which are replication defective. The murine endogenous retroviral sequences are stable genetic elements of the mouse chromosomes. It is not clear whether the mRNA encoded by such endogenous retroviral-related sequences would be processed and transported as are other cellular transcripts.

We have had a special interest in endogenous retroviral-related transcripts because of their unusual expression in murine lupus. Although expression of xenotropic- and ecotropic-related sequences is not associated with murine lupus, expression of endogenous mink cell focus-forming (MCF)-related sequences is associated with murine lupus (4). MCF is the third class of murine Type C retroviruses. Endogenous MCF-like *env* sequences are highly homologous to those of infectious MCF retroviruses. Full-length endogenous 8.4-kb MCF-related retroviral transcripts are highly expressed in the thymuses of autoimmune strains of mice but not nonautoimmune mice (4,

[1] *Abbreviations used in this paper:* LTR, long terminal repeat; MCF, mink cell focus-forming.

5). These 8.4-kb MCF-related RNA appear to be a primary feature of autoimmunity rather than a secondary effect, as this RNA is highly expressed from the first day of life in autoimmune NZB mice, long before the development of disease (5). Recent studies have shown that generalized autoimmune diseases are associated with antibodies to components of the splicing apparatus and abnormal splicing of cellular transcripts (6–8). It is, therefore, of great interest to study the distribution of endogenous spliced and unspliced transcripts that have been associated with generalized autoimmunity. Since our preliminary data have suggested that expression of the MCF-related transcripts is increased in CD4$^-$, CD8$^-$ thymocytes, we chose a CD4$^-$, CD8$^-$ thymoma cell line for assessment of nuclear cytoplasmic distribution of MCF-related transcripts. Although prior studies have addressed nuclear cytoplasmic distribution of other transcripts, to our knowledge, there are no reports of such an analysis of endogenous retroviral transcripts.

Materials and Methods

Cell Line and Tissue Culture. SL12.4 is a CD4$^-$, CD8$^-$ thymocyte cell line derived from an AKR thymoma (9). SL2 is a CD4$^-$, CD8$^-$ thymocyte cell line derived from a different AKR parent than the SL12.4 (C. MacLeod, personal communication). The cells were grown in DMEM (Biofluids, Rockville, MD) supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 0.2 mM L-glutamine, 50 μM 2-ME, penicillin, and streptomycin. Cells were cultured to a maximal concentration of 10^6/ml in 5% CO$_2$, 100% humidity at 37°C. In one experiment, rIL-1α (Genzyme, Boston, MA) at 8 U/ml was used to stimulate the cells in a 72-h culture.

Probes. MCFenv, mPT, and PT are oligonucleotide probes for the MCF envelope region and hybridize specifically to MCF, mPT, and PT transcripts, respectively (10). Oligonucleotide probes were synthesized on a DNA synthesizer (380B; Applied Biosystems, Inc., Foster City, CA). The sequences from 5' to 3' are as follows: MCF, GACACCCGAGTCCAGT; mPT, GTCCCAGGTTGTATAGAGG; PTenv, AAGGTGGGGCAGTCTCAGGGA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a 1.2-kb rat cDNA obtained from M. Piechaczyk (11). Mouse β actin and c-*myc* were kindly provided by Dr. J. Frederic Mushinski (NIH, Bethesda, MD). The constant region of the TCR β chain probe was a 0.6-kb Eco R1 fragment cleaved from the cDNA clone 8675 (12). The c-*myb* probe was a 0.5-kb Eco R1 fragment from a mouse cDNA library (13).

RNA Preparation and Northern Analysis. Total RNA was isolated via a modified version of the technique by Chomczynski and Sacchi (14) using RNAzol (Cinna/Biotecx, Friendswood, TX). Cytoplasmic RNA was isolated as described by M. Wilkinson (15). Nuclei recovered after cell membrane lysis were then processed by RNAzol for isolation of nuclear RNA. Poly (A)$^+$ RNA was selected by the use of oligo(dT) columns (Pharmacia Fine Chemicals, Piscataway, NJ). RNA was then denatured and subjected to electrophoresis on a 0.75% formaldehyde agarose gel, transferred to Nytran (Schleicher & Schuell, Inc., Keene, NH), prehybridized, hybridized, then washed as previously described for oligonucleotide probes (5). Prehybridization and hybridization for nonoligonucleotide probes was performed as previously described (5). Nonoligonucleotide probes were radiolabeled by random priming with ^{32}P-dCTP, and blots were washed twice at 55°C in 2× SSC, 0.1% SDS for 15 min and four times at 55°C in 0.2× SSC, 0.1% SDS for 15 min each. Oligonucleotide probes were purified through G-25 sephadex and nonoligonucleotide probes through G-50 sephadex columns (5'-3' Inc., Philadelphia, PA). Autoradiography was performed with Kodak XAR film at −70°C in the presence of Lightning Plus intensifying screens (Dupont Co., Wilmington, DE).

