JOURNAL OF VIROLOGY, Mar. 1987, p. 876–882
0022-538X/87/030876-07$02.00/0

Vol. 61, No. 3

Expression of Mink Cell Focus-Forming Murine Leukemia Virus-Related Transcripts in AKR Mice

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Received 22 September 1986/Accepted 3 December 1986

We used a synthetic 16-base-pair mink cell focus-forming (MCF) *env*-specific oligomer as radiolabeled probe to study MCF murine leukemia virus (MuLV)-related transcripts in brain, kidney, liver, spleen, and thymus tissues of AKR mice ranging from 5 weeks to 6 months (mo) of age. Tissue-specific expression of poly(A)$^+$ RNAs was seen: 6.0-kilobase (kb) transcripts were detected in the liver and kidney; 7.2- and 1.8-kb RNA species were present in the thymus. In addition, all the tissues tested contained 3.0-kb messages. The transcription of these MCF-related mRNAs was independent of the presence of ecotropic and xenotropic MuLVs. In general, expression of the MCF *env*-related transcripts appeared to peak at 2 mo of age; these messages were barely detectable in brain, kidney, liver, and spleen tissues after 2 mo and in thymus tissue after 4 mo of age. All of the subgenomic MCF *env*-related mRNAs (6.0, 7.2, 1.8, and 3.0 kb) appeared to contain the 190-base-pair cellular DNA insert, characteristic of the long terminal repeats associated with endogenous MCF *env*-related proviruses (A. S. Khan and M. A. Martin, Proc. Natl. Acad. Sci. USA 80:2699–2703, 1983). No genomic-size (8.4-kb) transcripts corresponding to endogenous MCF-related proviruses were detected. An 8.4-kb MCF *env*-related mRNA was first seen at 3 mo of age, exclusively in thymus tissue. This species most likely represents the first appearance of a recombinant MCF-related MuLV genome. The transcripts which were detected in thymus tissue might be involved in the generation of leukemogenic MCF viruses.

A high incidence of spontaneous thymomas occurs in AKR mice. Dualtropic, mink cell focus-forming (MCF) murine leukemia viruses (MuLVs), which have been isolated from preleukemic and leukemic mouse thymus tissues, have been implicated as the proximal causal agents of lymphomogenesis in AKR mice (12, 17, 35). Unlike ecotropic (25) and xenotropic (24) MuLVs, leukemogenic MCF viruses cannot be induced from the mouse germ line but arise by recombination among at least three different MuLV sequences: ecotropic, endogenous MCF-related, and xenotropic-related sequences (7, 23, 42). About two-thirds of the MuLV proviruses present in AKR mouse DNA are MCF related (36). One of the cloned endogenous MCF-related MuLV DNAs isolated from the AKR/J mouse genome, designated A-12, has been shown to be virtually identical in its 3′ *pol* and 5′ *env* regions to leukemogenic MCF MuLVs, suggesting that sequences present in A-12 or in another closely related proviral DNA might be involved in the generation of leukemogenic MCF viruses (20). The mechanism of recombination between different MuLV sequences resulting in MCF virus formation, however, is unclear. A model for recombinant virus generation involves interaction between viral genomic and mRNAs which have been copackaged into heterozygous virions (28). Accordingly, endogenous MCF-related transcripts might participate in the genesis of MCF MuLVs. The presence of subgenomic mRNAs of endogenous MuLV proviruses has previously been reported in BALB/c, AKR, and $129G_{1X+}$ mice (3, 27). However, the cloned DNA segments used as hybridization probes in those studies could not distinguish between endogenous MCF-related and xenotropic MuLV transcripts (3) or between MCF-related and ecotropic MuLV RNAs (27). In this paper, we used a radiolabeled 16-base-pair (bp) MCF *env*-specific oligomer, designated MCF_{env}, as a hybridization probe to study the expression of MCF-related transcripts in five different tissues of AKR mice, ranging in age from 5 weeks (wk) to 6 months (mo).

MATERIALS AND METHODS

Mice, RNAs, and DNAs. Weanling AKR/N mice were provided by the Frederick Cancer Research Facility, Frederick, Md. The mice were sacrificed at various ages ranging from 5 wk to 6 mo. RNAs were isolated from brain, liver, kidney, spleen, and thymus of 12 animals at each time point. RNAs, used as standards for determining hybridization specificity and molecular size, were prepared from cell lines infected with known MuLVs, which were generously provided by Janet Hartley (National Institutes of Health, Bethesda, Md.). These included NFS-Th-1 xenotropic MuLV-infected mink cells, MCF247 MuLV-infected mink cells, and an established line of NIH 3T3 cells producing AKR ecotropic MuLV obtained after transfection of the mouse cells with a molecularly cloned infectious ecotropic MuLV DNA designated pAKR623 (30). Total cellular RNAs were prepared from AKR mouse tissues and MuLV-infected cell lines by the guanidine-thiocyanate method (9, 40). Poly(A)$^+$ RNA was selected on oligodeoxythymidylate-cellulose (Collaborative Research, Inc., Waltham, Mass.; 1).

