

Rat α -lactalbumin has a 17-residue-long COOH-terminal hydrophobic extension as judged by sequence analysis of the cDNA clones

(mammary gland/lactose synthase/milk proteins/positive-hybrid selection/3'-end sequence analysis)

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Communicated by G. Gilbert Ashwell, May 4, 1981

ABSTRACT cDNA for rat α -lactalbumin has been cloned in bacterial plasmid, and its sequence has been analyzed. The DNA sequence analysis shows that rat α -lactalbumin has 17 extra residues beyond the COOH terminus of the α -lactalbumin isolated and sequenced to date from other species. The predicted COOH-terminal sequence is hydrophobic and proline rich and bears some resemblance to β -casein sequences.

α -Lactalbumin (α -LA) is a mammary gland-specific protein involved in lactose synthesis. It is a modifier protein that changes the substrate specificity of galactosyl transferase, an enzyme normally involved in the biosynthesis of glycoproteins. Specifically, it promotes the binding of glucose, otherwise a poor substrate, to galactosyl transferase, thereby permitting synthesis of lactose (for review, see refs. 1 and 2).

The complete amino acid sequences of human (3), bovine (4), goat (5), guinea pig (6), and rabbit (7) and the partial sequence of kangaroo (8) α -LA are known. These α -LAs are single polypeptides of 123 amino acid (except rabbit α -LA, which has 122) residues that have sequences somewhat similar to that of lysozyme (1, 2). α -LA from rat has been shown to be a glycoprotein (9, 10) and was separated on a DEAE-cellulose column into two molecular species (9). One of these has been shown by sedimentation analysis to have a higher molecular weight than any other known α -LA (9).

For studies of hormonal regulation of milk protein genes in normal and neoplastic tissue, we have fractionated individual RNA species from the total poly(A)⁺RNA of the lactating rat mammary gland and have identified and characterized rat α -LA mRNA (11, 12). It is difficult to obtain pure RNA absolutely free of other RNA species by conventional fractionation techniques. However, this problem can be resolved if a cDNA clone containing sequences complementary to the corresponding mRNA can be obtained. We now report the isolation of cDNA clone of rat α -LA gene sequences and present its nucleotide sequence.

This sequence encodes part of the known partial sequence for rat α -LA protein. From the DNA sequence, the remainder of the rat α -LA polypeptide sequence can be deduced; it is homologous with the other known α -LA sequences but contains a COOH-terminal 17-residue extension that has a sequence resembling that of a casein.

MATERIALS AND METHODS

Synthesis of Double-Stranded cDNA. Rat mammary gland poly(A)⁺RNA was prepared from 5-day lactating Sprague-Dawley

rats as described (12). cDNA was synthesized from 100 μ g of poly(A)⁺RNA by using 600 units of reverse transcriptase (RNA-dependent DNA nucleotidyltransferase, avian myeloblastosis virus, a gift of J. W. Beard, Life Sciences, St. Petersburg, FL) in a 1.2-ml reaction mixture of 50 mM Tris·HCl, pH 8.3/6 mM MgCl₂/1.5 mM dithiothreitol/40 mM KCl/400 μ M each dATP, dCTP, dGTP, and dTTP containing oligo(dT)₁₂₋₁₈ (12.5 μ g/ml), actinomycin D (300 μ g/ml), and 0.4 mCi of [³H]dATP (15 Ci/mmol; 1 Ci = 3.7 \times 10¹⁰ becquerels) (12). The yield of single-stranded cDNA was \approx 18 μ g. The DNA was fractionated on the basis of size by using an alkaline 10–30% sucrose density gradient containing 0.9 M NaCl/0.1 M NaOH and centrifuged in an SW 41 rotor at 40,000 rpm for 24 hr at 5°C (13). Fractions containing 300- to 850-nucleotide-long cDNAs (6 μ g) were pooled and considered enriched in α -LA cDNA sequences.

Double-stranded (ds) DNA was synthesized from the cDNA fraction by using 50 units of DNA polymerase I in a 200- μ l incubation mixture of 30 mM Tris·HCl, pH 7.2/4 mM MgCl₂/0.5 mM dithiothreitol/1 mM each dCTP, dGTP, dATP, and dTTP containing 10 μ Ci of [α -³²P]dATP at 25°C for 3 hr (13). The hairpin loops of the ds DNA (5 μ g) were clipped and made blunt ended by using nuclease S1 (50 units) in a 1-ml incubation mixture of 3 mM ZnSO₄/30 mM sodium acetate, pH 4.5/300 mM NaCl for 2.5 hr at 40°C. The sample was extracted with phenol/chloroform (1:1) and passed through a Sephadex G-75 column. The final yield of ds DNA was \approx 4 μ g.

