CHAPTER 3

STUDIES ON ALBUMIN mRNA DEGRADATION
PART-A: STUDY OF IN VIVO GENERATED CLEAVAGES OF MOUSE LIVER ALBUMIN mRNA

3.1 INTRODUCTION

The nature of mRNA degradation machinery is an important aspect of mRNA turnover that needs to be understood in greater detail and the information on in vivo generated mRNA degradation intermediates (Dis) would be important in this respect. Study of mRNA Dis is very useful in several ways.

(a) By mapping the ends of the Dis onto the mRNA, the cleavage sites can be localized and one can determine if the cleavages are endo- or exonucleolytic in nature.
(b) The sequences around the cleavage sites may give important additional information on the specificity of the cleavage process.
(c) If the formation and subsequent fate of the intermediates could be followed in a time dependent fashion, then the degradation pathway can also be worked out.

These information may be useful in identifying and purifying the enzymes responsible for the process.

Though several studies have been reported on mRNA turnover, the in vivo Dis of mRNAs have been studied only in a few eukaryotic cases (Ross et al., 1986; Cochrane and Deeley, 1989; Binder et al., 1989; Brown and Harland, 1990). In three of these studies (Cochrane and Deeley, 1989; Binder et al., 1989; Brown and Harland, 1990), short range (3-5 nucleotides long) primary structural homology has been observed between the various cleavage sites on the mRNA. However, the cleavage site sequences thus identified are different in each of these three cases.

Albumin mRNA is an abundant message expressed specifically in liver. Though it is known to be a very stable message (Peavy et al., 1985; Yamauchi et al., 1988), it is also known to be specifically destabilized under some conditions; e.g., in Xenopus liver after estrogen treatment (Kazmaier et al., 1985; Wolff et al., 1985; Schoenberg et al., 1989). Although the abundance of albumin mRNA may enable relatively easy detection of its in vivo Dis if any, such studies have not been reported so far. Pastori et al (1991b) have reported a specific fragment generated from albumin mRNA in vitro by an estrogen induced endonuclease activity. The expression of albumin mRNA is regulated during development and this has been well studied in rat and mouse systems. However, the role of mRNA turnover in this is not clear yet. Also, though albumin is believed to be liver specific, its mRNA was found to express at significant levels even in nonhepatic tissues like kidney, pancreas, heart, lung etc., at prenatal and early postnatal stages in rat (Nahon et al., 1988). We therefore, asked if an analogous situation exists in mice and aimed to study the following:
(a) Expression of albumin in several nonhepatic fetal tissues as compared to adult and fetal liver in mouse.
(b) In vivo generated Dls and cleavages, if any, of albumin mRNA in fetal and adult liver and various fetal nonhepatic tissues of mouse.
(c) Relationship of the cleavages with the developmental status of the tissue and cell type and its implication.

3.2 RESULTS

3.2.1 Albumin mRNA expression in nonhepatic fetal tissues of mouse

Equal amounts of total RNA isolated from 18-19 days old fetal mouse brain, heart, lung, kidney and liver and adult mouse liver were subjected to S1 protection assays using 5'-end labelled albumin cDNA probe (see section 2.1.2 and Fig.1) and the results are shown in Fig.2. Significant protection of the probe was observed with fetal and adult liver RNA samples as expected. However, RNA from fetal lung, kidney, brain and heart did not give any detectable signal. The absence of any signal with E. coli RNA used as control indicates the specificity of hybridization of the probe.

Rat and mouse are very closely related systems and therefore, this result is somewhat surprising. However, such differences in gene expression between these two systems have also been reported earlier. For example, alphafetoprotein (AFP) mRNA expresses at a significant level in rat fetal kidney (Sellem et al., 1984) but not in mouse fetal kidney (Dziadek and Andrews, 1983). Similarly, albumin mRNA expresses at significant levels in mouse yolk sac but not in rat yolk sac (Sellem et al., 1984).

3.2.2 Detection and characterization of albumin mRNA degradation intermediates and cleavages in mouse liver

In some of the experiments of the kind described above, fragmentation of albumin mRNA had been observed in fetal mouse liver. So, it was planned to study the Dls of albumin mRNA in more detail and the S1 nuclease protection analysis technique was used for such studies due to the following reasons:

(a) Dls of eukaryotic mRNAs are normally present in very low levels; so their detection and study would demand the use of sensitive techniques. S1 nuclease protection analysis involves nucleic acid hybridization in solution which is more sensitive than the filter hybridization employed in northern blot analysis.
(b) Some earlier studies had revealed the advantage of S1 nuclease protection analysis over other techniques like northern blot analysis for detection of mRNA Dls (Binder et al., 1989).
(c) S1 nuclease protection analysis is known to give much cleaner background than Northern.
(d) With the use of end-labelled probes for S1 nuclease protection analysis, ends of the Dls can be mapped quite accurately.
Fig. 2. Albumin mRNA expression in various fetal tissues of mouse. Total RNA from adult (lane 2) and fetal (lane 3) mouse liver and other fetal tissues (lanes 4-7) of mouse were subjected to S1 nuclease protection assay using 5' -end labelled albumin cDNA probe as described in Methods. The S1 nuclease protected DNA samples obtained were electrophoresed on a short denaturing polyacrylamide gel and autoradiographed as described in Methods. Control reaction performed with E. coli RNA is shown in lane 1. Amounts of RNA used were, 3 and 5 µg respectively in the case of adult and fetal liver total RNA and 30 µg in all other cases. Untreated albumin cDNA probe run along with the protected DNA samples is shown in lane 8.
Fig. 3 shows a diagrammatic representation of the detection of mRNA DIs and mapping of their ends by S1 nuclease protection analysis employing end labelled probes. When an end labelled cDNA probe corresponding to a particular mRNA is hybridized with an RNA sample containing that mRNA and the reaction mixture is subjected to S1 nuclease treatment, the undegraded mRNA molecules and those DIs whose both ends lie outside the region spanned by the probe, protect the entire length of the probe. Since the undegraded mRNA is always present at much higher levels compared to its DIs, the S1 nuclease protected DNA contains the full length protected probe as the major species. However, those DIs which are truncated in such a way that at least one of their ends is 'internal' (i.e., lie within the region spanned by the probe), protect the probe only partially, and hence, give rise to smaller (less than full length) protected DNA species. Since the probe is end-labelled, only those DIs which protect the labelled end of the probe, will give rise to protected DNA species detectable by autoradiography. The 'internal' ends of such DIs can be readily mapped onto the mRNA from a knowledge of the sizes of the smaller protected DNA species resulting from them and map position of the labelled end of the probe on the mRNA. The size (in nucleotides) of any smaller protected DNA species is equal to the distance between those points on the mRNA which the labelled end of the probe and the 'internal' end of the corresponding DI map to.

S1 nuclease protection analysis of adult and fetal mouse liver RNA with 5'-end labelled albumin cDNA probe

Fig. 4 shows the results of S1 nuclease protection analysis of 2 different pairs of fetal and adult mouse liver total RNA preparations performed with 5'-end labelled albumin cDNA probe. As expected, the full length protected probe ('A') was seen in both samples. Some truncated protected DNA species were also seen indicating the presence of fragments of albumin mRNA in the samples. Importantly, with fetal liver total RNA samples 3 prominent smaller protected DNA species (B, C and D) were observed which were almost undetectable in adult liver RNA samples. In these experiments, the amounts of fetal and adult liver RNA taken for S1 nuclease protection analysis were equal in each pair of samples. Moreover, after the experiment, equal amounts of protected DNA radioactivity obtained with fetal and adult liver RNA were used for gel electrophoresis. This is shown by the equality in the intensity of the full length protected species 'A' (which mainly represents the undegraded mRNA and hence would be expected to form the most predominant species in the S1 protected samples) in adult and fetal liver lanes of each pair. So, the differences in the intensities of species B, C and D between adult and fetal samples are unlikely to be due to differences in the amount of RNA used or amount of protected DNA loaded. Therefore, these results indicate that some specific DIs of albumin mRNA may be present in fetal liver but absent in adult liver.