MuLV DNAs used in the studies included cloned proviruses of AKR623 ecotropic (30), NFS-Th-1 xenotropic (4), MCF247 (designated MCF 1 in ref. 22), and 4070A amphotropic (8) MuLVs; endogenous MuLV sequences isolated from AKR/J and BALB/c mouse genomes (23); and Hirt DNAs obtained as previously described (18), 48 h after

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cocultivation of uninfected and MCF MuLV-infected mink cells (kindly provided by Janet Hartley).

Synthesis of MCF *env*-specific probe. A 16-bp oligomer, 5′ GACACCCGAGTCCAGT 3′, designated MCF_{env}, was synthesized by using the phosphotriester method (34) on a Vega Polynucleotide synthesizer (Vega Biotechnologies, Inc., Tucson, Ariz.) according to Vega protocol number 4. Briefly, 10-fold molar equivalents of the protected monomers (catalog numbers 10531, 10532, 10533, 10534; Vega Biochemicals, Tucson, Ariz.) were reacted sequentially with 50 mg of 5′-DMT-T-aminomethyl polystyrene resin (catalog number 10481; Vega Biochemicals) to yield the desired sequence. The dimethoxytrityl group of the resin or attached nucleotide was removed with 10% tricarboxylic acid in chloroform. Condensations were performed with a 3-fold molar excess of mesitylenesulfonyl-3-nitrotriazole (Vega Biochemicals) (15). Cleavage of the 16-mer from the support and removal of protecting groups were accomplished by treating the final support with 0.5 M syn-pyridine-2 carboxaldoxime tetramethylguanidium in pyridine-water (9:1, vol/vol) overnight, followed by incubation in 28% ammonium hydroxide for 3 h at 60°C. After vacuum evaporation, the residue was dissolved in 80% acetic acid for 20 min at room temperature and evaporated under vacuum (13). Final purification of the 16-mer was on a 20% polyacrylamide–50% urea gel. The slowest-moving band was cut out and eluted in distilled water by overnight incubation at 37°C. Urea and salts were removed by chromatography on a Sep-Pak column (Waters Associates, Inc., Milford, Mass.; 29).

DNA probes and Southern and Northern blot hybridizations. Long terminal repeat (LTR) probes included: X_{U3} DNA, which represented the *Pst*I-to-*Sma*I fragment encompassing almost the entire U_3 region of NFS-Th-1 xenotropic MuLV LTR (4), and $B\text{-}34_{162}$ DNA, which extended from *Alu*I to *Bgl*II located at positions 390 and 552, respectively, in endogenous B-34 MuLV proviral LTR (21). The latter DNA segment consisted of 162 nucleotides of the 190-bp cellular DNA insert, which is characteristic of the LTRs associated with endogenous MCF *env*-related proviruses. MuLV *env*-specific DNA probes included X_{env} derived from NFS-Th-1 xenotropic MuLV DNA (4), Ec_{env} isolated from AKR ecotropic provirus (6), and MCF_{env}, a synthetic 16-bp oligomer described above, consisting of sequences complementary to those extending from nucleotide position 262 to 277 from the 5′ end of MCF247 MuLV *env* (20).

RNA was denatured in 50% formamide–6% formaldehyde–20 mM MOPS (morpholinepropanesulfonic acid; pH 7.0)–5 mM sodium acetate–1 mM EDTA and fractionated by electrophoresis in 1% agarose gels containing 6% formaldehyde–20 mM MOPS (pH 7.0)–5 mM sodium acetate–1 mM EDTA (26). The gels were treated in 0.05 N NaOH–0.6 M NaCl for 30 min, followed by soaking in 0.5 M Tris hydrochloride (pH 8.7)–0.6 M NaCl for 30 min. RNA transfers were set up in 20× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M NaCl). For Northern blot hybridization experiments with the MCF_{env} probe, RNA blots were prehybridized overnight at 45°C in 6× SSC solution containing 50 mM Tris hydrochloride (pH 8.0), 0.05% sodium pyrophosphate, 10% dextran sulfate (Pharmacia Fine Chemicals, Piscataway, N.J.), 1 mg of denatured yeast RNA per ml (type 3; Sigma Chemical Co., St. Louis, Mo.), and 1% sodium dodecyl sulfate (SDS). Hybridizations were done overnight at 45°C by addition of the MCF_{env} oligomer end labeled with T4 polynucleotide kinase (P-L Biochemicals, Inc., Milwaukee, Wis.) and [γ-^{32}P]ATP (>5,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) at a final concentration of 5×10^6 cpm/ml of prehybridization mix. Filters containing transferred RNA were washed in a shaker with brisk agitation two times for 15 min each at 45°C in 6× SSC containing 0.05% sodium pyrophosphate–0.1% SDS and once for 2 min at 45°C in 1× SSC containing 0.025% sodium pyrophosphate–1% SDS. Northern blot hybridization studies with segments isolated from cloned MuLV DNAs as probes were done as described previously (38). The filters were prehybridized for 18 h in 5× SSC, 50% formamide (EM Science), 5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll plus 0.02% polyvinylpyrrolidone plus 0.02% bovine serum albumin), 0.1 M Tris (pH 7.5), 300 μg of denatured yeast RNA per ml, and 10% (wt/vol) dextran sulfate at 45°C. DNA segments purified from agarose gels were radiolabeled by nick translation (33) and added at a final concentration of 0.5×10^6 cpm/ml of prehybridization mix. After overnight hybridization at 45°C, the filters were washed twice at 50°C in 2× SSC–0.1% SDS for 10 min and twice at 50°C in 0.1× SSC–0.1% SDS for 15 min and then rinsed in 2× SSC.