Terminal Transferase Reaction and Construction of a Recombinant cDNA/pBR322 DNA. Terminal transferase homopolymer addition was carried out as described (14). The restriction endonuclease *Pst* I was used to linearize the pBR322 (Bethesda Research Laboratories, Rockville, MD). The linear plasmid DNA and ds cDNA were elongated with dGTP and dCTP, respectively, by using terminal deoxynucleotidyltransferase (Bethesda Research Laboratories). Approximately 18–20 nucleotides were added to each 3' end of the ds DNA. The oligo(dG)-tailed plasmid DNA and the oligo(dC)-tailed ds DNA were hybridized in a 1:1 molar ratio at 65°C in 10 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA for 2 hr. The reaction tubes were left at room temperature for 6 hr and then kept at 4°C until used for transformation of *Escherichia coli*.

Transformation and Colony Filter Hybridization. All procedures were conducted in accordance with the National Institutes of Health guidelines for recombinant DNA research. Hybrid plasmid was used to transform *E. coli* LE392 in the presence of CaCl₂ by a described procedure (15) and selected for tetracycline resistance and ampicillin sensitivity. The colonies containing a recombinant plasmid were grown in 2.5 ml

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Abbreviations: LA, lactalbumin; ds, double stranded; bp, base pair(s).

of L broth containing tetracycline (12.5 $\mu\text{g}/\text{ml}$). After 18 hr of incubation, 0.5 ml of culture was transferred to an Eppendorf tube and DNA was extracted and denatured (16). The DNA solutions were adjusted to 0.75 M NaCl/0.075 M Na citrate and spotted on nitrocellulose filters under suction. Filters were hybridized according to the Grunstein-Hogness procedure (17).

³²P-Labeled cDNA to Enriched α -LA mRNA. Poly(A)⁺ RNA was fractionated on a neutral sucrose gradient and α -LA mRNA fractions were identified by an *in vitro* translation system and immunoprecipitation with α -LA antiserum (11, 12). cDNA was synthesized from this enriched α -LA mRNA fraction as described (12).

Positive Hybrid Selection for Identification of cDNA Clones. The positive-hybrid selection procedure followed was a modification of similar procedures reported earlier (18–20). Cultures were grown to an OD at 590 nm of 0.7/ml and incubated overnight with chloramphenicol (100 $\mu\text{g}/\text{ml}$). Cells from 100-ml cultures were centrifuged, suspended in 2 ml of 15% sucrose/50 mM Tris·HCl, pH 7.5 containing lysozyme at 5 mg/ml and incubated at 22°C for 10 min. The cells were lysed by adding 2 ml of 50 mM Tris·HCl, pH 8.0/62.5 mM EDTA/0.4% Triton X-100 and centrifuging at 19,000 rpm for 1 hr at 4°C. The supernatant was removed and extracted once with phenol, and DNA was precipitated with ethanol and dissolved in 3 ml of 10 mM Tris·HCl, pH 7.6/1 mM EDTA. The solution was boiled for 10 min, treated with an equal volume of 1 M NaOH for 20 min at 22°C, and then with 35 ml of 1.5 M NaCl/0.15 M Na citrate/0.25 M HCl/0.25 M Tris·HCl, pH 8.0. DNA was immobilized on nitrocellulose filters and hybridized to 5 μg of poly(A)⁺ RNA in 300 μl of 65% formamide/0.4 M NaCl/0.2% NaDodSO₄/30 mM Pipes, pH 8.5, containing 50 μg of tRNA at 50°C for 2 hr. The filters were washed 10 times at 65°C with 25 ml of SET buffer (0.15 M NaCl/0.01 M Tris·HCl, pH 7.6/0.001 M EDTA) containing 0.5% NaDodSO₄ and twice with 25 ml of SET buffer alone. The filters were transferred to siliconized Eppendorf tubes and washed once with 300 μl of SET buffer diluted 1:10 at 65°C, and the supernatant was discarded. The hybridized RNA was eluted in 300 μl of water containing 20 μg of wheat germ tRNA at 100°C for 1 min. The eluate was extracted first with phenol and then with chloroform and then RNA was precipitated with ethanol. The mRNA obtained was translated in a wheat germ cell-free system in the presence of [³⁵S]cysteine as described (11, 12) with two modifications: (i) the wheat germ extract was treated with micrococcal nuclease (21) and (ii) the concentration of creatine phosphokinase was increased from 4 to 32 $\mu\text{g}/\text{ml}$. These changes increased the efficiency of translation (22). Aliquots of the resulting labeled products (2×10^5 cpm) were immunoprecipitated with rat α -LA antibody (9) in the presence and absence of 1 μg of unlabeled rat α -LA. The total proteins synthesized in the cell-free system and the immunoprecipitates were examined on 11.25% polyacrylamide gels (23).