Control experiments to examine the in vivo origin of degradation intermediates and S1 controls

RNA samples were used in all the experiments only after ensuring their integrity by agarose gel electrophoresis (Fig.5). However, in any RNA analysis, especially the study of cleavages of RNA, it is very important to ensure that the cleavages observed are not a result of experimental
Fig. 3. Mapping the termini of mRNA degradation intermediates by S1 nuclease protection analysis using end labelled probes. Thin lines indicate mRNA and its fragments. Solid bars represent cDNA probe and S1 nuclease protected DNA species with asterisks indicating their labelled ends. Vertical arrows indicate the cleavages, L, M, N, O and P which generate the various fragments of mRNA. K and Q are map positions of 3' and 5' ends of the probe on the mRNA. The mRNA fragments, v, w, x and y give rise to protected DNA species V, W, X and Y respectively during S1 nuclease protection analysis. Undegraded mRNA gives rise to full length protected probe species Z. Note that fragment z also gives full length protection to the probe and hence gets scored as full length mRNA because neither of its ends is 'internal' (see text). Fragments x and y go undetected since they give rise to protected species (X and Y) that lack the labelled end. Since Q corresponds to 5'-end of the probe, the sizes (in number of nucleotides) of V and W equal the distances (in number of nucleotides) of cleavage sites O and L respectively from Q, and hence, the map positions of O and L can be readily determined.
Fig. 4. S1 nuclease protection analysis of albumin mRNA in fetal and adult mouse liver using 5'-end labelled albumin cDNA probe. 3 µg each of 2 different total RNA preparations of fetal (lanes 2 and 4) or adult (lanes 1 and 3) mouse liver were subjected to S1 nuclease protection analysis with 5'-end labelled albumin cDNA probe and protected DNA fragments obtained were run on a long denaturing polyacrylamide gel (37 cm long, 0.4 mm thick) and autoradiographed as described in Methods. Radioactivity of protected DNA loaded was equal in the 2 lanes of each pair. Positions and sizes (in bp) of pBR322/Hind I marker DNA fragments (M) electrophoresed under identical conditions are shown with each pair of lanes. The full length protected DNA species A and the smaller protected DNA species B, C and D (which correspond to putative DIs) are indicated by arrows.
artifacts (during experimental steps like subcellular fractionation, S1 nuclease protection analysis etc). To this end, various control experiments were performed. The results of these controls and other observations given below indicated that species, B, C and D actually correspond to in vivo generated DI's and not experimental artifacts.

(a) B, C and D were very reproducibly observed in several preparations of fetal liver RNA (Fig.6).

(b) B, C and D were also detected with fetal liver cytoplasmic RNA (Fig.7). Cytoplasmic RNA preparation procedure is very different compared to total RNA isolation procedure (see section 2.2.2). Therefore, this result indicates that the RNA fragments corresponding to species B, C and D are not generated during RNA isolation procedure. Detection of same RNA degradation products in RNA samples made by different procedures has earlier been considered as a support for authenticity of the fragments observed (Meyer and Schottel, 1992).

(c) S1 nuclease protection analysis pattern of fetal liver total RNA did not change significantly even if it was isolated in the presence of high concentrations of RNase A (data not shown). Here, RNase A was added to the tissue before homogenization to get a concentration of 4 μg/ml of the enzyme in the homogenate. This indicates that artifactual degradation of RNA is unlikely to occur during any of the steps of the total RNA isolation procedure employed here.

(d) When fetal liver total RNA and poly (A)⁺ and poly (A)⁺ RNAs (made from fetal liver total RNA by oligo-dT column chromatography) were subjected to S1 nuclease protection analysis with 5'-end labelled albumin cDNA probe and the results compared, it was found that B, C and D could be detected with poly (A)⁺ RNA and total RNA but not with poly (A)⁺ RNA, and the levels at which they were detected were higher with poly (A)⁺ RNA than total RNA (Fig.8). This indicates that the RNA fragments that correspond to species B, C and D are present in poly (A)⁺ fraction which is in agreement with the results of Binder et al (1989). As expected, species A was detected with all 3 samples but the levels at which it was detected decreased in the following order: poly (A)⁺ RNA > total RNA > poly (A)⁺ RNA (Fig.8).

(e) Hybridization controls performed with same amount E. coli RNA instead of liver RNA consistently gave no or very faint signal (Fig.9a, lane 3) indicating that the hybridization by the probe is very specific under the conditions used.

(f) When the S1 nuclease protection analysis of fetal liver RNA was performed without addition of S1 nuclease during the S1 digestion step keeping all other steps of the procedure unchanged, only species A was observed (Fig.9a, lane 2) and B, C and D were not observed ruling out the origin of B, C and D from any spurious degradation of the probe during S1 nuclease protection analysis.

(g) Albumin mRNA levels are known to be higher in adult than in fetal mouse liver (Tilghman and Belayew, 1982; Sellem et al., 1984). So, one explanation for the inability to detect B, C and D with adult liver RNA could be that the higher concentration of full length mRNA competes out the smaller RNA fragments (that correspond to species B, C and D) from hybridizing to the probe. This is possible only when the concentration of the probe is limiting in the hybridization reaction. So, to confirm that the S1 nuclease protection analysis was done in DNA-excess condition for albumin mRNA, different amounts of adult liver RNA were
Fig. 5. Agarose gel electrophoretic pattern of some representative RNA samples used in this study. Cytoplasmic and total RNA samples are shown in the left and right lanes respectively. Positions of 23S, 18S and 4S RNA bands are indicated.

Fig. 6. Reproducibility of occurrence of B, C and D. Five different preparations of fetal mouse liver total RNA (lanes 1-5) were subjected to S1 nuclease protection analysis with 5'-end labelled albumin cDNA as described for Fig. 4. Protected DNA species A to D are indicated by arrows.
Fig. 7. Detection of B, C and D with fetal liver cytoplasmic RNA. S1 nuclease protection analysis was performed as in Fig. 4 using 5'-end labelled albumin cDNA probe with equal amounts of total (lane 1) or cytoplasmic (lane 2) RNA made from mouse fetal liver. Protected DNA species A to D are indicated by arrows.
Fig. 8. Comparison of S1 nuclease protection analysis patterns of albumin mRNA present in poly(A)$^+$ and poly(A)$^-$ fractions of fetal mouse liver RNA. Equal amounts of total (lane 1), poly (A)$^-$ (lane 2) and poly (A)$^+$ (lane 3) RNA samples from fetal mouse liver were subjected to S1 nuclease protection analysis with 5'-end labelled probe as in Fig. 4. Equal amount of radioactivity was loaded in lanes 1 and 2. Lane 3 received higher amount of radioactivity than lanes 1 and 2. Protected DNA species A to D are indicated by arrows.
Fig. 9. Controls for S1 nuclease protection analysis. Panel a, Equal amounts of fetal mouse liver total RNA subjected to S1 nuclease protection analysis with 5' -end labelled albumin cDNA probe as in Fig. 4 (lane 1) or hybridized to probe but not treated with S1 nuclease (lane 2). Control reaction performed with same amount of E. coli RNA is shown in lane 3. Protected DNA species A to D are indicated by arrows. Panel b, S1 nuclease protection analysis performed as described for Fig. 4 but with varying amounts (indicated above the lanes) of adult mouse liver RNA and constant amount of the 5'-end labelled albumin cDNA probe. Only top portion of the gel is shown.
subjected to S1 nuclease protection analysis with constant amount of probe DNA and the observed intensities of species A were compared. As shown in Fig. 9b, the signal intensity increased proportionately with increasing RNA concentration indicating that even at the maximum RNA concentration employed in the experiment, the probe concentration is not limiting for albumin mRNA. Since the RNA:DNA ratio employed in all the experiments of the present study falls well below the upper limit of this range, the above possibility was ruled out. In all the later experiments, it was ensured that the RNA:DNA ratio employed was either equal to or less (but never more) than this value.

(h) Moderate changes in the temperature at which S1 nuclease digestion was performed or the concentration of S1 nuclease employed for the digestion did not affect the pattern of protected species observed in the experiment though, the signal intensity reduced at very high S1 nuclease concentrations and temperatures of digestion, as expected (data not shown).

Considering all these results together with the observation that B, C and D are specifically observed only with fetal liver RNA but not with adult liver RNA, B, C and D are unlikely to be the result of experimental artifacts and they are inferred to represent in vivo generated DI's of albumin mRNA generated specifically in fetal liver. Further, other experiments described in part-B of chapter 3 ruled out the possibility that B, C and D might represent splicing intermediates rather than DI's.

S1 nuclease protection analysis of fetal and adult mouse liver RNA using 3'-end labelled albumin cDNA probe

The question whether the DI's observed here are generated by endo- or exonucleolytic activity can be answered using S1 nuclease protection analysis as described below.

Consider an RNA molecule that is cut by a single specific endonucleolytic cleavage. It gives rise to two fragments derived from the 2 parts of the RNA molecule present on either side (5’ and 3’) of the cleavage - the upstream and downstream DI’s respectively. They can be detected with 3'- or 5'-end labelled forms of the probe spanning the cleavage site by S1 nuclease protection analysis. Here, the sizes of these smaller protected DNA species (which correspond to the RNA fragments) detected using the 5'- and 3'-end labelled probes, together should be equal to the size of the full length probe. When the region of the RNA spanned by the probe is cleaved endonucleolytically at more than one specific site, still the above statement holds true for each pair of upstream and downstream fragments as long as, the cleavages occur independent of each other, i.e., they do not occur in any specific temporal sequence. So, under these conditions, every endonucleolytic cleavage gives rise to a pair of upstream and downstream RNA fragments which are 'contiguous' to each other and which, in turn, generate 'contiguous' protected DNA species that are detectable in S1 nuclease protection analyses performed with 5'- and 3'-end labelled probes.