DNA was electrophoresed on 0.6 or 1.4% agarose gels and transferred by the Southern procedure (41). Filters were prehybridized at 45°C in 6× SSC solution containing 10× Denhardt solution and 0.1% SDS for 3 h, followed by 1 h of incubation in 6× SSC solution containing 10× Denhardt solution, 0.1% SDS, 0.05% sodium pyrophosphate, and 50 μg of denatured salmon sperm DNA per ml. ^{32}P-MCF_{env} was added at a final concentration of 5×10^6 cpm/ml of prehybridization mix, and hybridizations were done overnight at 45°C. Filters were washed under the conditions described above for Northern hybridizations with the MCF_{env} oligomer probe.

RESULTS

Hybridization specificity of synthetic MCF_{env} oligomer. The location of MCF_{env} and comparison of nucleotide sequences present in analogous regions of MCF247, AKR ecotropic, and NFS xenotropic MuLV DNAs are shown in Fig. 1. The reactivity of ^{32}P-MCF_{env} with various MuLV proviruses was studied by Southern blot analysis. The results, shown in Fig. 2B, indicate that MCF_{env} hybridized specifically to the 5′ *env* segments of MCF247 (lane 4) and endogenous MCF-related MuLV proviruses, A-12, A-5, B-34, and A-1 (lanes 5 to 8, respectively) but not to AKR ecotropic, NFS xenotropic, or 4070A amphotropic MuLV DNAs (lanes 1 to 3, respectively). Furthermore, MCF_{env} probe annealed with the 8.8-kilobase (kb) linear DNA species present in Hirt DNAs isolated from six different MCF MuLV-infected mink cells (Fig. 2C). Weak hybridization of MCF_{env} with Cas 2S L4 MCF proviral DNA was seen (lane 14). This probably reflects nucleotide divergence in *env* since all the Hirt DNAs tested (lanes 9 to 14) contained about an equal amount of MCF linear DNAs based upon hybridization with an MuLV LTR probe (data not shown). The reactivity of MCF_{env} in Northern blot analyses with total cell RNAs isolated from cells infected with AKR ecotropic, NFS xenotropic, and MCF247 MuLVs is shown in Fig. 3A. ^{32}P-MCF_{env} hybridized to the 8.4- and 3.0-kb mRNAs transcribed from the MCF MuLV genome (lane 3). These transcripts represent retroviral full-length genomic and *env* mRNAs. No reactivity was seen with either ecotropic or xenotropic MuLV mRNAs (lanes 1 and 2, respectively). Since the MCF_{env} DNA probe also hybridized with 28S and 18S rRNAs, further experiments were performed with poly(A)$^+$ RNAs. To demonstrate that intact RNA was present in AKR ecotropic and NFS xenotropic MuLV-infected cellular RNA preparations,

TABLE 1. Expression of MCF-related mRNAs in AKR mice

Tissue	Size (kb) of MCF-related mRNAs at:				
	5 Wk	2 Mo	3 Mo	4 Mo	5 Mo
Brain	(3.0)[a]	(3.0)	(3.0)	(3.0)	(3.0)
Kidney	3.0	3.0	(3.0)	(3.0)	3.0
	(6.0)	6.0	(6.0)	(6.0)	6.0
Liver	3.0	3.0	(3.0)	(3.0)	(3.0)
	(6.0)	6.0	(6.0)	(6.0)	
Spleen	(3.0)	(3.0)	(3.0)	(3.0)	(3.0)
Thymus	7.2	7.2	7.2	7.2	
	3.0	3.0	3.0	3.0	3.0
	1.8	1.8	1.8	1.8	
			8.4	8.4	8.4

[a] Weakly detected transcripts or those seen upon long exposures of autoradiograms are indicated in parentheses.

Northern blot analysis was performed with ^{32}P-X_{env} and -Ec_{env} DNAs (panels B and C, respectively). As expected, the X_{env} DNA probe reacted with both the 8.4- and 3.0-kb mRNAs of xenotropic and MCF MuLVs (panel B, lanes 2 and 3, respectively), whereas the Ec_{env} DNA probe hybridized exclusively to AKR ecotropic MuLV mRNAs (panel C, lane 1).