Preparation of Plasmid DNA. Cells were grown in LB broth containing tetracycline (12.5 $\mu\text{g}/\text{ml}$) to an OD at 590 nm of 0.8/ml and harvested to prepare a clear lysate (24). DNA was extracted from the lysate with phenol/chloroform (1:1) and precipitated with ethanol. The supercoiled DNA was purified free of RNA and linear DNA by centrifugation three times in a CsCl gradient ($\rho = 1.5732$) containing ethidium bromide (100 $\mu\text{g}/\text{ml}$) in a VTi 50 rotor (Beckman) at 48,000 rpm for 12 hr.

Restriction Endonuclease Analysis. Plasmid DNA and *Pst* I inserts labeled at 3' ends were digested with restriction enzymes, and the resulting fragments were analyzed by agarose or polyacrylamide gel electrophoresis (25).

3' and 5' End Labeling of DNA Fragments. *Pst* I inserts were labeled with [α -³²P]-3'-dATP (cordycepin) by using ter-

minal deoxynucleotidyltransferase by a modification of the method described earlier (14). The reaction mixture of 100 mM sodium cacodylate/1 mM CoCl₂/0.1 mM dithiothreitol containing DNA to be labeled (50–150 $\mu\text{g}/\text{ml}$), 250 units of terminal deoxynucleotidyltransferase (P-L Biochemicals), and 0.5 mCi of 3'-[α -³²P]dATP (3000 Ci/mmol, New England Nuclear) at pH 6.9 was incubated at 37°C for 1 hr, and reaction was terminated by the addition of 500 μl of 10 mM Tris·HCl, pH 7.8/10 mM EDTA/200 mM NaCl containing 20 μg of tRNA. Restriction fragments with 5'-protruding staggers were labeled with [γ -³²P]ATP (3000 Ci/mmol, Amersham) by using polynucleotidylkinase (P-L Biochemicals) as described by Maxam and Gilbert (26).

DNA Sequence Analysis. End-labeled DNA was digested with the appropriate restriction enzyme. The fragments were separated on 6% polyacrylamide gels, eluted out of the gel, and subjected to base modification and cleavage as described by Maxam and Gilbert (26). Samples were subjected to electrophoresis on 0.4-mm polyacrylamide/8.3 M urea gels.

RESULTS

Construction of Recombinant Plasmids. cDNA was synthesized from the total poly(A)⁺ RNA of lactating rat mammary gland, which had at least 10% of α -LA mRNA activity (11, 12). The yield of cDNA was $\approx 20\%$ of the RNA used. The DNA was fractionated according to size on an alkaline sucrose gradient. Rat α -LA mRNA has been shown previously to be ≈ 800 nucleotides long (12). Thus, the cDNAs ranging from 300 to 850 nucleotides in length were combined and considered enriched for α -LA cDNA. Second-strand DNA was synthesized with *E. coli* DNA polymerase I by using the hairpin portions at the 3' terminus of the cDNA as primer. This DNA was blunt ended by nuclease S1 treatment, and an average of 15–20 dCMP residues were added at the 3' terminus by terminal transferase. Similarly, plasmid pBR322 was cleaved at its single *Pst* I site, and an average of 18 dGMP residues were added to the 3' terminus. Dimeric plasmids resulting from the hybridization of the tailed DNAs were used to transform *E. coli* LE392, and the transformants were monitored for their ability to grow in the presence of tetracycline (15). Ninety-five tetracycline-resistant ampicillin-sensitive transformants were isolated with a transformation efficiency of 4.5×10^3 transformants per μg of vector DNA. The transformants were grown in small volumes, and DNA was extracted and spotted on nitrocellulose filters. The filter was hybridized (17) with a ³²P-labeled cDNA probe synthesized from a mRNA fraction that was only $\approx 35\%$ enriched for α -LA mRNA as judged by translation and immunoprecipitation. Thirty-seven clones hybridized with this cDNA probe.