Sometimes, however, it may not always be possible to detect both upstream and downstream fragments for every endonucleolytic cleavage and this could be due to two reasons.
(a) Some cleavages depend on others for their occurrence (mentioned above).
(b) One of the 2 contiguous fragments is highly unstable by and hence is unable to accumulate
in vivo to an extent the other does.

When an RNA molecule is cleaved by an exonuclease, no specific intermediate would
accumulate more than the others. But exonucleolytic cleavages may also cause accumulation of
specific intermediates because the rate of movement of the exonuclease from one end of the
molecule to the other is not always constant; i.e., it slows down or pauses at certain sites. Such
accumulated DIs can be detected in S1 nuclease protection analysis using a probe spanning the
pause site and labelled at its 3'- or 5'-end (depending on whether they are generated by 3' → 5'
or 5' → 3' exonuclease activity). However, the DIs generated by exonucleolytic activities (unlike
those generated by endonucleolytic process) do not have contiguous partners. So, the DIs
produced by a 5' → 3' exonuclease activity can be detected only with 5'-end labelled probe and
the DIs produced by a 3' → 5' exonuclease activity can be detected only with 3'-end labelled
probe. This is diagrammatically illustrated in Fig.10.

The observation that albumin mRNA DIs were detected with 5'-end labelled albumin cDNA
probe (Fig. 4) rules out the involvement of a 3' → 5' exonuclease activity. So, the question to be
answered was whether these DIs are formed by endo- or 5' → 3' exonuclease activity. As
explained above, in S1 nuclease protection analysis performed with 3'-end labelled probe, it
should be possible to detect the contiguous upstream partners of these DIs only if the cleavages
are endonucleolytic. So, firstly, the sizes of B, C and D were determined graphically on the basis
of their electrophoretic mobilities with reference to the mobility of radioactively labelled
pBR322/Hind I marker DNA fragments, and they were approximately 468, 298 and 272
nucleotides, respectively. Therefore, the contiguous upstream partners of the DIs corresponding
to B, C and D would be expected to generate protected DNA species of sizes 184, 354 and 380
nucleotides, respectively in S1 nuclease protection analysis with 3'-end labelled albumin cDNA
probe, and like B, C and D, these species also would be expected to be detectable with fetal but
not adult liver total RNA. Therefore, S1 nuclease protection analysis was performed in exactly
the same way as before but using 3'-end labelled albumin cDNA probe. As shown in Fig.11, in
addition to the full length protected DNA species 'A', at least 4 prominent protected DNA species
could be detected, again, specifically in fetal liver but not in adult liver. These were designated E,
F, G and H. The biggest of them, 'H', could be resolved clearly from 'A', only upon running the
gel for twice as long as the usual period of time (compare lanes 1 and 3 in Fig.11). The sizes of
E, F, G and H were also determined (graphically, as before) and they were, 180, 355, 380 and
638 nucleotides, respectively. Thus, the sizes of E, F and G (180, 355 and 380 nucleotides) were
very close to the sizes predicted (184, 354 and 380 nucleotides) for the protected DNA species
from upstream DIs. Thus, it could be concluded that the DIs corresponding to each of the 3 pairs
of contiguous protected DNA species (B/E, C/F and D/G) result from a single common
endonucleolytic cleavage that occurs independent of the other two. The protected DNA species
B to H detected in the S1 nuclease protection analyses and the positions of the corresponding
endonucleolytic cleavages on the mRNA are diagrammatically shown in Fig.12. The protected
DNA species contiguous to 'H', is expected to be 14 nucleotides long. But in the experiments with

46
Fig. 10. Elucidation of endo- and exonucleolytic nature of RNA cleavages by S1 nuclease protection analysis. Thin lines represent RNA molecules. Solid and hatched bars represent DNA molecules labelled at 3' and 5' ends respectively. In panel a, K and L are two independently occurring site specific endonucleolytic cleavages. They give rise to contiguous RNA fragments W, X and Y, Z respectively. The upstream fragments W and Y can be detected only with 3'-end labelled probe with which they give rise to protected DNA species W' and Y'. Similarly, the downstream fragments X and Z can be detected only with 5'-end labelled probe with which they give rise to X' and Z'. w, x, y and z are lengths of W', X', Y' and Z' respectively. Since W', X', Y' and Z' correspond to RNA fragments produced by endonucleolytic cleavages, w+x and y+z equal the length of the probe. In panel b, K and L represent pause sites of 5'→3' and 3'→5' exoribonuclease activities respectively which result in accumulation of RNA fragments W and X respectively. W and X can be detected only with 5' and 3' labelled probes respectively. They give rise to protected fragments W' and X'. 
Fig.11. S1 nuclease protection analysis of albumin mRNA with 3'-end labelled probe. Equal amounts of fetal (lanes 1, 3) and adult (lane 2) mouse liver total RNA were subjected to S1 nuclease protection analysis as in Fig.4 but using 3'-end labelled albumin cDNA probe. In lanes 1 and 2, electrophoresis was performed till xylene cyanol crossed twice the gel length (74 cm) to resolve the largest protected fragment 'H' clearly. Radioactivity of protected DNA loaded was equal in lanes 1 and 2. The sizes (in bp) and positions of pBR322/Hind I marker DNA fragments (M) electrophoresed under identical conditions are shown. Protected DNA species A, E, F, G and H are indicated by arrows. E to H correspond to DIs.
Fig. 12. Protected DNA fragments observed in S1 nuclease protection analyses and the map positions of the corresponding cleavages in albumin mRNA. The line on top indicates mouse albumin mRNA. The positions corresponding to the Hind III sites of the mouse albumin cDNA (which define the ends of the probe) are shown. Solid lines represent various protected DNA species (B to H) observed in the S1 nuclease protection analyses performed with 3' and 5'-end labelled albumin cDNA probes. Sizes of the S1 protected fragments (in number of nucleotides) are indicated by the side of the solid lines. Approximate map positions of the cleavages (arrows) on the mRNA are also shown. Only a part of albumin mRNA sequence (coding for aminoacids 75 to 492) is reported in the literature (Minghetti et al., 1985). Hence the nucleotide numbering of the cleavage sites and the two Hind III sites (see above) was done by designating the 5'-nucleotide of the codon of the 75th aminoacid as 225th nucleotide. The position of the 3'-most cleavage (unlike the other three) was determined only from the results of the experiment with 3'-labelled probe.
5'-end labelled probe, such a species could not be detected. This could be because the complementarity is too short (14 nucleotides) for the efficient hybridization of the corresponding DI under the stringent hybridization conditions used in S1 nuclease protection analysis. However, it is also possible that the DI corresponding to H is formed by a 3' → 5' exonuclease activity. Since H is also detected (like B to G) only with fetal but not with adult liver total RNA, it appeared unlikely that the corresponding DI could have been produced by a process much different from the one that generates the other DIs and hence, it was assumed to be a result of endonucleolytic cleavage.

Apart from B to H, some other smaller protected DNA species were also observed in these experiments. But, most of these were either weak (close to background level intensity) or not detected reproducibly. Few of them, however, were quite reproducible and also prominent. But, they did not show any significant differences between their levels in fetal and adult liver suggesting that, they may correspond to DIs that are generated by a different mechanism. So, this study was restricted only to those DIs which correspond to B to H.

### 3.2.3 Changes in the levels of albumin mRNA degradation intermediates during development

Following the observation that albumin mRNA DIs are specifically present in fetal liver but not in adult liver, it was planned to study the levels of albumin mRNA DIs at different stages of development. Liver total RNA was prepared from 18 and 19 days old mouse fetuses and newborn mice at various stages after birth (0, 10, 20, 30, 60 and 90 days). Equal amounts of RNA were taken from each of these samples and subjected to S1 nuclease protection analysis with 5'-end labelled albumin cDNA probe. Since the concentration of albumin mRNA in liver is known to increase during prenatal and early postnatal life of mouse (Tilghman and Belayew, 1982; Sellem et al., 1984), the extent of protection of the probe as observed in the S1 nuclease protection analysis, also increased accordingly as expected. Therefore, for gel electrophoresis, equal amount of radioactivity was used from each S1 protected DNA sample, so that the autoradiographic signals of the smaller protected DNA species get normalized for that of full length protected species and hence could be compared between different samples. The results are shown in Fig.13a. The levels of B, C and D gradually decreased with further development from 18th day of gestation onwards and became undetectable by 20 days postpartum. When these RNA samples were subjected to S1 nuclease protection analysis in the same way but with 3'-end labelled probe, similar changes were observed in the levels of species E, F, G and H also (Figs.13b and c). These results indicate that the levels of albumin mRNA DIs decrease with development during the early postnatal period and reach adult levels (i.e., become undetectable) by about 20 days after birth.