Analysis of MCF MuLV-related RNAs expressed in AKR mouse tissues. The MCF_{env} oligomer probe was used in Northern blot analyses of poly(A)$^+$ RNAs isolated from brain, liver, kidney, spleen, and thymus tissues of AKR mice ranging in age from 5 wk to 6 mo. The results are presented in Fig. 4. Four mRNAs species were expressed at a low level at 5 wk of age (panel I). Tissue-specific expression of subgenomic messages was seen: 6.0-kb mRNA species was present in the kidney and liver; the thymus contained 7.2- and 1.8-kb mRNAs. A 3.0-kb mRNA was present in all five tissues, although expression in the brain and spleen was very low. An increased amount of these MCF-related transcripts was seen at 2 mo of age in all the tissues tested (panel II). A marked reduction in the level of MCF-related mRNAs was seen after 2 mo in brain, kidney, liver, and spleen. In contrast, the thymus maintained levels of the 7.2-, 1.8-, and 3.0-kb endogenous MCF-related mRNAs until 4 mo of age (panel IV). The 7.2- and 1.8-kb mRNAs were undetectable after 4 mo. The 3.0-kb mRNA species seen after 4 mo in thymus tissue probably contains spliced *env* mRNA of the 8.4-kb MCF viral RNA, since the level of both of these messages increased concurrently from 3 mo of age. An 8.4-kb MCF-related mRNA was first detected in the thymus at 3 mo of age (panel III). Since the expression of this message was restricted to the thymus even in 5-mo-old mice (panel V), it probably represented the genome of recombinant, thymotropic MuLVs. A summary of MCF-related messages present in the five AKR mouse tissues tested is shown in Table 1. The structure of these transcripts was analyzed with different MuLV subgenomic DNA probes and will be presented in another study (F. Laigret et al., manuscript in preparation). Northern blot analysis of bone marrow tissue obtained from a set of 3-mo-old AKR mice, different from the one used in panel III, indicated expression of the 3.0-, 7.2-, and 1.8-kb MCF_{env}-reactive RNA species (data not shown). All the tissues tested expressed 8.4- and 3.0-kb mRNAs of AKR ecotropic MuLVs from 5 wk, the level of which increased with age, indicating infectious virus production (Laigret et al., in preparation). No 8.4-kb X_{env}-reactive messages were detected in 5-wk and 2-mo-old AKR mouse tissues (Laigret et al., in preparation). The 7.2- and 1.8-kb MCF *env*-related RNA species were also detected in thymus tissues of BALB/c and NFS mice but not in DBA/2 and C57L mice (F. Laigret, unpublished data).

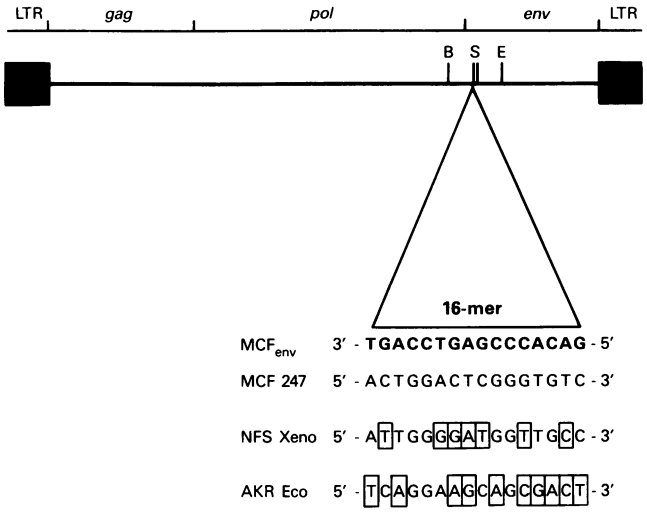

FIG. 1. Derivation of MCF_{env} oligomer. The location of the nucleotide sequence of MCF247 and its complementary sequence, which was used in synthesis of the MCF_{env} 16-mer, are indicated. The nucleotide sequences of NFS xenotropic and AKR ecotropic MuLVs in the analogous region are also shown. The boxed bases indicate nucleotide differences with respect to MCF247 MuLV genome. An incomplete restriction map of MCF247 proviral DNA (22) is shown at the top. Abbreviations: B, *Bam*HI; S, *Sma*I; E, *Eco*RI.