Densitometry. Densitometric analyses were performed with a scanning densitometer (1650; Bio-Rad Laboratories, Richmond, CA). The densitometry curves were traced onto paper of uniform composition, and the areas under each curve were cut out and weighed on a balance (Mettler Instrument Corp., Hightstown, NJ). Data are expressed as the weights relative to the weight of the curve for the band with the least amount of RNA detectable (this was 10 weight units, which is 0.001 g).

Results

RNA Isolation. RNA was quantified during fractionation and there was a relatively consistent recovery from unstimulated cells of ~39 μg total RNA per 10^7 cells, of which, approximately one fifth was present in the nucleus (an average of 7.1 μg). In contrast, IL-1-stimulated cells had increased cytoplasmic RNA but not nuclear RNA, 113 μg and 6.6 μg, respectively, with 6% of the RNA found in the nucleus (Table I). Results with poly(A)⁺ RNA were similar to those with total RNA (Table I).

Expression of MCF-related Retroviral Transcripts in SL12.4. Northern analysis of total RNA from the CD4⁻, CD8⁻ SL12.4 cell line demonstrated the presence of 8.4-kb (full-length) and 3.0-kb MCF-related transcripts (Fig. 1). To investigate whether these endogenous transcripts are readily transported into the cytoplasm, we fractionated the nuclear and cytoplasmic RNA. Northern blots of the nuclear and cytoplasmic RNA fractions demonstrated that the great majority of the MCF-related 8.4-kb band was present in the nucleus (Fig. 1). In contrast, the MCF-related 3.0-kb RNA demonstrated only a slight predominance in the nucleus. Of note, RNA derived from IL-1-stimulated cells had comparable nuclear/cytoplasmic distributions of 8.4- and 3.0-kb MCF-related transcripts despite a marked increase in the total amount of cytoplasmic RNA. When poly(A)⁺-selected RNA was studied, a comparable nuclear presence of the MCF-related RNA was observed (Fig. 2).

Studies Using MCF Subclass Probes. Fig. 3 shows the results of studies using the MCF *env* probe as well as the two subclass-specific probes PT and mPT. Endogenous MCF-related sequences have been subdivided into polytropic and modified polytropic subclasses that can be distinguished by specific oligonucleotide probes (16). Modified polytropic sequences have a 27-bp deletion in the *env* gene relative to PT sequences (16, 17). Using oligonucleotide probes specific for mPT and PT sequences, the 8.4-kb bands again were much darker in nuclear RNA than cytoplasmic, while the 3.0-kb bands were expressed at a slightly higher level in nuclear RNA than cytoplasmic. To quantitate the intensity of the bands, the blots were subjected to densitometric analysis. Table II demonstrates that the 8.4-kb RNA have a greater nuclear predominance than the 3.0-kb RNA.

TABLE I

Recovery of RNA after Separation into Nuclear and Cytoplasmic Fractions

RNA	No. of cells	RNA			
		N*	C‡	N/C§	N/T‖
Total RNA	5.0×10^7	40	200	0.20	0.17
Total RNA	3.3×10^7	30	167	0.18	0.15
Total RNA	3.3×10^7	38	166	0.22	0.19
Total RNA	8.3×10^8	567	2,550	0.22	0.18
Poly(A)⁺ RNA	8.3×10^8	5	17	0.29	0.22
IL-1-treated total RNA	3.8×10^7	25	430	0.06	0.05

* Total nuclear RNA isolated (μg).
‡ Total cytoplasmic RNA isolated (μg).
§ Ratio of nuclear to cytoplasmic RNA.
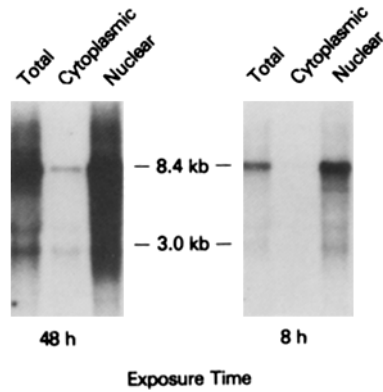‖ Ratio of nuclear to total (nuclear + cytoplasmic) RNA.