The levels of the DIs do not always start falling by 19th day of gestation as shown by the results in Fig.13. It was found to be slightly variable with the batch of RNA samples and the fall in levels can start anywhere from 19th day of gestation to 1st day postpartum (i.e., around the time of birth).
Fig. 13. Study of changes in the levels of albumin mRNA degradation intermediates during development. Mouse liver total RNA samples isolated on the day of birth ('B'), and on different days (indicated above the lanes) after birth or of gestation were subjected to S1 nuclease protection analysis using 5'-(panel a) or 3'-(panel b and c) end labelled albumin cDNA probe. In panel c, electrophoresis was performed till xylene cyanol crossed twice the gel length (74 cm) to resolve the largest fragment ('H') clearly and the portion of the gel with smaller fragments is not shown. The amount of radioactivity loaded was equal in all lanes of each panel. Lanes 1 and 9 received untreated 5'- and 3'-end labelled albumin cDNA probe samples, respectively. The various protected DNA species B to H are indicated by arrows.
3.2.4 Specificity of the changes in the levels of albumin mRNA degradation intermediates during development

The presence of albumin mRNA Di's at higher levels in fetal liver than in adult liver and the gradual decrease in their levels during early postnatal period could either be due to a process specific for albumin mRNA or a generalized process. If it were to be a general process, one would expect similar changes with respect to other mRNAs expressed in fetal liver also, perhaps, due to a higher level of general cytosolic RNase activity in fetal liver than in adult liver. In order to answer these questions, cytosolic RNase activity was studied in fetal liver as compared to adult liver and also the (AFP) mRNA and its Di's, if any, were studied by S1 nuclease protection analysis during early postnatal period of development.

Cytosolic RNase activity in fetal and adult mouse liver

The pancreatic type RNase activity accounts for majority of cytosolic RNase activity in most of the mammalian tissues and is believed to be normally present almost completely in a latent, inactive form complexed with the endogenous RNase inhibitor (RI; see section 4.1). Earlier reports have shown that a similar situation exists both in rat (Kumagai et al., 1983) and mouse liver (Trzaskos and Meyer, 1987) wherein, only very little cytosolic RNase activity could be detected without inactivating the endogenous RI. In agreement with this, the RNase activities as determined by gel assay procedure (see under 'gel assay' in section 2.2.15) of fetal and adult mouse liver cytosol were found to be very less and could be detected with not less than 3.5 μg total cytosolic protein/μl assay mix in both cases (Fig.14). This leads to the conclusion that there is no significant difference in the cytosolic RNase activity levels of fetal and adult mouse liver.

AFP mRNA degradation intermediates during development

AFP is a very close homologue of albumin. Its mRNA is expressed during both fetal and early postnatal life in mouse and this expression has been studied earlier by several workers. The AFP mRNA level is very high in fetal mouse liver but it decreases sharply after birth (Tilghman and Belayew, 1982; Sellem et al., 1984). This decrease has been shown to be due to the down regulation occurring at both transcriptional and posttranscriptional levels (Vacher et al., 1992). When liver total RNA prepared from 18 days old mouse fetuses and new born mice at various stages (0, 2, 4, 6 and 8 days after birth) after birth were subjected to S1 nuclease protection analysis using 5'-end labelled AFP cDNA probe (see section 2.1.2 and Fig.1) in the same way as described before (see section 3.2.3), some prominent smaller protected DNA species (shown by arrows in Fig.15a) presumably corresponding to AFP mRNA Di's were also observed apart from the full length protected AFP cDNA probe. However, the levels of these Di's did not show any significant changes during various stages of development studied. When the same set of RNA samples was subjected to S1 nuclease protection analysis using 3'-end labelled albumin cDNA probe, the results (Fig.15b) were consistent with the observation made for albumin mRNA in earlier experiments.
Fig. 14. Comparison of cytosolic RNase activity levels in mouse fetal and adult liver. 1 µg of E. coli RNA was incubated with increasing amounts of cytosolic protein from fetal or adult mouse liver (indicated above the lanes) in 1X assay buffer containing 5 mM DTT to assay for RNase activity by gel assay procedure as described in Methods. The positions of 23S, 16S and 4S RNA bands are indicated by arrows.
Fig. 15. Study of AFP mRNA degradation intermediates during development. Mouse liver total RNA samples made from 18 days old fetuses (F) or new born animals on the day of birth (B) or on different days (indicated above the lanes) after birth were subjected to S1 nuclease protection analysis with 5'-end labelled AFP cDNA probe (panel a) or 3'-end labelled albumin cDNA probe (panel b). Amount of radioactivity loaded was equal in all the lanes of each panel. In panel a, the protected DNA species corresponding to AFP mRNA DI's are indicated by arrows. In panel b, species H is indicated by arrow and the portion of gel containing species E to G is not shown.
Thus, the studies on RNase activity levels and AFP mRNA DIIs together indicate that the higher levels of DIIs in fetal liver may not be due to a general process but a process specific to albumin mRNA.

3.2.5 Albumin mRNA degradation intermediates in regenerating liver?

Hepatocyte cell division in mouse is known to decrease after birth and reach the base line by around 20 days (Tilghman and Belayew, 1982). The levels of albumin mRNA DIIs also follow a similar course as shown by the experiments of the present study. If the accumulation of albumin mRNA DIIs in fetal liver is due to the dividing nature of the tissue, it might be expected to occur in regenerating adult liver also. Therefore, total RNA samples prepared from adult regenerating liver at 18 and 30 hours after hepatectomy were studied by S1 nuclease protection analysis using 5'-end labelled albumin cDNA probe. However, as shown in Fig.16, regenerating adult liver did not contain any of the albumin mRNA DIIs in detectable levels. Its S1 nuclease protection analysis pattern very much resembled that of adult liver RNA. This indicates that cell division status per se may not be responsible for accumulation of albumin mRNA DIIs in fetal liver.

3.2.6 Distribution of albumin mRNA between membrane bound and free ribosomes in fetal and adult liver

Some of the earlier studies have shown that mRNA degradation can be affected by changes in the relative distribution of the mRNA between membrane-bound and free polysomes (Zambetti et al., 1987; Mason et al., 1988). So, the relative distribution of albumin mRNA between membrane-bound and free polysomes in fetal and adult liver was studied. RNA was made from free and membrane-bound polysome fractions of adult and fetal mouse liver and equal amounts of RNA from each pair were subjected to S1 nuclease protection assay using 3'-end labelled albumin cDNA probe. The radioactivity of the protected probe DNA was taken as a measure of the amount of albumin mRNA present in the sample. The results showed that both in fetal and adult liver, the ratio of the amount of albumin mRNA in membrane-bound polysome fraction to that in free polysome fraction was about 2.0 (Fig.17). So, the relative distribution of the albumin mRNA between the two populations of ribosomes does not appear to be different in fetal and adult liver.

3.2.7 Search for other condition(s) involving mouse albumin mRNA fragmentation

As already mentioned in section 3.1, albumin mRNA is known to get destabilized in Xenopus liver following estrogen treatment. Straus and Takemoto (1987) have shown that subjecting rat hepatoma cells to nutritional stress (specifically, the deficiency of essential amino acids) also results in posttranscriptional down regulation of albumin mRNA. If, albumin mRNA degradation occurred in mouse also under similar conditions, it would give an opportunity to see if there are similarities between the cleavages occurring under such conditions and the cleavages observed in fetal liver. This would reflect on the relationship between the degradation machineries operating under these conditions. So, it was planned to study the adult liver RNA made from fasted and estradiol treated animals by S1 nuclease protection analysis.
Fig.16. Analysis of albumin mRNA from regenerating liver. Two preparations each of mouse liver total RNA isolated at 18 (lanes 1 and 2) and 30 (lanes 3 and 4) hours after partial hepatectomy were subjected to S1 nuclease protection analysis using 5'-end labelled albumin cDNA probe as described for Fig.4. Sizes (in bp) and positions of pBR322/Hind I marker DNA fragments (M) electrophoresed under identical conditions are shown. S1 nuclease protection analysis of fetal and normal adult liver RNA are shown in lanes 5 and 6 respectively for the purpose of comparison. Protected DNA species A to D are indicated by arrows.
Fig. 17. Relative distribution of albumin mRNA between membrane-bound and free polysomes. Equal amounts of RNA from free (open bars) and membrane bound (hatched bars) polysomal fractions of fetal (F) or adult (Ad) mouse liver were subjected to S1 nuclease protection assays using 3'-end labelled albumin cDNA probe. The radioactivity of the protected DNA obtained with each RNA sample was taken as a measure of the level of albumin mRNA in that sample.
Liver RNA made from adult male mice fasted for 48 hours before sacrificing was subjected to S1 nuclease protection analysis using 5'- or 3'-end labelled albumin cDNA probes. The results (Fig.18) indicated the absence of the DI(s) corresponding to B to H in these RNA samples - no significant difference could be observed between the S1 nuclease protection analysis patterns of the RNA samples made from the fasted and normal adult animals.

To study the effect of estrogen treatment, liver total RNA was prepared from adult male mice at 6, 12, 24, or 48 hours after estradiol injection and control animal that received vehicle injection. These RNA samples were subjected to S1 nuclease protection analysis with 3'-end labelled albumin cDNA probe. Again, the results shown in Fig.19 indicate that the S1 nuclease protection analysis patterns of liver RNA from estradiol treated mouse and vehicle injected control mouse are very similar.