Detection of endogenous MCF *env*-related transcripts in the absence of MuLV expression. To investigate whether transcription of endogenous MCF-related messages depends on ecotropic MuLV expression, we analyzed RNAs isolated from AKR *Fv-4^r* congenic mice (kindly supplied by H. Ikeda) which restrict the spread of AKR ecotropic viruses because of the presence of the *Fv-4^r* gene (19). As a consequence, AKR *Fv-4^r* mouse tissues do not contain any detectable AKR ecotropic MuLV transcripts (19). Northern blot analyses were performed with X_{U3} DNA as a radiolabeled probe under hybridization conditions which could detect LTR sequences associated with the MCF-related and the ecotropic MuLV transcripts. The results indicated that 7.2- and 3.0-kb mRNAs were expressed in thymus tissue of 2-mo-old AKR *Fv-4^r* mice, even in the absence of 8.4-kb ecotropic MuLV RNAs (Fig. 5, lane 2); a 1.8-kb RNA species was faintly detected upon longer exposure of the autoradiograph (data not shown). Thymus tissue of 2-mo-old AKR/N mice contained 8.4-kb transcripts representing the ecotropic MuLV genome in addition to the 7.2-, 3.0-, and 1.8-kb messages (lane 1). The subgenomic RNA species also hybridized to the MCF_{env} oligomer probe (data not shown).

Analysis of LTR sequences of MCF-related transcripts. We have previously shown that most endogenous MCF *env*-related MuLV proviruses contain a 190-bp cellular DNA insert in the U_3 region of the LTRs (21). However, this sequence is absent in the LTRs of known infectious MuLV proviruses. We analyzed the LTR sequences associated with the MCF-related messages present in AKR mouse tissues. Poly(A)$^+$ RNAs isolated from the kidneys of 2-mo-old mice (containing 3.0- and 6.0-kb messages) and from the thymuses of 3-mo-old mice (containing 3.0-, 7.2-, 1.8-, and 8.4-kb mRNAs) were subjected to Northern blot analysis with ^{32}P-labeled B-34_{162} DNA, which hybridizes specifically with

