A NOVEL CYTOSKELETAL ASSOCIATED PROTEIN: GENOMIC ORGANIZATION AND PROTEIN PATTERN*

Haralabos ZORBAS, Ernst PÖSCHL, Antonia PÖSCHL and Peter K. MÜLLER

Max-Planck-Institut für Biochemie, 8033 Martinsried, F.R.G.

Received May 21st, 1986

c-DNA clones were constructed from chicken sternal m-RNA and screened for recognizing those m-RNA species the amount of which decreases during chondrocyte dedifferentiation. One such clone was used to screen a chicken genomic library and, furthermore, to deduce peptide sequences suitable for raising antibodies. Initial results on the genomic structure and on some biochemical and immunochemical properties are presented.

Chondrocytes synthesize and secrete a variety of macromolecules which provide the structural framework for the biomechanical properties unique to cartilageneous tissues[1]. Chondrocytes maintained in culture change their morphology and alter their pattern of synthesis of extracellular matrix proteins. Most studied is the switch from synthesis of type II collagen to synthesis of type I and type III collagen[2]. In order to analyse this process of dedifferentiation on a molecular level we constructed c-DNA clones from chicken sternal m-RNA, screened a genomic library and also used the c-DNA to deduce peptide sequences which were suited as peptide immunogens after coupling to a carrier protein.

EXPERIMENTAL

In order to isolate m-RNA we used 6M-guanidinum hydrochloride and repetitive alcohol precipitation[2]. Northern blot analysis, construction of genomic libraries, and radioactive labeling of c-DNA probes are described in detail by Maniatis et al.[3]. Quantitation of m-RNA was carried out by means of the S1 nuclease technique according to Berk and Sharp[4]. Selection of potentially immunogenic sites was based on criteria of hydrophilicity and hydrophobicity which is described by Walter and Doolittle[5]. The detailed description of the p550 clone is given in ref.[6]. The peptides (11 and 9 amino acid residues) were synthesized with an additional N-terminal Cys residue and coupled via MBS (N-maleimidobenzoyl-N-hydroxysuccinimide ester) to keyhole limpet hemocyanin (KLH). These conjugates were used for immunisation of rabbits. Immunoblot analysis and immunofluorescence studies were carried out according to published methods[7,8].

* Presented on the occasion of the 20th anniversary of the Institute of Rheumatology in Prague (13th of March 1986).

RESULTS AND DISCUSSION

Chondrocytes were maintained in culture for prolonged times so that their dedifferentiation had proceeded to such an extent that the majority of cells had assumed a fibroblast like morphology[8]. At several weekly time intervals m-RNA was isolated and analyzed by Northern blot hybridization using c-DNA probes specific for a1(I), a2(I), and a newly constructed c-DNA clone, named p550. While a1(I) and a2(I) specific m-RNA species increase with time in culture, the p550 clone recognizes a m-RNA species which decreases during the time of in vitro culture[2]. Using S1--nuclease resistance as an experimental approach to quantify m-RNA by hybridization technique we determined the amounts of p550 specific m-RNA in various tissues at different gestational ages. As summarized in Table I, p550 specific m-RNA occurs abandontly in chondrocytes, tendons, and in calvaria of 12 day old chicken. Calvaria, however, show a significant decrease during development of the chicken embryo since p550 specific m-RNA is virtually absent in 19 day old calvaria. Little is found in parenchymal tissues such as liver or brain. Furthermore, it is surprising that neither the long bones nor the articular cartilage contains appreciable amounts of the p550 specific m-RNA.