FIGURE 1. Northern blot containing 5 μg of total, cytoplasmic, or nuclear RNA from SL 12.4 cell line. The RNA was hybridized to the MCFenv probe and exposed for 8 or 48 h.

Comparison of MCF Transcripts with Those of Other Genes. Generally, nonretroviral transcripts are present at a much higher level in the cytoplasm than in the nucleus (18). To compare the compartmentalization of these endogenous retroviral-related transcripts with that of other cellular transcripts, we probed the blots with other genes encoding various sized RNA. The blot shown in Fig. 3 was stripped and re-probed as shown in Fig. 4 *B*. In all cases, the cytoplasmic bands were darker than the nuclear, as was expected from previous studies (18). To assess fully mature transcripts, poly(A)$^+$ RNA was purified and hybridized to the same probes and similar results were obtained (Fig. 4 *A*).

Densitometric analysis was performed as in Table II for several additional genes. In all cases, nuclear/cytoplasmic ratios were <0.5 for nonretroviral-related cellular genes, whereas the MCF-related retroviral ratios were always >1.0. Data for several genes from two different blots are shown in Table III. It should be noted that poly(A)$^+$-selected RNA gave high nuclear/cytoplasmic ratios for the MCF tran-
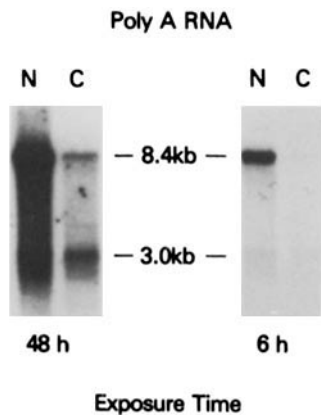

FIGURE 2. Northern blot containing 2.5 μg of poly(A)$^+$ selected cytoplasmic or nuclear RNA from the SL 12.4 CD4$^-$, CD8$^-$ cell line hybridized to MCFenv and exposed for 6 or 48 h.
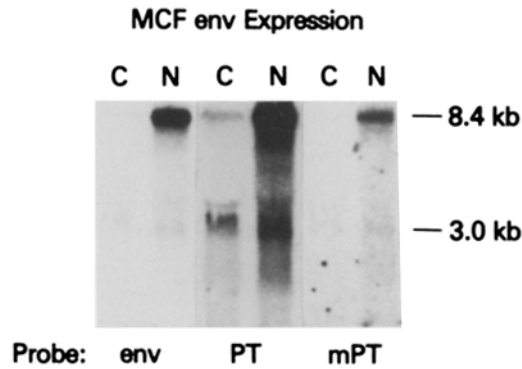
MCF env Expression

FIGURE 3. Northern blot containing 20 μg of cytoplasmic or nuclear RNA from the CD4⁻, CD8⁻ cell line. The blot was probed initially with the MCFenv probe, then stripped and reprobed.

scripts and that the 3.8-kb c-*myb* gene had a greater cytoplasmic presence than the 3.0-kb MCF-related transcript.

To determine whether or not the SL12.4 cell line was unique in demonstrating large amounts of nuclear retroviral transcripts, the studies were repeated using a different CD4⁻, CD8⁻ thymoma cell line, SL2, with very similar results (data not shown).

