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Age-Dependent Changes Are Observed in the Levels of an Enzyme Mediator of Interferon Action: A (2′-5′)A_n-Dependent Endoribonuclease (42815)

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Abstract. A (2′-5′)oligoadenylate-dependent endoribonuclease (RNase L) is an important mediator of interferon's antiviral actions. Levels of this enzyme were determined in spleen, lung, liver, and kidney of mice at different times after birth. The levels of RNase L were found to be relatively low in newborn kidney, lung, and spleen. RNase L levels rise 2- to 10-fold in these three tissues as mice approach 5 days of age. In the spleen, levels of RNase L remain high as mice reach adult life. In the lung and kidney, however, RNase L levels decrease after 14 days. RNase L levels in the liver are highest from birth to 5–7 days and then decrease subsequently and remain low in adult mice. These changes in RNase L levels with postnatal development may be important with regard to age-specific susceptibility to some virus infections.

Mammalian cells and various mammalian tissues contain an endoribonuclease (RNase L) that is normally latent unless activated by (2′-5′)oligoadenylates ((2′-5′)A_n (n = 2–15)) (1, 2). The activated enzyme cleaves single-stranded RNAs preferentially 3′ to UA, UG, and UU sequences (3, 4). The (2′-5′)A_n molecules are produced in cells by another enzyme, a (2′-5′)$A_n$synthetase, which is in turn activated by double-stranded RNA (5). The (2′-5′)$A_n$synthetase, normally present at low levels in cultured cells, is induced 10- to 1000-fold by interferon treatment (1, 5) and by changes associated with a decrease in cell growth rates (1, 6). RNase L, like the (2′-5′)$A_n$synthetase, is generally present at low levels in actively growing cultured cells but can be induced by interferon treatment (7–10) and has been shown to increase as the rate of cell growth decreases (8–10). RNase L and the (2′-5′)$A_n$synthetase have been implicated as antiviral proteins which inhibit translation by increasing the rate of mRNA degradation in interferon-treated, virus-infected cells (1, 11). Increased levels of (2′-5′)A_n activation of RNase L are observed in interferon-treated mouse L cells infected with encephalomyocarditis virus (12). Conversely, microinjection of an analog inhibitor of (2′-5′)A_n restores encephalomyocarditis viral RNA accumulation in interferon-treated cells (13). Recently, Chebath *et al.* (14) have shown that Chinese hamster ovary cells transfected with cDNA encoding the (2′-5′)$A_n$synthetase constitutively express this gene which renders these cells resistant to infection with mengovirus and other picornoviruses in the absence of interferon treatment. Additional roles for the (2′-5′)$A_n$synthetase and RNase L as potential regulators of cell growth rates and as mediators of interferon's cell growth inhibitory actions have been proposed (15–17); however, these have yet to be clearly defined or demonstrated (18).

RNase L levels in murine embryonal carcinoma cells have been found to be low in undifferentiated cells and to rise sharply when cells are induced to differentiate (10). Low levels of RNase L in undifferentiated cells are correlated with resistance to the antiviral and cell growth inhibitory (10) activity of interferons. Induction of RNase L in embryonal carcinoma cells following differentiation suggests that expression of the gene encoding this enzyme is regulated during changes associated with development and/or differentiation, as well as changes in cell growth rates.

Regulation of RNase L levels in several tissues of Balb/c mice has been investigated (19). RNase L is found in murine tissues at levels comparable to those seen in cultured cells at saturation density, with some tissues, i.e., spleen and lung, having relatively high

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levels of this enzyme and others, i.e., liver, having lower levels. RNase L is not induced significantly following interferon treatment; however, RNase L levels are depressed in mice receiving injections of rabbit anti-interferon immunoglobulin (19) suggesting that high levels of RNase L are maintained in animals by continuous production of endogenous interferon. These data suggest that RNase L levels are regulated *in vivo* as well as *in vitro.*

The current studies were undertaken to determine whether RNase L levels are regulated during postnatal development of Balb/c mice. Previously Galabru *et al.* (20) had shown that constitutive levels of the (2′-5′)$A_n$synthetase are very low in mice aged 8–18 days, rising in mice aged 19–57 days, and then falling off in older mice. Results of this investigation suggest that RNase L levels, like those of the (2′-5′)$A_n$synthetase, are regulated temporally during postnatal development.