Using the p550 clone as a probe we screened a genomic library constructed from both sternal and calvarial DNA and found a number of overlapping clones which we used to establish the physical map of the p550 gene spanning a range of about 28 kbp in length (Fig. 1). Since the size of the p550 specific m-RNA was determined by Northern blot analysis as 4·4 and 6·5 kb in length, the gene contains a number of introns which brings its coding capacity to about 20% (ref.[6]). At present we attempt to localize and to characterize the 5′ end of the gene in order to analyse

TABLE I
Relative amount of p550 specific m-RNA

Tissue	Age of chicken (days)	Amounts of m-RNA	Tissue	Age of chicken (days)	Amounts of m-RNA
Sternum	12	+++++	Tendon	16	+++++
	16	+++++	Articular cartilage	16	+
	19	+++++			
Calvarium	12	+++++	Long bone	16	+
	16	+++	Brain	16	−
	19	+	Liver	16	−

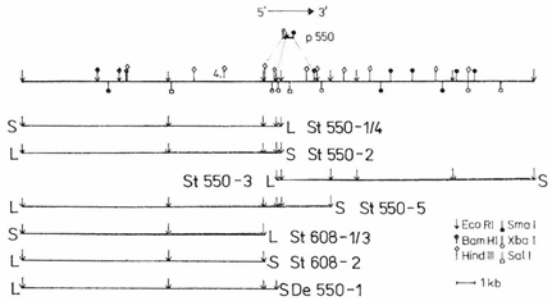

FIG. 1
Physical map of the p550 specific gene

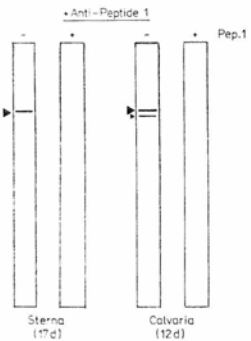

FIG. 2
Immunoblot analysis of calvaria and sterna extracts. The bands were identified by reaction with synthetic peptide probes

FIG. 3
Immunofluorescence of chicken chondrocytes maintained in an in vitro culture. The immunological reaction was specific to the use of antibodies directed against synthetic peptide probes. Magnification: 40×

the regulating motifs which could account for the developmentally controlled and tissue specific expression of this gene.

In the second line of experiments we raised antibodies against synthetic peptides the sequence of which has been deduced from the p550 clone and selected for potentially immunogenic sites by computer assisted analysis. Using immunoblot analysis of whole sterna and calvaria extracts we visualized with the peptide specific antibodies a protein band of an apparent molecular weight of 155 000 (Fig. 2). This reaction could be completely suppressed by preincubation with the highly purified peptide demonstrating the specificity of the immunological response. The same antisera were used for indirect immunofluorescence studies of cultured mesenchymal cells. A thin intracellular network lights up which showed a close association with intermediate size filaments[9] (Fig. 3). It was clearly distinguishable from the tubuline and actin networks which constitute the two other cytoskeletal scaffolds. Again, the specificity of the reaction was demonstrated by preincubation with the purified peptide which completely suppressed the immunofluorescence staining with the anti-peptide antibody. Because of the size of the p550 coded protein and since the sequences show no homology to any published DNA or protein sequence we assume that this protein belongs to the growing group of cytoskeletal associated proteins the function of which is still not clear as yet. Further studies on both the protein and genomic levels are required in order to establish its function which could be of importance for a better understanding of the tissue specificity and developmental control of genes expressed in mesenchyme.

*We thank Prof. K. Kühn for encouraging this work and for helpful discussions. The excellent technical assistance of H. Kadner and A. Pavlovic is gratefully appreciated. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (*Mu *378/13—3).*

REFERENCES

1. Caplan A. J.: Sci. Am. *251*, 82 (1984).
2. Duchene M., Sobel M. E., Müller P. K.: Exp. Cell Res. *142*, 317 (1982).
3. Maniatis T., Fritsch E. F., Sambrook J.: *Molecular Cloning.* Cold Spring Harbor Laboratory 1982.
4. Berk A. J., Sharp P. A.: Cell *12*, 721 (1977).
5. Walter G., Doolittle R. F. in the book: *Genetic Engineering* (J. K. Setlow and A. Hollaender, Eds), Vol. 3, p. 61. Plenum Press, New York 1983.
6. Pöschl E.: *Thesis.* University of Munich, Munich 1984.
7. Towbin H., Staehelin T., Gordon J.: Proc. Natl. Acad. Sci. U.S.A. *76*, 4350 (1979).
8. Müller P. K., Lemmen C., Gay S., Gauss V., Kühn K.: Exp. Cell Res. *108*, 47 (1977).
9. Geiger B.: Biochim. Biophys. Acta *737*, 305 (1983).