3.3 DISCUSSION

In the present study, four in vivo generated DI(s) of mouse albumin mRNA have been detected and the endonucleolytic cleavages giving rise to them have been mapped onto the mRNA. These studies also reveal the following.

(a) The albumin mRNA DI(s) are present at significant levels in fetal mouse liver but are undetectable in adult mouse liver.
(b) The levels of the DI(s) start decreasing around the time of birth, become undetectable by 20 days after birth and remain so till adult stage.

The implications of these observations are discussed in this section.

3.3.1 Specificity of mouse liver albumin mRNA cleavages

Various observations indicate that the DI(s) studied in this work are formed by a specific process. As already mentioned, in addition to the prominent protected species B to H which were considered in this study, few other prominent species were also observed. However, their levels did not show any developmental stage-related variations suggesting that the DI(s) corresponding to these species are probably produced by a different process. Moreover, during the same period of development in which the albumin mRNA DI(s) show a gradual decrease in their levels, the mRNA DI(s) of AFP, which is a very close homologue of albumin, did not show any significant change in their levels. These observations together with the finding that the cleavages observed are relatively small in number (only 4 over a length of 650 nucleotides of the mRNA) may imply that the DI(s) corresponding to the protected species B to H are formed by a specific regulated degradatory process causing functional inactivation of the albumin mRNA in a developmental stage specific manner.

Studies on the degradation of eukaryotic mRNAs like apolipoprotein (apo) II mRNA, histone mRNA and homeobox mRNA (Ross et al., 1986; Ross and Kobs, 1986; Binder et al., 1989; Brown and Harland, 1990) have shown that specific initial cleavage(s) of their decay occur in their
Fig. 18. Study of the effect of fasting on albumin mRNA. Equal amounts of liver total RNA made from 18 days old mouse fetuses (lanes 1 and 4) and normal (lanes 2 and 5) and 48 hours fasted (lanes 3 and 6) adult mice were subjected to S1 nuclease protection analysis with 5'- (panel a) or 3'-(panel b) end labelled albumin cDNA probe. Amount of radioactivity loaded was equal in all lanes of each panel. The protected DNA species A to H are indicated by arrows.
Fig. 19. Study of the effect of estradiol treatment on albumin mRNA. Adult male mice were injected intraperitoneally with 1 mg estradiol dissolved in 100 μl injection vehicle (9:1 mixture of polypropylene glycol and dimethyl sulfoxide) or 100 μl of vehicle alone. Equal amounts of liver total RNA made from estradiol treated mice at 6, 12, 24 and 48 hours after injection (lanes 1, 2, 4 and 5 respectively) and vehicle injected control (lane 3) were subjected to S1 nuclease protection analysis with 3'-end labelled albumin cDNA probe as described for Fig.11.
3'-untranslated regions (UTRs). In the case of histone mRNA, the cleavages have been shown to be rate limiting in the decay process (Ross and Kobs, 1986; Ross et al., 1986). Cleavages have been shown to occur even in the coding region for apoll mRNA (Cochrane and Deeley, 1989). A putative nuclease activity involved in estrogen-induced destabilization of albumin mRNA in Xenopus liver was shown to make specific cleavages in vitro, in the 5'-half of the albumin mRNA (Pastori et al., 1991b). The results obtained in the present study indicate the occurrence of cleavages in the middle, coding region of albumin mRNA. The other regions of this mRNA have not been examined in this study and it is possible that specific cleavage(s) occur even in those regions. Whereas the histone mRNA was found to be mainly degraded by an exonucleolytic activity (Ross et al., 1986; Ross and Kobs, 1986), the chicken liver apoll mRNA and Xenopus albumin and homeobox mRNAs were found to be cleaved endonucleolytically (Cochrane and Deeley, 1989; Binder et al., 1989; Brown and Harland, 1990; Pastori et al., 1991b) as observed for albumin mRNA in the present study.

Three RNase activities have been reported to be present in mouse liver cytosol (Mavrothalassitis and Georgatsos, 1984). Of these, the alkaline and neutral activities prefer UpN and CpN bonds (where N is mostly a purine). The acid RNase activity is specific to NpU bonds (where N is A, G or C). In addition, it was also found to cleave ApG bonds present in the sequence GAG. The 2'-5'A dependent RNase L, which is present in several cell types and implicated in message degradation, has a preference for UU and UA sequences (Wreschner et al., 1981). Such short range (di- or trinucleotide) specificities alone, however, will not be adequate to explain the specific cleavages observed in the present study. The nucleotide sequence of a part of albumin mRNA, which includes the entire region spanned by the probe used in the present study, is known (Minghetti et al., 1985). So, in order to see if the sites of the cleavages detected bear any similarity to already known mRNA cleavage site sequence elements, 20 nucleotide long stretches of albumin mRNA primary sequence spanning each of the four cleavages (whose map positions were determined from the data on the sizes of 51 protected species B to H; see Fig.13) were examined. None of these four cleavage site flanking sequences (CSFSs) contained the sequence motifs, UAA/AAU and GAUG/CAGU present at the endonucleolytic cleavage sites of apoll mRNA (Cochrane and Deeley, 1989; Binder et al., 1989) or the sequence motif, ACCUA of Xenopus homeobox mRNA (Brown and Harland, 1990). However, interestingly, homology comparisons revealed the presence of a sequence motif, CCAN1-3CUGN0-1UGAU (Fig.20) in 3 of the 4 CSFSs, i.e., those corresponding to the species, F/C, G/D and H. The CSFS corresponding to the fragment B/E, had only the CCA segment, and no clear explanation could be given for this at present. Importantly, the sequence motif, CCAN1-3CUGN0-1UGAU or even just the segment CUGN0,gUGAU was not found anywhere else in the region of the mouse albumin mRNA spanned by the probe used in the present study. The presence of such a unique sequence motif in three of the CSFSs and the absence of some simpler trinucleotide motifs like AAU/UAA (present in apoll mRNA cleavage sites) in all the four CSFSs (which together comprise a total length of 80 nucleotides) again underscore the specificity with which the cleavages might have occurred.

These cleavages and the common sequence motif present in three of the CSFSs may be useful to identify and study the nuclease(s) responsible. It will be interesting to see if there are
Fig. 20. Cleavage sites and cleavage site flanking sequences of albumin mRNA. Thin line indicates mouse albumin mRNA. Vertical arrows indicate positions of the endonucleolytic cleavages observed in this study. The CSFS (boxed) and protected DNA species (in parentheses) corresponding to each cleavage are also shown. Nucleotides in upper case letters indicate common sequence segments present in various cleavage sites.
any similarities between the cleavages observed here and those specifically made in *Xenopus* liver albumin mRNA by the estrogen-induced nuclease activity (Pastori et al., 1991b).

In order to see if there are any secondary structural characteristics that are common among the different CSFSs, computer prediction of albumin mRNA secondary structure was made with the help of RNA FOLD program of GCG package using the primary sequence published by Minghetti et al. (1985). The results (Fig. 21) showed the absence of significant secondary structural similarity among the four CSFSs. All the four CSFSs had double stranded parts interrupted with single stranded regions. In two of the CSFSs (those corresponding to species H and C/F), the segment CUGN1UGAU was present in single stranded form. However, the primary sequence used here as input is only partial because, the complete sequence of albumin mRNA is not known and moreover, the RNA FOLD program of GCG can fold only a maximum length of 1200 nucleotides. So, these secondary structure predictions need to be treated as tentative.

3.3.2 Variation in the levels of albumin mRNA degradation intermediates and the turnover rate of albumin mRNA

Specific mRNA degradation has been suggested to occur by a two step process (Brawerman, 1987). It has been proposed that the first step could involve interaction of the degradation machinery with some unique sites on the mRNA resulting in some specific rate limiting cleavages leading to the generation of the DIs and in the second step, these DIs may be rapidly degraded by more general RNA degradation mechanisms. Thus, according to this model, the specificity of mRNA degradation and regulation lies in the first step and any regulation of specific mRNA decay also occurs at the level of the first step, which, hence, is the rate-limiting step in specific mRNA decay. Here, it is important to note that though the first step involves only partial (not complete) degradation of the mRNA, it might be adequate to cause functional inactivation of the mRNA and meet the cellular demands. Thus, if the DIs are formed by the rate limiting step, they should accumulate to higher levels when the mRNA decays rapidly and vice versa. The levels of mRNA DIs have been studied in relation to the changes in mRNA decay rates in the case of H4 histone and apoll mRNAs and the results of these studies indeed are consistent with this expectation. The DIs of both H4 histone and apoll mRNAs accumulate to higher levels when the decay rates of the respective mRNAs increase as a result of blockage of DNA synthesis and estrogen withdrawal respectively (Ross et al., 1986; Binder et al., 1989). The DIs of prokaryotic *ompA* mRNA also behave similarly (Melefors et al., 1988; Lundberg et al., 1990).