Screening for α -LA cDNA-Containing Plasmids. Since the ds cDNA used for transfection was not pure and the ³²P-labeled cDNA probe used to light up the clones was only 35% pure, we screened the clones for α -LA cDNA with the positive translation analysis (18–20). For such analysis, we pooled the plasmid DNA in sets of nine from the clones that hybridized with our ³²P-labeled cDNA probes. The DNA was fixed on nitrocellulose filters and then hybridized with total poly(A)⁺ RNA under R-loop conditions. The RNA was eluted and translated *in vitro* by using [³⁵S]cysteine. If the translation product was immunoprecipitated by rat α -LA antiserum and immunocompeted by pure rat α -LA antigen, the plasmid DNA was considered to have the α -LA cDNA sequences. The pooled plasmid DNAs that were positive by this assay were then individually analyzed by the same assay. Fig. 1 B and C shows the polypeptides synthesized when the cell-free translational system was programmed with mRNA selected by hybridization to the recombinant DNA (plasmids p18 and p58) immobilized on nitrocellulose mem-

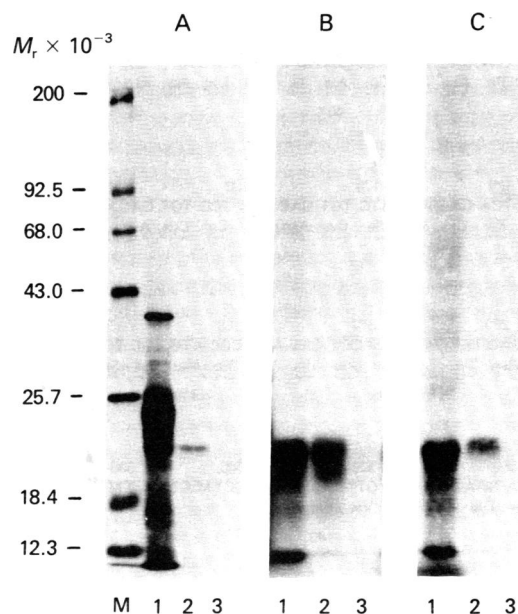


FIG. 1. Autoradiographs of 11.25% polyacrylamide gels showing [³⁵S]cysteine-labeled polypeptides synthesized in wheat germ cell-free system programmed with poly(A)⁺ RNA (A), RNA selected by hybridization to p18 DNA (B), and to p58 DNA (C). Tracks: 1, lactoproteins; 2 and 3, immunoprecipitates and immunocompetitions (with 1 μg of rat α-LA) of the translation products, respectively; M, ¹⁴C-labeled markers.

branes. The *in vitro*-synthesized protein was immunoprecipitated by α-LA antiserum and immunocompeted with pure rat α-LA. Total poly(A)⁺ RNA from the lactating gland synthesizes several [³⁵S]cysteine-labeled lactoproteins (22) (Fig. 1, track 1), but only one of these was immunoprecipitable with α-LA antiserum and immunocompeted by pure rat α-LA (Fig. 1 B and C). For some as yet unknown reasons, the sharpness of the *in vitro*-synthesized α-LA protein band varies from one gel to another. Five out of 95 recombinant plasmids, p1, p5, p17, p18, and p58, were identified as positive clones by this assay and contain α-LA cDNA sequences.