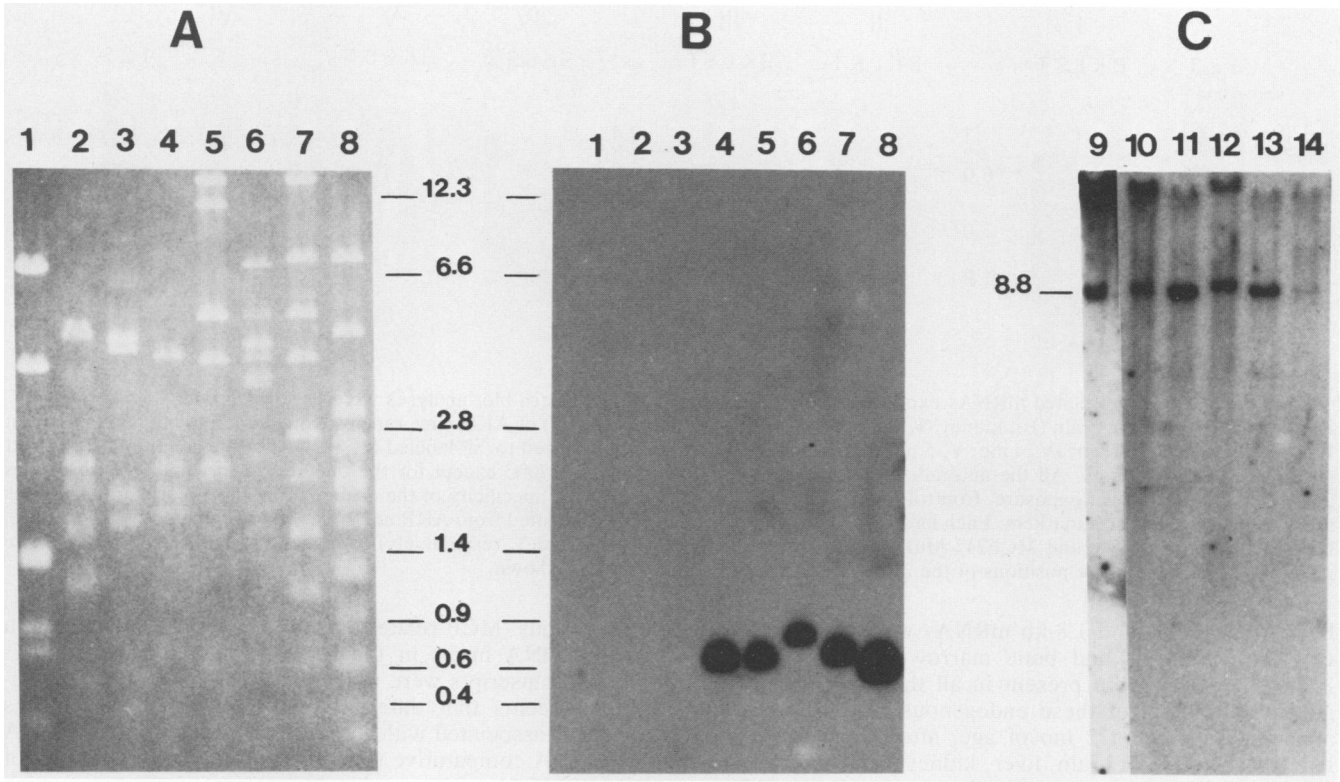

FIG. 2. Hybridization of MCF_{env} oligomer with ecotropic, xenotropic, and dualtropic MuLV DNAs. Cloned MuLV proviruses were singly or doubly digested with various restriction enzymes to generate 0.0144 μg of similar-size (about 0.6-kb) 5′ *env* segments from the different DNAs. The restriction enzymes used for each cloned provirus were as follows: AKR 623 ecotropic, *Bgl*II; NFS-Th-1 xenotropic, *Eco*RI and *Bgl*II; 4070A amphotropic, *Eco*RI and *Sma*I; MCF 1 (MCF247-derived), *Eco*RI and *Bam*HI; A-12, *Eco*RI and *Bam*HI; A-5, *Eco*RI and *Pvu*II; B-34, *Eco*RI and *Bam*HI; and A-1, *Pst*I and *Bam*HI. These DNAs, which are shown in lanes 1 to 8, respectively, were visualized by ethidium bromide staining of the agarose gel (panel A), transferred by the Southern procedure, and hybridized with ^{32}P-MCF_{env} oligomer (panel B) as described in Materials and Methods section. The autoradiogram of Hirt DNAs obtained from MCF MuLV-infected mink cells with ^{32}P-MCF_{env} is shown in panel C. The amount of Hirt DNAs applied to the gel was adjusted (10 to 40 μg) so that each lane contained approximately the same amount of linear DNAs. The Hirt DNAs which are present in lanes 9 to 14 are as follows: AKR 13 (11, 12), Akv-1-M111A (32), Akv-1-MB311 (Akv-1-C311 in reference 31), Akv-1-36 (11, 17), BALB/c CB208 (12), and Cas 2S L4 (isolated by Janet W. Hartley from a mesenteric lymph node of an NFS mouse which had developed myelogenous leukemia upon injection with Cas 2S wild-mouse ecotropic MuLV). Molecular sizes (in kilobases) are indicated.

the 190-bp cellular DNA segment, characteristic of the majority of endogenous MuLV LTRs (21). The results, shown in Fig. 6, indicate that radiolabeled $B\text{-}34_{162}$ DNA hybridized with all the subgenomic MuLV RNA species present in both kidney and thymus tissues (lanes 1 and 2, respectively). However, the 8.4-kb MCF_{env}-reactive mRNA species present in thymus tissue (shown in Fig. 4, panel III) did not hybridize with ^{32}P-labeled $B\text{-}34_{162}$ DNA.

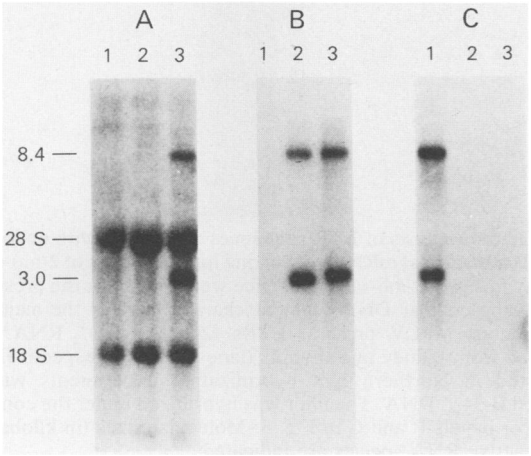

FIG. 3. Specificity of the MCF_{env} oligomer. Total cellular RNAs (5 μg) isolated from AKR ecotropic MuLV-infected NIH 3T3 cells (lane 1), NFS xenotropic (lane 2), and MCF247 (lane 3) MuLV-infected mink cells were subjected to Northern blot analysis and hybridized to ^{32}P-labeled MCF_{env}, X_{env}, and Ec_{env} DNAs (panels A, B, and C, respectively) under the conditions described in Materials and Methods. Sizes (indicated in kilobases) were assessed by comparison with 28S and 18S rRNA bands visualized by ethidium bromide staining of the marker lane, which contained total cellular RNA. The positions of the full-length genomic and *env* mRNAs as well as the 28S and 18S rRNAs are indicated.