In the case of albumin mRNA, its relative turnover rates in fetal and adult mouse liver are not known. This is mainly due to the serious difficulties in measuring the mRNA half lives in whole animal systems (as compared to measurement in cell lines or cell preparations). However, the results of the present study have indicated that the levels of its DIs are high in fetal liver, start decreasing around the time of birth, become undetectable by 3rd week after birth, and remain so till the adult stage. So, in the light of the correlation between DIs and turnover rate discussed above, the changes in the levels of albumin mRNA DIs may be interpreted in terms of albumin mRNA turnover rate and it can be concluded that the turnover rate of albumin mRNA is higher in
Fig. 21. Secondary structure of albumin mRNA at the cleavage sites. The 5'-1200 nucleotides of the partial sequence of mouse albumin mRNA (Minghetti et al., 1985) was analyzed using RNA FOLD program of GCG package and secondary structure predicted. The secondary structure of only those parts of the mRNA which contain the CSFSs (bold letters) is shown in the figure. The protected DNA species corresponding to each cleavage site are indicated in parentheses on the left of the respective secondary structure diagrams.
fetal liver than in adult liver, and it decreases during the first 3 weeks of postnatal life.

3.3.3 Role of posttranscriptional control mechanisms in the developmental regulation of albumin gene expression

Previous studies on the hepatic expression of albumin mRNA in mouse during development (Tilghman and Belayew, 1982; Selle et al., 1984) show that, it increases from 15th day of gestation reaches a plateau by 3rd week postpartum and remains at that level till adult stage (Tilghman and Belayew, 1982). These studies (Tilghman and Belayew, 1982) also show that this up regulation has two phases. Between 15th to 19th days of gestation, there is a steep increase in the mRNA level (first phase) and from 19th day of gestation to 3rd week after birth, the rate of increase lowers resulting in a gradual plateauing of the mRNA levels (second phase).

As discussed in the previous section (3.3.2), the observations made in the present study suggest that the turnover rate of albumin mRNA starts decreasing around the time of birth (from 18th day of gestation to 1 day after birth) and with a gradual fall, reaches the adult state by 20 days after birth. In this study, the stages earlier than 18th day of gestation have also been studied and these intermediates have been detected even in those stages (results not shown). But significant changes in their levels start occurring only around the time of birth (Figs. 13 and 15b), indicating that the decrease in albumin mRNA turnover occurs mainly during the 2nd phase (see above) of its upregulation. During the same period of development, the levels of AFP mRNA are also known to be regulated (Tilghman and Belayew, 1982; Selle et al., 1984). Though this regulation is known to be partly posttranscriptional (Vacher et al., 1992), the exact posttranscriptional step at which control operates is not known. Nevertheless, in the case of AFP, it is down regulation (unlike albumin mRNA) and hence, if it is occurring at the level of mRNA stability, it would only be expected to cause an increase in the mRNA turnover rate. So, levels of its DI would be expected to increase if the mRNA stability is regulated or remain unchanged. The results shown in Fig. 15a reveal some putative DI of AFP mRNA; but no detectable changes in the levels of any of the cleavage products could be observed during this period contrary to the observation with albumin mRNA. This observation is consistent with the absence of any change in the AFP mRNA turnover during this phase of development. Since AFP is a close homologue of albumin, these results also imply that the changes observed in the turnover rate of albumin mRNA may be specific to that mRNA. Although the AFP cDNA probe used in these experiments spans a significant portion (more than 50%) of the AFP mRNA, one cannot rule out the possibility that changes in the turnover rate of AFP mRNA could not be detected because, its rate limiting cleavages occur at sites that are not spanned by this probe.

Earlier studies of Tilghman and Belayew (1982) have indicated that developmental regulation of both AFP and albumin occurs primarily at the level of transcription. However, posttranscriptional regulation has also been later shown to operate for AFP gene expression (Vacher et al., 1992). The results of the present study, now suggest that in the case of albumin, posttranscriptional control at the level of mRNA stability may exist at least during the 2nd phase (see above) of its upregulation. Control of mRNA stability has not been ruled out in the developmental regulation of albumin gene expression in rat (Nahon et al., 1982) and hence, it
may be interesting to check on such a possibility in rat also using an approach similar to that used in this study.

As far as the physiological signal responsible for this process is concerned, it may not be cell division per se, because, the DIs are absent in regenerating liver (Fig.16). However, it is likely that the signal is related to development.

One of the causes for the difference in albumin mRNA turnover between fetal and adult liver could be a change in the mRNA distribution between membrane-bound and free ribosomes as has been shown for some mRNAs (Zambetti et al., 1987; Mason et al., 1988). However, no significant difference, could be observed in the relative distribution of albumin mRNA among the two classes of ribosomes from fetal and adult liver (Fig.17). On the other hand, since poly (A) tail length is very well known to influence mRNA turnover (see under 'poly (A) tail' in section 1.4.1), it is likely that the albumin mRNA molecules in fetal and adult liver differ in the length of their poly (A) tails and this difference accounts for the difference in their turnover rates. The role of poly (A) tail length in this process has not been examined yet and hence, this possibility cannot be ruled out in the differential degradation of albumin mRNA in fetal and adult liver. For determining mRNA poly (A) tail length, a cDNA probe spanning the 3′-most region of the mRNA (covering up to the site of polyadenylation) is required and further studies using such a probe would answer this question.

3.3.4 Study of mRNA degradation intermediates as an approach to understand the regulation of mRNA turnover in whole animals

Regulation of gene expression at the level of mRNA stability is usually demonstrated by directly measuring the decay rates of the mRNA under control and experimental conditions. However, there are several difficulties in doing this since each of the currently available procedures has some drawback. These are discussed below.

(a) The pulse-chase method has 2 problems. Firstly, it is difficult to label the mRNA of interest to high specific activity and hence, the method is useful only with abundantly expressed or rapidly transcribed messages. Secondly, after the pulse period, it is not easy to chase out the labelled nucleotide rapidly. So, only stable mRNAs (with half-lives in excess of the time required for the chase) can be studied. Nevertheless, the advantage of this procedure is that it allows measurements in the most natural condition, because it affects cellular metabolism minimally.

(b) The method for RNA half-life measurement from the rate of fall of the mRNA level following inhibition of transcription by drugs like actinomycin-D, has been employed in many studies. However, such drugs are known to affect other metabolic processes of the cell besides transcription; e.g., actinomycin-D has been suggested to both increase and decrease RNA decay rate (Greenberg, 1972; Cavalieri et al., 1977). Moreover, since this procedure involves general transcription block, it cannot be used for stable mRNAs. An alternate procedure is to express the mRNA from a promoter that can be experimentally induced (like hsp 70 promoter which can be induced with heat shock). With an inductive stimulus, the
mRNA is allowed to accumulate and its decay is followed after removal of the stimulus. Unlike actinomycin-D treatment, here the transcription of the mRNA of interest is specifically blocked. Here, the possibility of the induction stimulus *per se* affecting the turnover has to be ruled out by proper controls. However, this procedure involves lot of preparatory work of making and transfecting the appropriate gene constructs for each mRNA.

(c) In approach-to-steady-state method, mRNA half-life is determined from the time taken for steady state labeling of the mRNA during continuous exposure of cells to constant levels of labelled nucleoside. Here, transcription rate must be assumed to be constant during labeling time, and the procedure is not sensitive enough to use with scarce mRNAs.

Moreover, though all these procedures are convenient to use with cultured cells or cell suspensions, they are difficult to be employed for determination of decay rates of specific mRNAs in organs of whole animals. In whole animals, it is difficult to maintain uniformity for all cells with respect to any of the treatments (like radiolabeling, actinomycin-D treatment etc.) employed in these procedures. When conditions for induction or deinduction of the mRNA are known, then, using principles of approach to steady-state method mRNA half-life can be determined in whole animals (Cochrane and Deeley, 1988) from the time taken for the mRNA to go from one steady-state to another. But for this, one must first of all be sure that, changes in transcription and/or decay rate of the mRNA (which cause the induction or deinduction) are established very early during the response so that they can be assumed to remain constant over the period during which new steady-state is reached.

Because of such difficulties, the mRNA half-life measurements have been made in whole animals only in a few cases. These difficulties get further exacerbated while dealing with embryonic stages. Often, due to these problems, conclusions on changes at the level of mRNA stability are drawn indirectly by excluding changes at other levels like transcription, nuclear transport and splicing etc.

