polymerase-8/ml in 8mM-Mg2+/120mM-KCl/50μM-dNTP (containing [3H]TPP, 560 c.p.m./mol of nucleotide). Excision for 10 min created a gap of 9 nucleotides/pyrimidine dimer, which was filled by the DNA polymerase, converting it into a single-strand break (Fig. 1, curve a), ligatable by Escherichia coli DNA ligase (loss of priming sites for E. coli DNA polymerase).

Their combined repair action in vitro is dependent on ionic strength and Mg2+ concentration, which were varied to make the exonuclease limiting. Thus at 4mM-Mg2+ and 70mM-KCl the activity of the exonuclease was one-sixth that of the DNA polymerase. Under these conditions the combined enzymes catalyzed incorporation of nucleotides into incised u.v.-irradiated phage-T7 DNA (Fig. 1, curve b) concurrent with breakdown shown in a parallel experiment with radioactively labelled template (Fig. 1, curve c). This reached a plateau of 20 nucleotides/pyrimidine dimer, which was not significantly increased by addition of fresh enzyme. This forms the basis for a nick-translation system (Lehman, 1967) for repair of u.v. damage in human cells, although the limitation of 20 nucleotides/pyrimidine dimer has yet to be explained.

This work was supported by National Science Foundation grant PCM-79-14191, and National Institute of Environmental Health Sciences grant 5 Pol ES00454.


Hogeboom, G. H., Schneider, W. C. & Stiebich, M. J. (1952) J. Biol. Chem. 196, 111-120


---

The α-amanitin-binding subunits of eukaryotic RNA polymerase II

ERIK BATEMAN AND BRUCE NICHOLSON

Department of Physiology & Biochemistry, University of Reading, Whiteknights, Reading RG6 2AJ, U.K.

Eukaryotic DNA-dependent RNA polymerases are multimeric enzymes consisting of 10-15 polypeptides (Roeder, 1976). Information about the role of subunits in transcription of deproteinized DNA or chromatin is limited (Sentenac et al., 1977). One way to examine subunit function is to study the interaction of transcription inhibitors with the subunits of RNA polymerase (Nixon et al., 1972; Wu & Wu, 1974). α-Amanitin is a naturally occurring non-competitive inhibitor of RNA polymerase II, suitable for such an investigation (Cochet-Meilhoc & Chambon, 1974).

RNA polymerase II was purified from wheat germ as described by Jendrisak & Burgess (1975). The purified enzyme was shown to be of high purity by polyacrylamide-gel electrophoresis under non-denaturing and denaturing conditions (Laemmli, 1970). The subunit composition agreed with that previously reported (Jendrisak & Burgess, 1977).

RNA polymerase (50μg) was incubated with various concentrations of α-amanitin (Boehringer) at 37°C for 10 min in a buffer containing 50mM-Tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine), pH 7.9, 0.15mM-NaCl, 10mM-2-mercaptoethanol and 25% (v/v) glycerol. The samples were placed on ice, NaBH4 (Amersham; 424mCi/mmol) was added to a final concentration of 1mM, and the mixture was incubated for 2h at 0°C. Reactions were terminated by addition of 1ml of ice-cold 10% trichloroacetic acid. After a further 10 min at 0°C the samples were centrifuged at 10000 g for 7min. The precipitates were rinsed twice with acetone, to remove trichloroacetic acid, and dissolved in electrophoresis sample buffer (Schwartz & Roeder, 1974). RNA polymerase subunits were separated by electrophoresis on 13% polyacrylamide slab gels as described by Laemmli (1970). Labelled subunits were identified by fluorography (Bonner & Laskey, 1974). To aid in identification of subunits, RNA polymerase labelled with succinimidyl [2,3-3H]propionate was run on the same gel. Fluorographs were scanned at 600nm with a Pye-Unicam spectrophotometer fitted with a linear transport device (Fig. 1).

α-Amanitin binds predominantly to subunits IIA and IIB. There is some labelling of these subunits in the absence of α-amanitin, but in the presence of 0.1μg of α-amanitin/ml the labelling increases 2-fold. This concentration is sufficient to cause around 5% inhibition, with the polymerase concentration at around 1mg/ml. Polymerase is usually assayed at a concentration of 20μg/ml, at which concentration 50% inhibition is obtained with α-amanitin at 0.025μg/ml. At higher concentrations (100μg/ml) there is a 10-fold increase in the label attached to subunits IIA and IIB. Subunit IIA* is also labelled (results not shown). This subunit is a minor component of wheat-germ RNA polymerase II arising from proteolytic cleavage of subunit IIA (Guilfoyle & Jendrisak, 1978). At this concentration, a radioactive peak also appears at the dye front.