Methods. *Materials.* The (2′-5′)A_3 from Pharmacia (Piscataway, NJ), T4 RNA ligase from New England Biolabs (Beverly, MA), and [5′-^{32}P]cytidine-3′,5′-bisphosphate from Dupont/NEN Research Products (Boston, MA) were used to synthesize (2′-5′)A_3-[^{32}P]pCp as previously described (21). Other reagents were from standard commercial suppliers.

Mice and preparation of tissue extracts. A breeding colony of Balb/c mice was maintained at the Animal Research Center at Arizona State University and used as a source of juvenile and adult mice. Cytoplasmic tissue extracts were prepared from the kidney, liver, lung, and spleen. These were initially placed in ice-cold wash buffer (140 m*M* NaCl, 2 m*M* KCl, 10 m*M* Na–K phosphate buffer, pH 7.5), dried briefly on paper towels, weighed, placed in dishes containing homogenization buffer (80 m*M* KCl, 5 m*M* $MgCl_2$, 0.3 m*M* $CaCl_2$, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 m*M* 2-mercaptoethanol, 250 m*M* sucrose, 20 m*M* Tris–Cl, pH 7.5), and minced to about 1 mm^3. Tissues from littermates were pooled for newborn and 1-, 2-, 5-, 7-, and 14-day-old mice prior to homogenization. Tissues from older mice were prepared from individual animals. The minced tissues were homogenized using a Potter Elvehjem-type mechanical homogenizer with Teflon pestle at a tissue to homogenization buffer ratio of 1:10 (w/v). The crude nuclei were removed by centrifugation at 600*g* for 5 min and a postmitochondrial supernatant fraction (S30) was obtained by centrifugation at 30,000*g* for 30 min. These extracts were stored in aliquots at −70°C.

Assays for RNase L. Assays for (2′-5′)A_n-dependent endonuclease activity have been described (21–23). RNase L is characterized by two activities, an endonuclease activity dependent on the presence of (2′-5′)A_n (21, 24) and a (2′-5′)A_n (24) or (2′-5′)A_3pCp (21, 22) binding activity. These activities copurify during chromatography on ion exchange columns, gel filtration, adsorption onto hydroxylapatite, differential precipitation with ammonium sulfate, and affinity chromatography using poly(A) agarose (4, 7, 21) and are associated with the presence of a single polypeptide (mol wt 80,000). The (2′-5′)A_3pCp binding assay has proven to be the most quantitative method currently available for assaying the levels of this protein (21). Previous studies established optimal conditions for this binding assay using murine tissue extracts (19).

RNase L can be crosslinked to (2′-5′)-A_3pCp by exposure to high-intensity UV light (21, 23). The crosslinked product is a single polypeptide (mol wt 80,000) that has been found in murine cultured cells (23) and in various murine tissues (19). In murine cultured cells and in murine tissues, the 80,000 molecular weight protein is the major protein specifically labeled by (2′-5′)A_3pCp (8, 19, 23). A filter binding assay for the presence of RNase L in tissue extracts which measures the amount of labeled (2′-5′)A_3-[^{32}P]pCp, a derivative of (2′-5′)A_n, retained on nitrocellulose filters is the primary assay used in these studies (21).