The conclusions drawn in the present study on the modulation of albumin mRNA turnover are based on the positive correlation between accumulation of Dis and increase in mRNA turnover rate which has been shown in literature. Nevertheless, they need to be substantiated by other lines of evidence. In studies concerning the regulation of gene expression, most of the time, the question one encounters is regarding the level at which regulation of expression of an mRNA occurs and, under such circumstances, the information on relative turnover rates is sufficient to find out if any regulation operates at the level of message stability or not and this can be achieved by determining the relative levels of mRNA Dis in a given experimental condition as compared to the control condition following the experimental approach used here. In the present study, end-labelled probes have been used in order to map cleavage sites. But, when this is not required, relative levels of the mRNA Dis may be studied with uniformly labelled cDNA or cRNA probes, which can be made with several fold higher specific activity than end-labelled probes. With such probes, the detection of Dis of less abundant mRNAs may be easier. In any case, more future studies are needed to establish the general applicability of this procedure to different kinds of systems or mRNAs.
PART-B: STUDY OF ALBUMIN mRNA DEGRADATION IN VITRO

3.4 INTRODUCTION

Study of mRNA decay in mammalian cells have several experimental limitations. Genetic selections are difficult with mammalian cells. Moreover, none of the currently available procedures for mRNA half-life measurement are reliable for use at all the different physiological conditions and with all kinds of mRNAs. These difficulties are exacerbated when working with whole animals as model systems. Because of these problems, identification of trans-acting factors involved in mRNA turnover has been quite difficult. In an attempt to enable a convenient analysis of the various factors (especially trans-acting factors), in vitro mRNA decay reactions were attempted and developed by Ross et al (1986). Later similar cell free mRNA decay systems were developed by others also. These in vitro reactions, employ polysomes (Ross and Kobs, 1986), polusome extracts (Pastori et al., 1991b) or crude cell lysates (Wager and Assoian, 1990; Brown et al., 1993; Hua et al., 1993) as the source of nuclease. Both polysomes and naked RNA have been used in these studies as substrate (Ross and Kobs, 1986; Pastori et al., 1991b). The accuracy with which results obtained using in vitro systems reflect the in vivo situation has been shown in several ways.

(a) The relative decay rates of different mRNAs observed in cell-free decay reactions are same as that observed in vivo (Ross and Kobs, 1986).
(b) In the case of histone mRNA, the degradation pathway was shown to be similar in vivo and in vitro (Ross et al., 1986).
(c) The specificity of mRNA decay which is observed in vivo is maintained in in vitro reactions also (Ross and Kobs, 1986).
(d) In vitro reactions also mimic the regulated mRNA decay observed in vivo (Wager and Assoian, 1990; Pastori et al., 1991b). E.g., liver polysomes from estrogen treated Xenopus degrade albumin mRNA faster than the polysomes made from control animals, reflecting the estrogen dependent destabilization of Xenopus liver albumin mRNA observed in vivo (Pastori et al., 1991b).

A major advantage of setting up cell-free mRNA decay systems is that they enable the development of an assay for nuclease(s) involved in specific mRNA decay and hence would be of great use in purifying and characterizing them. Therefore, some preliminary in vitro mRNA decay experiments were tried with the objective to study the formation of albumin mRNA Dls in vitro. In the present system, the degradation of albumin mRNA in the in vitro decay reaction would be expected to produce, Dls that are same as the in vivo generated Dls which were observed in the earlier experiments (see PART-A of this chapter). Additionally, the cell-free reaction mix made from fetal liver would be expected to be more efficient in generating these Dls from albumin mRNA than the reaction mix made from adult liver.
3.5 RESULTS

3.5.1 Degradation of albumin mRNA in vitro in the presence of fetal liver postnuclear supernate

For in vitro mRNA decay experiments, adult liver total RNA was chosen as substrate as it does not contain any of the Dls observed. Postnuclear supernate (PNS) was used as the source of nuclease. Adult mouse liver RNA was incubated with PNS made from fetal mouse liver at 10°C or 25°C for various lengths of time. At the end of the reaction, samples were phenol extracted and RNA was precipitated. The precipitated RNA samples were subjected to S1 nuclease protection analysis with 3'-end labelled albumin cDNA probe. Control reactions involved incubation of E. Coli RNA with fetal liver PNS. Fig.22, shows results of a typical experiment. They indicated that all the 4 Dls detected with 3'-end labelled probe in earlier experiments, were generated from adult liver RNA in the in vitro decay reaction (as shown by the appearance of the four protected DNA species E to H).

The Dls detected in the cell-free reactions cannot be explained as coming from the RNA present in fetal liver PNS because, the levels of the protected species E to H (which correspond to the Dls) increase in a time dependent fashion (Fig.22, lanes 3-5). Moreover, control reactions involving incubation of E. Coli RNA with higher concentrations of PNS protein (4 µg/µl reaction mix as compared to 500 ng/µl reaction mix in the experimental reactions), produced only a faint signal, if at all, corresponding to species 'A' and no signals corresponding to any of the smaller protected DNA species which represent the Dls (lane 1 in Fig.22).

3.5.2 In vitro degradation in the presence of adult liver postnuclear supernate

Since the earlier experiments (see PART-A this chapter) have indicated the accumulation of albumin mRNA Dls in fetal liver but not in adult liver, the fetal liver PNS would be expected to be more efficient than adult liver PNS in generating the Dls in vitro. So, in vitro decay reactions were performed exactly as before but using both fetal and adult mouse liver PNS proteins at equal concentrations (200 ng/µl reaction mix) in separate reactions. As shown in Fig.23, the intensity of the protected DNA species E to H observed after the S1 nuclease protection analysis was comparable for both fetal and adult liver PNS treated RNA samples (compare lanes 3 and 4). This result indicates that the adult liver PNS also is as efficient in generating all the Dls in vitro as the fetal liver PNS.
Fig. 22. Reaction, *in vitro*, of albumin mRNA with fetal liver postnuclear supernate. 20 µg of adult mouse liver RNA were incubated at 10°C (lanes 3-5) or 25°C (lanes 6-8) for 0, 15 or 30 minutes with 25 µg of mouse fetal liver PNS proteins as described in Methods and subjected to S1 nuclease protection analysis using 3'-end labelled albumin cDNA probe as described in Fig. 11. Lanes 1 and 2 represent control reactions performed as above at 25°C for 30 minutes with either 20 µg of *E. coli* RNA and 200 µg of mouse fetal liver PNS proteins or, same amount of adult mouse liver total RNA in the absence of PNS proteins. The protected DNA species A and E to H are indicated by long arrows. Other protected species are indicated by short arrows. The top portion of the gel at lower exposure is shown on the right so that, species H can be seen clearly.
Fig. 23. Comparison of in vitro degradation of albumin mRNA by adult and fetal liver postnuclear supernates. 20 μg of adult mouse liver total RNA were incubated at 25°C for 15 minutes without (lane 2) or with 10 μg of PNS proteins from fetal (lane 3) or adult (lane 4) mouse liver as described in Methods and subjected to S1 nuclease protection analysis with 3'-end labelled albumin cDNA probe as described in Fig. 11. The S1 nuclease protection analysis pattern of fetal liver RNA is shown in lane 1 for comparison. The protected DNA species A and E to H are indicated by long arrows. Other prominent protected species are indicated by short arrows. The top portion of the gel at lower exposure is shown on the right so that, species H can be seen clearly.
3.6 DISCUSSION

The occurrence in the in vitro reactions (Figs. 22 and 23) of all the four in vivo cleavages of albumin mRNA observed in earlier experiments rules out two important alternative explanations for the appearance of B to H in the S1 nuclease protection analysis of fetal liver RNA discussed in PART-A.

One explanation for the appearance of B to H in the S1 nuclease protection analysis of fetal liver total RNA could be that they are formed as a result of hybridization of the probe not to the Dis of albumin mRNA but, to the incompletely spliced forms of albumin pre-mRNA (present in the total RNA samples) and hence, the ends of the protected species actually correspond to the intron-exon junctions of albumin primary transcript rather than the mRNA cleavage sites. The precise locations of intron-exon junctions in mouse albumin gene are not available yet. However, the formation of the Dis in in vitro reactions employing adult liver RNA in a time dependent fashion (Fig. 22, lanes 3-5), rules out this possibility since, the Dis are known to be undetectable in adult liver RNA (see Figs. 4 and 11). Moreover, the change in levels of A and H with increasing time of incubation exhibited a precursor-to-product relationship as seen in Fig. 22 (lanes 3-5 and 6-8). This could not have been observed if the above possibility were true.

Because AFP mRNA disappears from mouse fetal liver by 8-10 days after birth and the disappearance of in vivo Dis of albumin mRNA was also found to occur almost at the same time, a strong argument could be that, B to H are formed as a result of hybridization of the albumin cDNA probe to the partially homologous AFP mRNA in S1 nuclease protection analysis (that is, their ends do not correspond to regions of mismatch between AFP and albumin mRNA). However, this is again ruled out by the ability to detect E to H with adult liver RNA subjected to in vitro decay reaction since, it is known that the expression of AFP mRNA in adult liver is almost undetectable.

The fetal liver specific degradation of albumin mRNA could be because of either the expression or the activity of the nuclease (responsible for the degradation) being specific to fetal stage. The results of Fig. 23 show that the adult liver PNS is as efficient as the fetal liver PNS in generating the specific albumin mRNA Dis in vitro indicating that, the nuclease responsible for the degradation exists in adult liver also, but, acts on albumin mRNA (leading to the generation of the specific Dis) in vivo only in fetal liver. This could be due to two reasons.