DISCUSSION

Our data show that four subgenomic MCF *env*-related mRNAs, 7.2, 6.0, 3.0, and 1.8 kb in size, are temporally expressed in young AKR mice ranging from 5 wk to 5 mo of age. The expression of these messages was independent of the expression of ecotropic and xenotropic MuLVs. Three of the MCF-related RNAs were expressed in a tissue-specific manner: 6.0-kb transcripts were detected only in kidney and

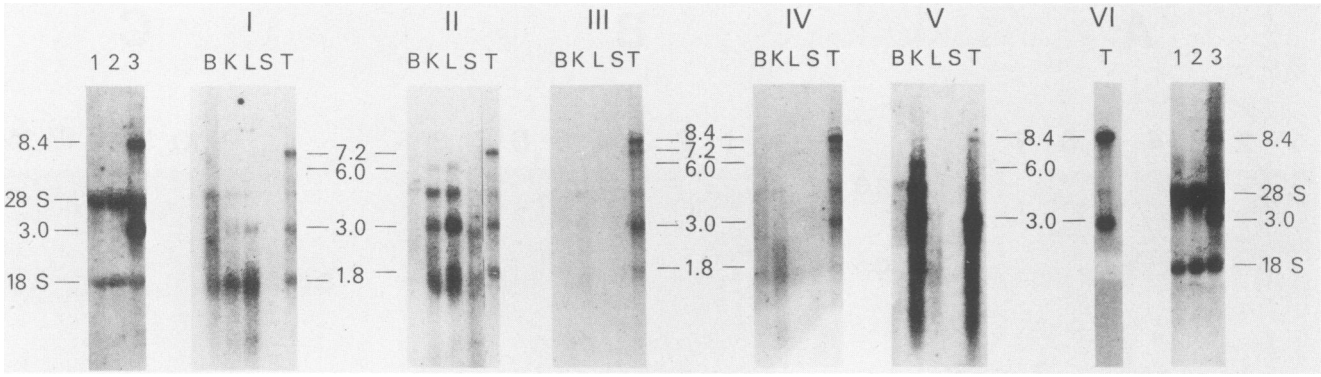

FIG. 4. MCF MuLV-related mRNAs expressed in AKR mouse tissues. Northern blot analyses were performed with 1 μg of poly(A)$^+$ RNAs isolated from the brain (B), kidney (K), liver (L), spleen (S), and thymus (T) of AKR mice ranging from 5 wk to 6 mo of age. Panels: I, 5 wk; II, 2 mo; III, 3 mo; IV, 4 mo; V, 5 mo; VI, 6 mo. The filters were hybridized to ^{32}P-labeled MCF$_{env}$ under the conditions described in Materials and Methods. All the autoradiograms were exposed overnight at −70°C except for the one shown in panel VI, which was developed after 15 min of exposure. Control RNAs were included to demonstrate specificity of the probe during the experiment as well as to serve as molecular-size markers. Each lane contained 5 μg of total cell RNA isolated from AKR ecotropic MuLV-infected NIH 3T3 cells, NFS xenotropic MuLV-, and MCF247 MuLV-infected mink cells (lanes 1, 2 and 3, respectively). The sizes of the MuLV mRNAs are indicated in kilobases. The positions of the 28S and 18S cellular rRNAs are also shown.

liver tissues; 7.2- and 1.8-kb mRNAs were expressed specifically in thymus and bone marrow cells. Endogenous 3.0-kb messages were present in all the tissues tested. In general, the level of these endogenous MuLV transcripts appeared to peak at 2 mo of age, after which they were barely detectable in brain, liver, kidney, and spleen tissues but continued to be seen in thymic tissue until 4 mo of age. The variation seen in the levels of the endogenous MCF-related transcripts at different ages suggests that transcription of endogenous MCF-related proviruses might be regulated by the state of differentiation of particular tissues. Since LTRs have been shown to be involved in tissue tropism (5, 14) and can respond to cellular factors (16, 37), it is possible that the LTRs present in endogenous MCF-related proviruses can determine which sequences will be expressed. Analysis of the LTR sequences indicated that the subgenomic MCF-related mRNAs were transcribed from endogenous MCF-related proviruses which contained a 190-bp DNA insert in their LTRs (21). Since the MCF-related transcripts were expressed in a tissue-specific manner, it seems that different endogenous LTR sequences might be associated with the 3.0-, 6.0-, and 7.2/1.8-kb RNA species. A comparative sequence study of the 3′ termini of cloned cDNAs will provide insight into sequences which might be involved in the tissue-specific expression of the endogenous MCF-related DNAs. Although most of the endogenous MCF *env*-related DNAs which have been isolated from the mouse genome are incomplete because of the presence of internal restriction sites used in cloning or deletion of viral sequences (23), a retroviral length MuLV provirus of this gene family has previously been described (2). However, since we were unable to detect full-length

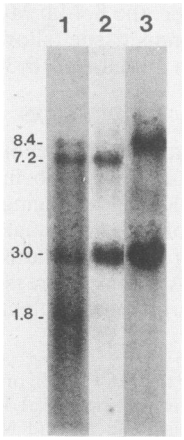

FIG. 5. Expression of endogenous MCF *env*-related mRNAs in AKR *Fv-4*^{r} mice. Northern blot analyses were performed with 5 μg of total cellular mRNA obtained from the thymuses of 2-mo-old AKR/N and AKR *Fv-4*^{r} mice (lanes 1 and 2, respectively). The filter was hybridized to ^{32}P-labeled X_{U3} DNA under the conditions described in Materials and Methods. Control RNA consisted of 5 μg of total cellular RNA isolated from MCF247 MuLV-infected mink cells (lane 3). MuLV mRNA sizes are indicated in kilobases.