Discussion

Using the SL12.4, CD4⁻, CD8⁻ cell line, we have studied the nuclear vs. cytoplasmic compartmentalization of MCF-related transcripts. While 80% of cellular RNA was found within the cytoplasm, <10% of the (unspliced) 8.4-kb MCF-related RNA was cytoplasmic. The spliced 3.0-kb MCF-related messages were present at slightly higher levels in nuclear when compared with cytoplasmic RNA. Similar results were obtained for both MCF subclasses, PT and mPT. In contrast to the preferential nuclear localization of endogenous MCF RNA, nonretroviral cellular transcripts, showed a marked predominance in cytoplasmic RNA. Comparable results also were obtained with the SL2 thymoma cell line, suggesting that such intracellular com-

TABLE II

Densitometric Analysis of the Bands in Fig. 3

RNA size	Probe	N*	C‡	N/C§
8.4	MCFenv	892‖	25	35
8.4	PT	2,574	81	31
8.4	mPT	734	<10	>73
3.0	MCFenv	92	87	1.1
3.0	PT	1,022	432	2.4
3.0	mPT	38	<10	>3.8

* Nuclear.
‡ Cytoplasmic.
§ Nuclear to cytoplasmic ratio.
‖ Relative amount of specific message as determined by densitometric analysis (see Materials and Methods).
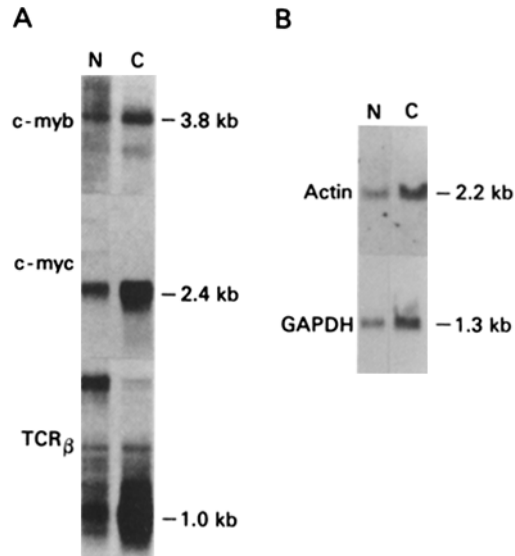
A

B

FIGURE 4. Northern blot of cytoplasmic or nuclear RNA from the CD4⁻, CD8⁻ cell line. (A) 2.5 µg of poly(A)⁺ was loaded onto gel; (B) 20 µg total RNA load. The same blot was reprobed after stripping for each indicated RNA.

partmentalization is not unique to a particular cell line. Thus, MCF-related endogenous retroviral transcripts are distributed differently from other cellular transcripts. Although the MCF-like sequences are, like other genes, inherited in the DNA as stable elements, their retroviral-like structure may confer upon them unusual properties. Lawrence et al. (19) have developed a nonisotopic in situ hybridization procedure to directly visualize specific primary transcripts within interphase nuclei. They found that labeled transcripts synthesized from an integrated Epstein-Barr viral genome form specific curved linear "tracks" within the nucleus. The authors hypothe-

TABLE III

Nuclear/cytoplasmic (N/C) Ratios for Total RNA and poly(A) Selected RNA Determined by Densitometry

Probe	Size	RNA	N*	C‡	N/C§
	kb				
GAPDH	1.3	Total	404‖	968	0.42
Actin	2.2	Total	164	410	0.40
TCRbeta	1.0	Poly(A)⁺	124	1,665	0.07
c-myb	3.8	Poly(A)⁺	267	865	0.31
c-myc	2.3	Poly(A)⁺	389	1,388	0.28
MCFenv	8.4	Poly(A)⁺	859	27	32
MCFenv	3.0	Poly(A)⁺	129	43	3.0
MCFenv	8.4	Total	1,014	18	56
MCFenv	3.0	Total	128	97	1.3

* Nuclear.
‡ Cytoplasmic.
§ Nuclear to cytoplasmic ratio.
‖ Relative amount of specific message as determined by densitometric analysis (see Materials and Methods).

size that these transcripts are specifically organized within the nucleus and are actively transported from their site of synthesis to nuclear pores for exit from the nucleus into the cytoplasm. Since endogenous MCF transcripts of the 8.4-kb size localize to the nucleus, they may be limited in ability to traverse and exit the nucleus because of molecular size. Smaller 3.0-kb MCF transcripts might be capable of more rapid transport to nuclear pores. Smaller transcripts also may be better able to be transported through nuclear pores. Such a transport mechanism can show sequence specificity as demonstrated by the finding that the adenoviral 55-kD polypeptide E1B can increase transport of late viral mRNAs to the cytoplasm without affecting transport of other mRNA (20).