The first possibility is that difference(s) in the nature of cis-acting element(s) (like poly (A) tail length) of albumin mRNA in fetal and adult liver may lead to the differential exposure of the mRNA to the nuclease, making the adult liver albumin mRNA a poor substrate for the nuclease compared to the fetal liver albumin mRNA. However, since, in all the in vitro reactions (Figs. 22 and 23), adult liver RNA was used as the substrate and all the specific Dis of albumin mRNA could be generated in such reactions, the above possibility appears to be very unlikely.
The second possibility is that the developmental stage specificity might be imparted to the nuclease by another trans-acting factor(s) which regulates the degradatory process either by interacting with the nuclease itself or by interacting with the mRNA. In the former case, it may negatively regulate the putative nuclease in adult liver; whereas, in the latter case, it may change the accessibility of the mRNA to the putative nuclease. The inability to see the action of the trans-acting factor in vitro may be because it is very labile and loses its activity during the process of preparation of PNS and/or under the conditions of the in vitro degradation reaction.

In addition to the protected species E to H, some more prominent protected DNA species (indicated by short arrows in Figs.22 and 23) were observed when in vitro treated adult liver RNA was subjected to S1 nuclease protection analysis. This indicates that additional decay products which were not observed in the earlier experiments were also generated in the in vitro reactions. This could have been due to some unrelated nuclease activities that are different from the one which is responsible for the developmentally regulated specific in vivo cleavages of albumin mRNA, and which got somehow activated during the process of cell lysis (to make PNS) or under the conditions of the cell free decay reaction. Almost the whole of intracellular RNase activity that acts at neutral pH is RI-sensitive. But many earlier studies have shown that specific mRNA degrading enzymes are insensitive to RI (Ross and Kobs, 1986; Pastori, et al., 1991b; Brown et al., 1993; Hua et al., 1993). So, by including RI in the reaction mix, it might be possible to bring down the activity of unrelated RNases in the in vitro reactions to a large extent. This might avoid the appearance of the species other than E to H in the in vitro reactions. However, it is also possible that under the in vitro reaction conditions, additional sites on the mRNA may be accessible for more cleavages. Further, in the in vitro reactions, the fetal liver specific degradation could not be observed. This could be because the putative negative regulator is polysome associated and hence its effect could not be detected in the in vitro reactions which employ naked RNA as the substrate. In that case, by using polysomes as the substrate it may be possible to reduce the occurrence of other cleavages and also generate in vitro conditions that are as closer to the in vivo situation so as to mimic the fetal liver specific generation of the DIs observed in vivo.
PART-C: POSSIBLE MODEL TO EXPLAIN THE MECHANISM OF REGULATION OF ALBUMIN mRNA TURNOVER DURING MOUSE LIVER DEVELOPMENT

Experiments to study the status of albumin mRNA in regenerating adult mouse liver have indicated that cell division per se is unlikely to be the signal responsible for its degradation. So, it is possible that some development related stimuli are responsible for regulating the degradation of this mRNA. Since the results of in vitro decay reactions are suggestive of negative regulation, the enhanced degradation of the albumin mRNA observed in fetal liver may be occurring by default mechanism while keeping the nuclease under repression in adult liver. Since the fetal liver specific action could not be seen in in vitro reactions employing naked adult liver RNA as substrate, it is likely that the negative regulator is polysome associated.

Thus, in fetal liver, where the putative polysome associated negative regulator is inactive or absent, the nuclease acts on the mRNA turning it over at a higher rate. As the animal develops, either the activity (of already existing negative regulator) or expression of the negative regulator gets stimulated in a developmentally regulated process so that, by about 20 days after birth, it reaches the activity in adult liver and represses the nuclease almost completely. In any case, the question whether the negative regulator influences the nuclease action by acting on the latter directly or by making the cleavage sites on the mRNA inaccessible to the nuclease by (affecting the secondary/tertiary structure of the mRNA) is still open now. All these possibilities are diagrammatically represented in Fig.24.

During early prenatal period, when the albumin mRNA induction starts, the nuclease action may not be significant due to low concentration of the mRNA and transcription may be the sole determinant of the mRNA level. However, as the mRNA accumulates with time, the degradation rate of the mRNA may become appreciable leading to a gradual fall in the rate of further accumulation of the mRNA. This could be the reason behind the biphasic nature of upregulation of albumin mRNA expression during development (see section 3.3.3).
Fig. 24. Model proposed to explain the possible mechanism by which degradation of mouse liver albumin mRNA could be regulated during development. The negative regulator could prevent the degradation of the mRNA either by blocking the nuclease action (A) or by reducing the accessibility to the nuclease of the cleavage sites on the mRNA (B).
PART-D: SUMMARY OF THE RESULTS AND CONCLUSIONS

(a) Specific fragments of albumin mRNA - as shown by the appearance of smaller protected DNA species B/E, C/F, D/G and H in S1 nuclease protection analysis performed with albumin cDNA probe - are present in fetal liver RNA but undetectable in adult liver RNA.

(b) The protected DNA species B to H are neither experimental artifacts nor represent any nonspecific fragmentation of the mRNA occurring during the isolation or further handling but, correspond to four specific in vivo generated DI s of albumin mRNA.

(c) The albumin mRNA DI s are generated by independently occurring endonucleolytic cleavages.

(d) Three of the cleavage sites contain the unique sequence motif CCAN\textsubscript{1-3}CUGN\textsubscript{0-1}UGAU.

(e) The levels of albumin mRNA DI s (which are significant in fetal liver) start decreasing progressively around the time birth, become undetectable by around 20 days after birth and remain at that state till adult stage.

(f) These changes are specific for albumin mRNA because, during the same period of development, the levels of AFP mRNA DI s do not change and moreover, the cytosolic RNase activity is roughly equal in adult and fetal mouse liver.

(g) Regeneration, fasting or estradiol treatment fail to induce the generation of albumin mRNA DI s in adult liver.

(h) Generation of albumin mRNA DI s in a time dependent manner from adult liver RNA in vitro upon incubating it with fetal liver PNS and the presence of precursor-to-product relationship between the species A and H in such experiments shows that the ends of the protected species do not correspond to the intron-exon junctions of albumin pre-mRNA or the regions of mismatch between AFP and albumin mRNA.

(i) Ability of adult liver PNS also to generate the DI s from adult liver RNA in vitro indicates that the nuclease does occur in adult liver also but probably fails to act in vivo due to negative regulation.

(j) Together, the results lead to the conclusion that the albumin mRNA turnover rate is high in fetal liver and it starts decreasing gradually and specifically around the time of birth to reach the adult state by 20 days after birth and this mRNA stabilization process, which seems to be developmentally regulated, also contributes (apart from transcriptional activation) to the upregulation of albumin mRNA expression which is known to occur during this period of development.
On the basis of the results obtained from the studies on the *in vivo* and *in vitro* degradation of albumin mRNA, a model has been proposed to explain the possible mechanism by which the albumin mRNA degradation could be regulated *in vivo* during development.
PART-E: SUGGESTED FUTURE LINE OF WORK

(a) Firstly, the in vitro reactions have to be optimized thoroughly in order to use them to the fullest possible extent for the identification and characterization of the degradation machinery involved in albumin mRNA cleavage. The different ways in which optimization can be done have been discussed in section 3.6. The in vitro reaction can be utilized as an assay for the purification of the nuclease activity. However, for this, the optimized in vitro reactions need to be simplified into rapid assay procedures by which the specific cleavages of albumin mRNA could be monitored so that the nuclease activity of interest is specifically detected and assayed. It may be convenient to follow the occurrence of just one (rather than all) of the three cleavages (which occur at the sites near the common sequence element $CCAN_1$-$3$CUGN$_0$-$1$UGAU), using a probe designed in such a way that the smaller protected species corresponding to the cleavage can be well separated by a rapid short gel run.

(b) Initially, different subcellular fractions of liver can be assayed to identify the cellular compartment in which the nuclease resides and then further purification could be done. However, it is likely that the specificity and nuclease functions remain in more than one polypeptide, in which case, at some step in purification, specificity of cleavage might be lost from the nuclease activity. At that stage, one might have to perform in vitro reconstitution experiments in order to identify and assay for the specificity factor.

(c) Identification of the features of albumin mRNA that act as specificity determinants for the in vivo cleavages observed in this study will be another important aspect for further work. One way to do this would be to study the in vitro degradation pattern of mutant transcripts (made by in vitro transcription). Since, three of the four CSFSs have a reasonably large sequence element in common, it is likely that this primary structural element is one of the specificity determinants (for at least these 3 cleavages). So, site-directed mutations that alter this sequence element will be of great use. It will be interesting to see if albumin mRNA DIs similar to the ones observed in this study in fetal liver are produced in any of the mouse fetal liver cell lines. If such cell lines are available, it will be more preferable to study the effect of mutations in those cell lines (by transfecting them with the appropriate gene constructs) rather than in cell free in vitro decay reactions.
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