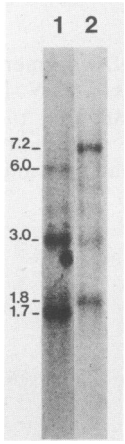

FIG. 6. Analysis of LTR sequences associated with endogenous MuLV transcripts. mRNAs occurring in the kidneys of 2-mo-old and the thymuses of 3-mo-old AKR mice were tested for the presence of the 190-bp cellular DNA segment characteristic of the majority of endogenous MuLV proviral LTRs (21). Poly(A)$^+$ RNA (1 μg) isolated from kidney and thymus (lanes 1 and 2, respectively) was analyzed in Northern blot hybridization experiments with ^{32}P-labeled B-34$_{162}$ DNA. The filter was hybridized under the conditions used for panels B and C in Fig. 3. Molecular sizes (in kilobases) of the reactive RNA species are indicated.

transcripts corresponding to endogenous MCF-related proviruses, our data suggest that the endogenous MCF-related MuLV DNA family is defective for virus expression.

The 6.0-, 7.2-, and 1.8-kb transcripts are unique, since they do not correspond in size to any known virus-associated mRNA. These subgenomic MuLV messages might have resulted from a splicing event or from transcription of deleted endogenous MuLV DNAs. MuLV proviruses which contain deletions in *pol* and *env* have previously been described (23, 39) and might be potential templates for the subgenomic transcripts. It is unknown whether each RNA species is homogeneous and transcribed from a single locus or consists of messages transcribed from different endogenous MuLV DNAs which contain deletions of similar size. RNAs transcribed from the latter DNA templates would appear as a single RNA species in Northern blot analysis, albeit heterogeneous. The 3.0-kb transcripts correspond in size to retroviral *env* messages and most likely represent complete endogenous *env* mRNAs. We also detected 7.2- and 1.8-kb MCF_{env}-reactive transcripts in thymus tissues of 2-mo-old BALB/c mice. Messages of similar sizes were previously identified by Boccara et al. in BALB/c mouse thymus tissue by using an MuLV DNA probe which could react with MCF as well as xenotropic MuLV sequences (3). However, the relatedness between the RNA species in the two mouse strains is unclear, since their expression seems to be regulated differently: 7.2- and 1.8-kb RNAs were not detectable in AKR thymus tissue after 4 mo of age and in MCF-induced thymomas (Laigret et al., in preparation), whereas these RNA species were seen at an increased level in X-ray-induced thymoma cell lines of 9-mo-old BALB/c mice.

The role of the MCF-related mRNAs detected before MCF virus isolation in MuLV-induced leukemogenesis is unclear. The generation of thymotropic MCF viruses, which have been implicated as the proximal causal agent in T-cell lymphomas in AKR mice, has previously been shown to involve ecotropic, xenotropiclike, and endogenous MCF-related sequences (20, 42). Since recombination might occur via interaction between viral messages (28), the subgenomic MCF-related transcripts which are expressed in AKR thymus tissue, such as the 7.2-, 3.0-, and 1.8-kb mRNAs, might participate in the formation of thymotropic recombinant MCF MuLVs. The involvement of the endogenous MuLV messages in MCF virus generation is suggested by expression of the subgenomic MCF-related messages before (5 wk to 2 mo) as well as concurrent with (3 to 4 mo) the appearance of 8.4-kb MCF-related transcripts. The nature of the latter full-length transcripts, which were detected before MCF virus isolation (about 5 mo of age; [7, 17]), is unknown. Several possibilities can be entertained regarding the origin of the 8.4-kb pre-MCF mRNAs. (i) The RNAs might have been generated by recombination between different endogenous MuLV sequences, including endogenous MCF-related sequences, and might represent intermediate structures in the formation of MCF MuLVs. (ii) The 8.4-kb RNA species might be transcribed from a novel family of endogenous MuLV proviruses or might correspond to the genomes of the recently described MuLVs designated ERVs (10). The latter virus family is unique in that the recombinant MuLVs isolated from 3- to 4-mo-old AKR mouse thymus tissue appear to be MCF-related in *env* but display an ecotropic host range. (iii) Another interesting possibility is that the 8.4-kb RNA species might represent mRNAs of endogenous MCF-related DNAs from which the 190-bp insert in the LTR has been deleted. Since expression of the full-length pre-MCF transcripts was restricted to the thymus, MuLVs associated with this RNA species might be involved in MCF-induced thymic leukemias. We are currently analyzing cloned cDNAs of the MCF-related transcripts to determine their molecular structure and relatedness to lymphomogenic MCF viruses.

ACKNOWLEDGMENTS

We thank Marcia L. Meltzer and Willard W. Davis for excellent technical assistance and Susan K. Rosenfeld for typing the manuscript.

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