Methods for preparation of the derivative of (2′-5′)A_n, [^{32}P](2′-5′)A_3pCp (sp act 3000 Ci/mmole at the date specified by the manufacturer (Dupont/NEN)) have been described (22). The specific activities for (2′-5′)A_3[^{32}P]pCp on the dates of the experiments were calculated according to the formula given by the manufacturer and were

between 1500 and 4000 Ci/mmole. Photocrosslinking of RNase L to (2′-5′)A_3[^{32}P]pCp by ultraviolet light was performed essentially as described (21, 23) and the labeled protein identified by its electrophoretic mobility on 10% polyacrylamide gels containing sodium dodecyl sulfate autoradiography of the desiccated gel (21, 23). Protein concentrations were determined by the Bradford method (25) using the protein assay kit from Bio-Rad Inc. (Richmond, CA). The relative intensity of autoradiographic exposure as well as the amount of protein as revealed by Coomassie brilliant blue staining in each lane of the gel was determined using a transmission densitometer.

Results. Levels of RNase L in murine tissue extracts were determined using the (2′-5′)A_3pCp binding assay and photocrosslinking assay. Extracts consistently contained a protein (mol wt 80,000) that could be photocrosslinked to (2′-5′)A_3pCp. Figure 1 shows the proteins from lung and spleen which were photocrosslinked to (2′-5′)A_3pCp in the presence or absence of unlabeled (2′-5′)A_3. Almost all of the (2′-5′)A_3pCp was specifically crosslinked to one protein (mol wt 80,000) which had been previously shown to copurify with RNase L (23). Lesser amounts of (2′-5′)A_3pCp were bound to three other proteins (mol wt 62,000, 45,000, and 40,000) present in tissue extracts. The 45,000 and 40,000 molecular weight proteins were previously shown to be proteolytic degradation products of RNase L (mol wt 80,000) (10, 19). Preparation of murine tissue extracts in the presence of 1 m*M* PMSF was found to prevent formation of the 40,000 and 45,000 molecular weight proteins. A 62,000 molecular weight protein was seen in photocrosslinking experiments in which a (2′-5′)A_3pCp is present at 0.25 to 0.5 n*M*, but not seen in photocrosslinking reactions in which (2′-5′)A_3pCp was present at around 0.05 n*M*. It is not known whether or not the 62,000 molecular weight protein is a degradation product of RNase L or if this protein has a specific function related to the binding of (2′-5′)A_3pCp. Previously, a protein of the same molecular size was found in nuclear extracts from EAT cells (26). When autoradiograms were scanned using a transmission densitometer the 80,000 molecular weight protein was generally shown to contain 90–95% of the radioactivity. Some extracts showed higher levels of the 40,000 and 45,000 molecular weight proteins than others. Extracts prepared from murine intestine have relatively large amounts of the 40,000 and 45,000 molecular weight proteins (19) as do extracts prepared in the absence of PMSF (8). In the current studies, the relative amounts of the 40,000 and 45,000 molecular weight proteins were low for all tissue extracts except for those prepared from livers of older mice which showed a slight increase (10–20%) in the relative amounts of the 40,000 and 45,000 molecular weight proteins.

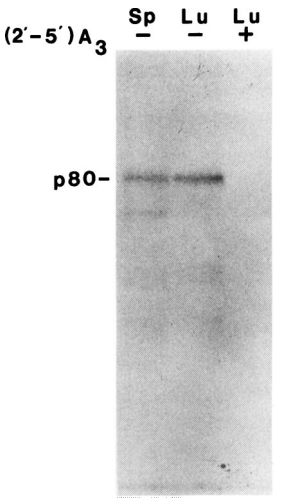

FIG. 1. Photocrosslinking of (2′-5′)A_3pCp to protein from murine lung and spleen tissues. Aliquots of tissue extracts containing 150 mg protein from 8-week-old murine spleen and lung were incubated in 0.1-ml assay mixtures containing 20 fmole (2′-5′)A_3[^{32}P]pCp and photocrosslinked as described under Methods. One micromolar (2′-5′)A_3 was present (+) or absent (−) during incubation and photocrosslinking as indicated. Photocrosslinked proteins were observed by electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate followed by autoradiography as described previously (21). The mobility of RNase L (mol wt 80,000) is indicated.