It has previously been shown (Cochet-Meilhoc & Chambon, 1974) that 1 molecule of α-amanitin/molecule of RNA polymerase is sufficient to cause inhibition. The results imply that the α-amanitin-binding site is shared between subunits IIA and IIB. By using chemical cross-linking reagents, it has been shown that these subunits are in contact, and the use of competitive inhibitors indicates that subunits IIA and IIB are involved in DNA and nucleotide binding (E. Bateman, unpublished work). Since α-amanitin inhibits both initiation and elongation (Cochet-Meilhoc & Chambon, 1974), the binding of α-amanitin to subunits IIA and IIB is consistent with their having a direct role in transcription.
Sulphated-glycosaminoglycuronan–collagen interactions in developing rat tail tendon

JOHN E. SCOTT, CONSTANCE R. ORFORD and EIRA W. HUGHES

Department of Medical Biochemistry, University of Manchester Medical School, Oxford Road, Manchester M13 9PT, U.K.

In the laying-down of connective tissue, interactions between fibres (collagen) and interfibrillar polymers (proteoglycans, etc.) are of prime importance. Our integrated electron-microscopic biochemical investigation was performed to identify the important interactions during the development of tendon.

Developing tail tendons from rats of various ages (19-day foetal to 126 days post partum) were examined by electron microscopy after staining for proteoglycan with a cationic copper phthalocyanin dye, Cuprolinic Blue, in a ‘critical point’ metalisation method (Scott, 1980). In parallel, tendons were digested with papain. Glycosaminoglycuronans were isolated and fractionated as cetylpyridinium complexes, than as calcium salts in aqueous ethanol, followed by electrophoresis in 0.1 M-calcium acetate or -barium acetate, and in 0.1 M-HCl. Fractions were analysed colorimetrically for uronic acid and hydroxyproline (mglg dry wt.; A. Jendrisak, J. J. (1978) Biochemistry 17, 1860–1866; Jendrisak, J. J. & Burgess, R. R. (1975) Biochemistry 14, 4639–4645; Jendrisak, J. J. & Burgess, R. R. (1977) Biochemistry 16, 1959–1964).

Mean collagen-fibril diameters increased more than 10-fold with age according to a sigmoid curve (Fig. 1), the rapid growth phase being from 30 to 90 days after conception. Fibril periodicities were considerably smaller (50–55 nm) in phase 1 and phase 2 than in phase 3 (>62 nm).

Transverse sections extended previous results obtained on longitudinal sections (Scott, 1980) to give a three-dimensional picture of proteoglycan around, but not inside, collagen fibrils. Proteoglycan filaments may encompass several thin fibres in very young tissue, but did not usually link larger, mature fibres. Transfibrillar proteoglycan filaments were spaced along the vertical axis of the fibril at distances equal to those of the collagen periodicity, implying specific interactions between proteoglycan and collagen.

Dermatan sulphate is the main glycosaminoglycuronan in mature tendon, but chondroitin sulphate and hyaluronate preponderate in foetal tissue. The changes are largely complete early in phase 2. Proteoglycan (proportional to length of stained filaments) and collagen (proportional to cross-sectional area of fibrils) were quantified from electron micrographs. Their ratios showed the same behaviour with age as those of uronic acid: hydroxyproline and hyaluronate:hydroxyproline, which decreased rapidly immediately after birth and then reached a plateau. The ratios reached their low plateau coincident with the onset of rapid growth (phase 2) in collagen fibril diameter.

A collagen cylinder surrounded in a regular fashion by proteoglycan implies the relationship, ‘proteoglycan/hydroxyproline is proportional to the reciprocal of the fibril diameter’, since proteoglycan = $k_1 \times$ circumference = $k_2 \times \pi r$, and hydroxyproline = $k_3 \times$ fibril cross-sectional area = $k_4 \times r^2$. The ratio $2k_1/k_2 = \text{proteoglycan/hydroxyproline}$. Dermatan sulphate/hydroxyproline follows the prediction throughout the age range (Fig. 2), confirming that the proteoglycan orthogonal array around the mature fibril is largely dermatan sulphate. Hyaluronate (and less certainly chondroitin sulphate) fits the relationship from the beginning of phase 2 onwards, but not in phase 1, where both are in large excess over that expected to be bound to collagen. The amount of fibril-associated dermatan sulphate is about 20 times that of fibril-associated hyaluronate (Fig. 2).

The results contribute to the following view of fibrillogenesis and fibre maturation.

Phase 1 (0–40 days after conception). Collagen synthesis leads to the formation of increasing numbers of thin fibrils, rather than...