Retroviral sequences in the genome differ from other genes in that they lack noncoding introns characteristic of most cellular genes. As a result, mRNA transcribed from endogenous retroviral sequences may be translated without undergoing splicing. If transcript size is a factor in transport of RNA to the cytoplasm, splicing of a transcript to generate a smaller sized mRNA would be expected to increase the movement of such a transcript to the cytoplasm. A full-length retroviral transcript of 8.4 kb, by virtue of its size, might be preferentially retained in the nucleus. However, spliced 3.0-kb MCF transcripts were retained in the nucleus to a much greater extent than other transcripts, including the 3.8-kb c-*myb* transcripts. Therefore, factors other than mere size may decrease the ability of endogenously encoded retroviral-like transcripts to leave the nucleus. Since the 8.4- and 3.0-kb transcripts both contain the LTR and *env* regions, sequences in one of those regions could serve to inhibit transport from the nucleus. Although specific sequences inhibiting splicing have not been described for MCF-like sequences, such sequences have been described in other systems (18, 21). For example, a *cis*-acting sequence (*nrs*) within RSV RNA acts as a negative regulator of splicing (18).

In addition to possible effects on splicing, retroviral sequences could alter transport from the nucleus. Specific sequences within *env* or the LTRs (both shared by 8.4- and 3.0-kb transcripts) could alter their interactions with transport mechanisms. This possibility is highlighted by the observation that a 6- and a 7-bp deletion in a pre-mRNA can markedly alter transport to the cytoplasm (22). Moreover, it has been hypothesized that there may be specific nuclear pores that target certain transcripts to specific regions of the cytoplasm (23). In such a scheme, pores that allow transport of retroviral-related transcripts may have different capacities or rates than other pores.

The increased relative nuclear presence of retroviral-like transcripts could result from their rapid degradation in the cytoplasm. Consistent with this possibility is the observation that frameshift mutations in the *gag* gene of the Rous sarcoma virus causes premature termination of gene translation by destabilizing unspliced cytoplasmic RNA (18). Moreover, the 8.4-kb message could be more quickly degraded than the 3.0-kb transcript on the basis of size, although Belasco et al. (24) have found similar decay rates for the *Escherichia coli bla* gene and a truncated form that differed in size by 50% (24). On the other hand, the retroviral-like transcripts are polyadenylated, which provides protection against exonuclease digestion (25, 26). In addition, our group previously has found that 3.0-kb MCF-related transcripts can be expressed independently of 8.4-kb transcripts after in vivo injection of mice with mitogens (27), suggesting that transcript-specific stabilization mechanisms may exist.

It also has been observed that MMTV *env* transcripts are much more stable in the spliced than the unspliced form (L. King, unpublished observations).

Human and murine autoimmune diseases are characterized by the presence of autoantibodies directed toward specific components of the splicing apparatus (6–8), which could contribute to disease. In fact, a splicing defect has been reported for autoimmune C3H-*gld/gld* mice (28, 29). Since the autoantibodies reactive with splicing components have been reported to crossreact with retroviral proteins (30–33), it is not clear whether retroviral products or self-antigens might be immunogens for such antibodies. Our laboratory has described an association of thymic full-length 8.4-kb MCF-related RNA with autoimmunity. We now demonstrate that a large amount of such 8.4-kb MCF message is nuclear. Abnormalities of the intracellular physiology of endogenous retroviral RNA could contribute both to the thymic 8.4-kb MCF-related transcripts and to the autoimmune state.

Summary

Endogenous mink cell focus-forming (MCF)-like retroviral sequences in the murine genome are stable, inherited sequences analogous to other chromosomal genes. As such, it is thought that they are transcribed and translated in a manner analogous to other genes. However, when the SL12.4 CD4$^-$, CD8$^-$ thymoma cell line was studied for nuclear/cytoplasmic distribution of endogenous MCF-related transcripts, there was a nuclear predominance. The great majority of full-length 8.4-kb endogenous MCF-related transcripts were nuclear. Even the smaller, spliced 3.0-kb transcripts were at least as prominent in the nucleus as the cytoplasm, whereas cellular RNA was 80% cytoplasmic and other cellular transcripts were represented in the cytoplasm to a much greater extent than the nucleus. Size cannot fully account for the nuclear presence of MCF-related endogenous transcripts, because the 3.0-kb MCF transcripts occurred in the nucleus to a much greater relative extent than 3.8-kb c-*myb* transcripts. These studies point to retroviral-like structures of these transcripts as influencing their intracellular compartmentalization.

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