Age-dependent Changes in RNase L levels. RNase L levels in tissues obtained from mice at different ages were determined using the (2′-5′)A_3pCp binding assay and the (2′-5′)-

A_3pCp photocrosslinking assay. Figure 2 shows the effect of age on the amount of (2′-5′)A_3pCp bound to extracts prepared from four tissues. The amount of (2′-5′)A_3pCp bound depended upon both the tissue type and the age of the mice. The mechanism for regulating levels of RNase L in kidney, liver, lung, and spleen appeared to be different.

The level of RNase L present in extracts of murine kidney tissue as determined using the (2′-5′)A_3pCp binding assay was highly variable; however, age-related changes were clearly observed (Fig. 2). Kidney tissue from newborn mice had relatively low levels of RNase L. The levels rose to a peak between 2 and 14 days and then dropped off in older mice (up to 6 months old). These results were consistent with data obtained when the amounts of radioactive (2′-5′)A_3pCp cross-linked to the 80,000 molecular weight protein were compared (Fig. 3, Table I). The autoradiogram shown in Fig. 3 and the corresponding desiccated polyacrylamide gel were scanned using a transmission densitom-

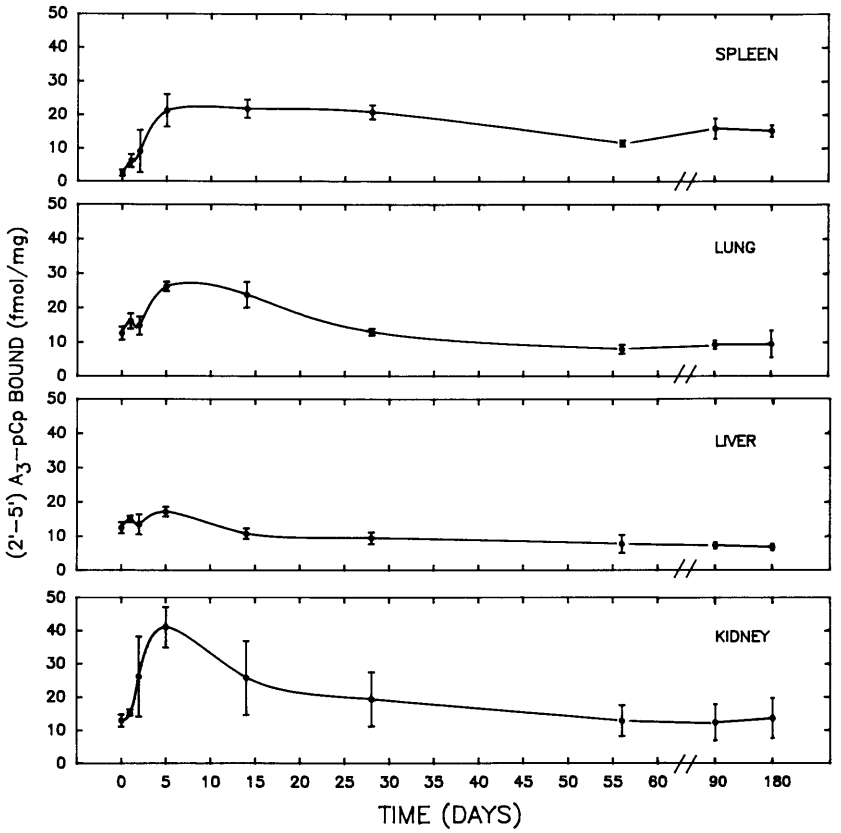

FIG. 2. RNase L levels in murine tissues prepared at varying times after birth. The postmitochondrial supernatant fractions from murine spleen, lung, liver, and kidney were prepared from Balb/c mice at birth (0 days) and at varying times subsequent to birth. Tissue preparations from mice aged 0–5 days were pooled from four mice within the same litter. Mice aged 14 days were pooled from two mice within the same litter. Tissues from older mice were prepared individually. The tissue extracts were incubated with 6 fmole (2′-5′)A_3pCp in 0.1-ml assay mixtures as described previously for the (2′-5′)A_3pCp binding assay. Protein concentrations were determined according to the method of Bradford (25). The amount of (2′-5′)A_3pCp bound is expressed as femtomoles per milligram protein. For each determination, four tissue preparations were used, each of which was assayed in duplicate. The values are given as the means ± SD (ordinate) for the four determinations.