Restriction Endonuclease Mapping. The plasmids p18, p17, and p58 when cleaved with *Pst* I and analyzed on a polyacrylamide gel were found to contain α-LA cDNA inserts of 630, 300, and 280 base pairs (bp), respectively. The DNA inserts of p1 and p5 were of 280 bp and their restriction maps were the same as that of p58. The 630-bp insert of p18 contains cleavage sites for the restriction enzymes *Ava* II, *Alu* I, *Eco*RI, *Hinf*I, *Hha* I, *Msp* I, *Sst* I, and *Xba* I (Fig. 2). Many restriction enzymes did not cleave the insert, including *Bam*HI, *Bgl* II, *Hind*II, *Hind*III,

Kpn I, *Sal* I, and *Xho* I. The insert of p17 was cleaved by *Eco*RI and *Sst* I but not by *Xba* I. Similarly, the insert of p58 was cleaved by *Xba* I but not by *Eco*RI and *Sst* I. DNA sequence analysis of each of these clones established the restriction map as shown in Fig. 2.

DNA Sequence Analysis. DNA sequence analyses of p18, p17, and p58 were undertaken to establish whether or not these plasmids contained DNA coding for rat α-LA. The strategy used in determining the complete nucleotide sequences of the *Pst* I inserts is shown in Fig. 2. Inserts of the plasmids were labeled with terminal transferase and [^{α-32}P]dATP (cordycepin) and the 3'-terminal *Pst* I/*Eco*RI or *Pst* I/*Xba* I fragments were sequenced by the method of Maxam and Gilbert (26). Plasmids were also digested with *Eco*RI/*Xba* I, and the fragments were labeled with polynucleotide kinase and [^{γ-32}P]ATP. The 5'-terminal *Eco*RI/*Pst* I or *Xba* I/*Pst* I fragments were then sequenced. The sequence was determined on both strands and with different clones to ensure accuracy. The complete nucleotide sequence of the coding strand of the p18 insert is shown in Fig. 3. The cDNA insert is 623 nucleotide long, which includes the G-C tails and sequences of the *Pst* I site. The 3' end of the coding strand is oriented toward the *Eco*RI site of the pBR322. On the contrary, 3' ends of the p17 and p58 inserts are oriented in the opposite direction with respect to the *Eco*RI site of pBR322. The nucleotide sequence of the p17 insert aligns with the sequence of nucleotides 195–452 of the p18 insert whereas the sequence of the p58 insert aligns with the sequence of nucleotides 405–596 of p18 (Fig. 2). The p58 insert has, after nucleotide 596, a sequence G-T-C-T-C followed by an A₃₂ tail at the 3' end. All these inserts are flanked by G-C tails of 15–20 bp at the 5' and 3' ends.

Nucleotides 28–129 correspond to amino acid residues 25–58 of rat α-LA, whose sequence has been determined by Prasad and Ebner (27), with the exception of the nucleotides at residues 39 and 44. The amino acid sequence from position 58 onward to the COOH-terminal end of rat α-LA, unknown so far, has now been derived by reading the nucleotide sequence in triplets from the arginine codon at position 58 (Fig. 3). The protein sequence extends to 140 residues, well beyond the 123 or 122 residues normally assigned to other α-LAs. The termination codon, UAA, is located next to the codon that encodes proline as the COOH-terminal residue of the 140-residue protein. This 17-amino acid COOH-terminal extension contains five prolines and nine hydrophobic residues.

DISCUSSION

Rat α-LA is a glycoprotein (9, 10) and separable into two molecular forms that show differences in their molecular weights by both sedimentation and polyacrylamide/NaDodSO₄ (Laemm-