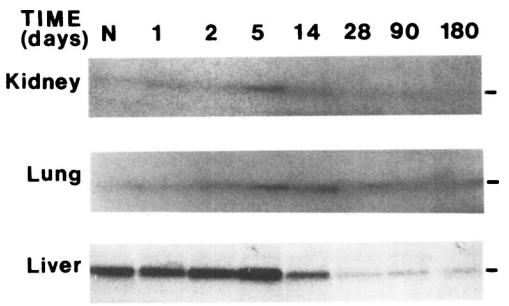

FIG. 3. Photocrosslinking of (2′-5′)A_3pCp to protein in postmitochondrial supernatant fractions prepared from murine tissues obtained at the ages indicated. Aliquots of tissue extracts from mice of varying ages were incubated in 0.1-ml assay mixtures containing 20 fmole (2′-5′)A_3[^{32}P]pCP and photocrosslinked as previously described (21). The amount of protein photocrosslinked was 0.3 mg for liver extracts and 0.15 mg for lung and kidney extracts. Proteins were separated by SDS–polyacrylamide gel electrophoresis and labeled proteins were visualized by autoradiography. Time (N = newborn to 180 days) is indicated across the top. The dash indicates RNase L (mol wt 80,000).

eter. The relative intensity of autoradiographic exposure for the (2′-5′)A_3pCp photocrosslinked 80,000 molecular weight protein in kidney tissue extracts was found to rise two- to threefold in young mice and then fall in older mice to levels comparable to those seen in newborn mice.

Liver extracts of newborns, unlike kidney extracts of newborns, had relatively high levels of RNase L as did liver extracts prepared from 1- to 14-day-old mice (Fig. 2). The levels of RNase L in liver extracts prepared from older mice were about half those seen in mice aged 2–14 days as determined by the (2′-5′)A_3pCp binding assay. Relative autoradiographic exposure of the 80,000 molecular weight protein photocrosslinked to (2′-5′)A_npCp also showed age-specific changes (Fig. 3). When the autoradiogram shown in Fig. 3 and the corresponding desiccated gel were scanned with a transmission densitometer (Table I), peak RNase L levels were seen in mice aged 2 days and the lowest levels were seen in older mice. The levels of RNase L as determined by the (2′-5′)A_3pCp photocrosslinking assay were 5- to 10-fold higher in 2-day-old mice than in 28–180 day old mice. The age-dependent changes in RNase L levels as detected by the (2′-5′)A_3pCp photocrosslinking assay (Fig. 3) were more pronounced than the changes detected by the (2′-5′)A_3pCp binding assay (Fig. 2). Low levels of (2′-5′)A_3pCp photocrosslinked to the 80,000 molecular weight protein in liver extracts from older mice were also associated with a slight increase (not shown) in the amount of (2′-5′)A_3pCp photocrosslinking to 45,000 and 40,000 molecular weight proteins which were previously shown to be a proteolytic degradation product of RNase L (10, 19). Increased degradation of RNase L to a product of 45,000 as well as smaller products which also bind (2′-5′)A_3pCp could result in a decrease in the levels of the 80,000 molecular weight protein as detected by the photocrosslinking assay without a concomitant decrease in overall (2′-5′)A_3pCp binding activity. It was not clear whether increased proteolysis of RNase L might have occurred prior to preparation of tissue extracts or during preparation of extracts. An age-dependent change in the *in vivo* protein turnover

TABLE I. RELATIVE INTENSITY OF AUTORADIOGRAPHIC EXPOSURE FOR 80,000 MOLECULAR WEIGHT PROTEIN IN TISSUES FROM MICE AT DIFFERENT AGES

Age (days)	N	1	2	5	14	28	90	180
Kidney	100[a]	140	297	237	253	120	140	93
Liver	100	253	354	287	161	76	43	36
Lung	100	125	169	225	344	181	194	194
Spleen	100	796	756	810	610	1316	1189	729

[a] Relative optical density was determined by scanning autoradiograms and the corresponding lanes of a Coomassie brilliant blue stained gel using a transmission densitometer. The ratio of the relative autoradiogram intensity to relative intensity of protein stains was determined. All values are given as a percentage of that observed for newborn mice.

rate for RNase L could account for lower levels of the protein in the livers of older mice. Further experiments would be needed to verify or refute this possible mechanism for age-dependent changes in RNase L levels in the liver.

In murine lung tissue, RNase L levels rose rapidly following birth then decreased gradually as mice matured. Newborn to 2-day-old mice had low levels of RNase L whereas 5- to 14-day-old mice had two- to fourfold higher levels of RNase L activity (Fig. 2). These changes in the level of RNase L seen in the binding assay are reflected in photocrosslinking experiments as shown in Fig. 3. When the autoradiogram shown in Fig. 3 and the corresponding desiccated gel were scanned with a transmission densitometer, 14-day-old mice were found to have three to four times the amount of 80,000 molecular weight protein as newborn mice (Table I).

RNase L levels in murine spleen increased at least 5- to 10-fold during early postnatal growth as shown in Figs. 2 and 4 and Table I. Extracts prepared from spleens of newborn mice contained very little RNase L. The levels of RNase L in newborn spleen was often below the level of sensitivity for the assay. One- to two-day-old mice had a variable amount of RNase L that was within the range detectable using the (2′-5′)A_3pCp binding assay. After 5–14 days, the levels of RNase L in murine spleen rose sharply and remained high in mice up to 4 weeks old. Thereafter the amount of RNase L decreases somewhat as mice approach 180 days. In order to more closely examine changes occurring in RNase L levels during early postnatal development of the spleen, spleens from mice aged 6–12, 12–24, and 24–28 hr were compared to 3-, 4-, 5-, and 7-day-old mice as shown in Fig. 4. In this experiment, the most rapid changes appeared to occur between the 6–12 and 12–24 hr with a total increase of about 10-fold. Of all four tissues examined, the spleen showed the greatest consistent change in RNase L levels with postnatal development and aging as determined by these assays.

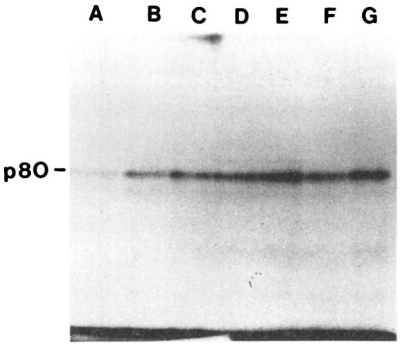

FIG. 4. Photocrosslinking of (2′-5′)A_3pCp to protein from postmitochondrial supernatants from murine spleen. Photocrosslinking of (2′-5′)A_3pCp to 0.3 mg spleen extract was performed as described in the legend to Fig. 3. The 80,000 molecular weight protein is indicated. Lane A, newborn, 6–12 hr old; lane B, 12–24 hr old; lane C, 36–48 hr old; lane D, 3 days old; lane E, 4 days old; lane F, 5 days old; lane G, 7 days old.

Discussion. The observed changes in RNase L levels in murine tissues during postnatal development and aging of mice provide evidence that RNase L levels are modulated *in vivo* as well as *in vitro.* The functional significance of this age-dependent variation in RNase L levels has yet to be investigated. The (2′-5′)$A_n$synthetase and RNase L have been characterized as antiviral proteins in cultured cells (1, 2) and presumably these proteins have a protective antiviral function *in vivo.* Age-dependent susceptibility to viral infections have been observed in mice (27, 28) and in humans (28, 29) and it is possible that increased susceptibility to some viral infections may be due to low levels of the (2′-5′)$A_n$synthetase and/or RNase L.