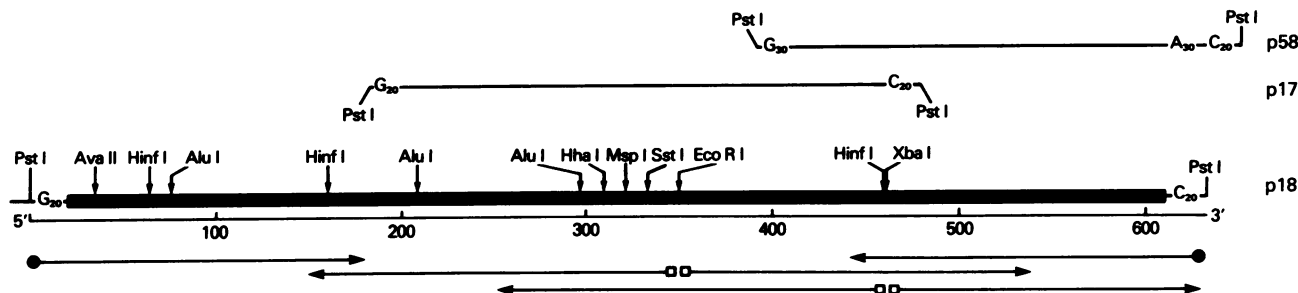


FIG. 2. Restriction endonuclease map and sequence strategy for p18, p17, and p58 cDNA inserts. *Pst* I inserts were labeled at the 3' ends (●) and digested with *Eco*RI. *Eco*RI/*Xba* I-digested plasmids were labeled at the 5' ends (□) and then again digested with *Pst* I. The lengths of the arrows show the extent of sequence determined for a particular fragment. In p18, the coding sequence is 5' to 3' toward the *Eco*RI site of pBR322, but in p17 and p58, the coding sequence is in the opposite direction. For comparison, all here are shown as 5' to 3', according to mRNA sequence.

long. It is possible that during evolution, a change has occurred in this region that has resulted in the extension of rat α -LA. For example, transversion of T \rightarrow G in the termination codon TGA, possibly present in the α -LA gene of other species at codon 124, would result in the GGA codon. This codon codes for glycine, the amino acid found at position 124 in rat α -LA (Fig. 3). This single mutation would abolish the termination and allow a read through of α -LA mRNA up to the next termination codon after residue 140. It has also been suggested that a single mutation in a leucine codon normally present at position 123 in α -LAs of other species will generate a termination codon, resulting in the 122-residue-long rabbit α -LA (7). Taken together, these arguments suggest that the DNA region corresponding to residues 122–124 of the α -LA gene sequence is amenable to more frequent mutational changes, generating small or large α -LAs. However, there are other possibilities worth considering. For example, one possibility is that the 123-amino acid-long α -LA is synthesized by another α -LA mRNA, for which the cDNA clone has as yet not been identified. We have, for these very reasons, analyzed the sequences of the DNA inserts of more than one plasmid DNA (p17 and p58). The nucleotide sequences of p17 and p58 are identical to the sequence of p18 for nucleotides 195–452 and 405–596, respectively (Fig. 3). We have also isolated 12 additional α -LA cDNA clones; none of them shows any difference in the restriction sites present in the nucleotide sequence coding for the COOH-terminal extension part of the protein (*Msp* I, *Sst* I, and *Eco*RI sites in Fig. 3; unpublished results). Thus, the only class of mRNA detected thus far has a COOH-terminal extension sequence, which makes the possibility of separate mRNA for the 123-amino acid-long α -LA less likely. Nevertheless, the possibility of two mRNAs should be left open. A second possibility is that all α -LAs may well be synthesized to a length longer than 123 amino acids with the extensions at the COOH-terminal end as in the rat and that during the secretory process, the COOH-terminal extension is cleaved off in a manner similar to a presequence. All the NH₂-terminal presequences of the secretory proteins (signal peptide), including the α -LAs, are hydrophobic (and some are also proline rich) and are cleaved during the secretory process (34, 35). The COOH-terminal 17-residue extension of rat α -LA is also hydrophobic and proline rich. In the rat, however, this COOH-terminal extension somehow escapes cleavage. The sequences of α -LA cDNA clones from other species should unequivocally answer such questions. The cleavage of presequences (signal peptide) of rat α -LA in the presence of microsomal membranes from dog pancreas has been studied by Lingappa *et al.* (31). In these studies, the sequences of the COOH-terminal end before and after the treatment with microsomal membranes was not investigated.

Caseins from all the species sequenced to date are proline-rich milk proteins. It is worth noting that the COOH-terminal proline-rich extension of rat α -LA contains sequences ¹²⁹Val-Val-Pro and ¹³⁸Pro-Val-Pro, which are also present in β -casein from bovine and ovine sources but at positions 83–85 and 172–174, respectively (36). The sequences of rat caseins have not yet been analyzed. Rat casein cDNA clones have been isolated in our laboratory, but their sequences have not been determined.

Now that the sequences of α -LA cDNA clones are known, it should be possible to probe the molecular mechanisms of the hormonal regulation of α -LA mRNA synthesis and the genomic organization of the α -LA gene sequences in normal as well as neoplastic tissues.

We thank M. Siegel and B. Stubblefield for their expert technical

assistance, Drs. R. Dhar and P. Reddy for introducing us to DNA sequence analysis techniques, and Ratna Qasba and Drs. P. N. Kaul and E. Appella for their help in the preparation of this manuscript.

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