Temporal modulation of RNase L levels in tissues could hypothetically be a consequence of changes in the relative proportion of different cell types due to differentiation *in situ* or in migration of cells from another tissue of origin. Murine liver is the site of hemopoiesis during fetal life and in the early neonatal period, but ceases to function in this capacity as the animal matures (30). The spleen becomes a site of hemopoiesis just prior to birth and continues to function as a hemopoietic tissue into adult life (30, 31). Since reticulocytes, precursors to mature red blood cells, are known to have relatively high levels of RNase L (32), the relatively high levels of RNase L in newborn liver could be a

consequence of the large proportion of blood-forming cells present in liver at that time. Likewise the RNase L levels in spleen may rise as this tissue assumes a role in hemopoiesis. Murine spleen also contains many different cell types that arise *in situ* through differentiation of precursor stem cells or which migrate to the spleen from other sites, i.e., the thymus (31). The dramatic rise in RNase L levels in the spleen could also be associated with development of the reticuloendothelial system and formation of lymphatic tissue. Tumor cells derived from lymphocytes also have relatively high levels of RNase L (33). In the future, it should be possible to resolve which cells within a tissue contain high levels of RNase L using antibody to RNase L (34) in combination with immunohistochemical staining (35). These studies would be able to show whether age-related changes in RNase L levels are the result of changes in the relative abundance of different cell types within tissues or occur as a result of temporal changes within cells.

The rise in RNase L levels seen in young mice may be a consequence of cell growth regulation and/or differentiation taking place within various tissues. RNase L is apparently induced when cultured cells become growth arrested (8, 9) or when undifferentiated embryonal carcinoma cell lines are induced to differentiate (10). When one embryonal carcinoma cell line, PC 13, was induced to differentiate by culturing in media containing $O^{2'}$-dibutyryl-adenosine-3',5'-monophosphate, 3-isobutyl-1-methylxanthine, and retinoic acid, the levels of RNase L rose during the 7-day treatment (10). These studies using embryonal carcinoma cells suggest that RNase L levels are modulated by differentiation and/or developmental processes.

The functional consequences of changes in RNase L levels with postnatal development are unknown; however, it is possible that these changes may affect the ability of the mouse to mount an antiviral response against RNA viruses. The (2'-5')$A_n$synthetase and RNase L have been shown to inhibit picornovirus replication at the level of RNA degradation in tissue culture cells (12–14). Several genera of the picornoviridae have also been shown to affect newborn or suckling mice more severely than adult mice (28). Inoculation of mice aged 3–7 days with several species of Coxsackie virus results in paralytic infection whereas 12-day and older mice are not susceptible (36). Several enteroviruses replicate and produce fatal lesions in 1- to 3-day-old mice, but do not have any detectable effect on older mice (28, 37). Low levels of RNase L in several tissues of neonatal and very young mice as well as low levels of the (2'-5')$A_n$synthetase in mice less than 18 days old (20) may partially explain their increased susceptibility to these infections.

Regulation of RNase L levels *in vivo* during postnatal development of mice occurs within the context of complex changes required for the development of host defense systems, including the immune system. Future studies should determine how regulation of RNase L levels fit within this context and whether changes in RNase L levels reflect changes in the distribution of specific cell types within tissues, or changes associated with cell differentiation during development. Further experiments to test the hypothesis that low RNase L and (2'-5')$A_n$synthetase levels in very young mice account for increased susceptibility to enterovirus infection should help to define a role for these enzymes *in vivo*. A measurement of the levels of (2'-5')A_n (12) in control and virus-infected tissues from neonatal and older mice would provide additional evidence that the (2'-5')$A_n$synthetase–RNase L pathway is an important murine antiviral enzyme system.

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