Nucleic Acids Research, Vol. 19, No. 18 ***4975–4982***

Association of a change in chromatin structure with a tissue-specific switch in transcription start sites in the α2(I) collagen gene

Kristen M.Beck, Alfred H.Seekamp, G.Roger Askew, Zhu Mei, Catherine M.Farrell, Sandia Wang and Lewis N.Lukens*

Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06459, USA

Received June 6, 1991; Revised and Accepted August 16, 1991

ABSTRACT

Chick embryonic sternal chondrocytes do not synthesize α2(I) collagen until they are shifted by treatment with 5-bromo-2′-deoxyuridine (BrdUrd) to a fibroblastic phenotype, yet they transcribe this gene as rapidly as BrdUrd-treated cells. To examine further this transcription, the DNase I hypersensitive sites were mapped in the 5′ region of this gene in chondrocytes, BrdUrd-treated chondrocytes, fibroblasts and three types of non-transcribing cells. A DNase I hypersensitive site at −200 bp, previously shown to be associated with the active transcription of this gene in fibroblasts, is not present in chondrocyte chromatin. The chondrocyte α2(I) gene contains, however, a novel major hypersensitive site in the DNA region corresponding to the fibroblast intron 2, near the chondrocyte-specific transcription initiation site of this gene. This novel hypersensitive site is associated with the use of this alternate start site by chondrocytes, since it is lost when BrdUrd treatment causes these chondrocytes to switch to the initiation of transcription at the fibroblast start site. The BrdUrd-treated chondrocytes contain the same α2(I) hypersensitive sites as fibroblasts, except that fibroblasts have an additional, previously unreported, site at −1000 bp.

INTRODUCTION

The two most abundant types of collagen in vertebrates are type I collagen, found in major proportions in bone, skin, ligaments and tendons, and type II collagen, found almost exclusively in hyaline cartilage. The regulation of synthesis of these molecules is extremely important in development and in a number of physiological and pathological conditions (for reviews see references 1 and 2). A promising system for investigating how this regulation is achieved is provided by the exposure of cultured chick embryonic chondrocytes to 5-bromo-2′-deoxyuridine (BrdUrd) (3, 4, 5). During 7–8 days of culture in the presence of this thymidine analogue, the initially floating chondrocytes become attached to the culture dish and shift from a chondrocytic to a fibroblastic phenotype. Untreated chondrocytes synthesize type II collagen and other cartilage specific products, but do not make type I collagen. The BrdUrd-shifted chondrocytes, on the other hand, synthesize type I collagen and other products characteristic of fibroblasts, but do not make type II collagen. Since the mesenchymal cells that are the precursors of chondrocytes synthesize type I collagen (6), the BrdUrd may be causing a 'de-differentation' back to the mesenchymal phenotype. A similar shift in phenotype is also induced by the tumor promoter, phorbol-12-myristate-13-acetate (7, 8), or by repeated subculture (reviewed in reference 6), or by transformation with Rous Sarcoma virus (9, 10, 11).

Although chondrocytes do not synthesize type I collagen, a heterotrimer of chain composition $[\alpha1(I)]_2\alpha2(I)$, our previous studies indicated that they transcribe the α2(I) gene. The steady state level of α2(I) mRNA in the cytoplasm of untreated chondrocytes, measured with cDNA probes derived from the 3′ end of the mRNA, was found to be surprisingly high, about one third of the level in the cytoplasm of the BrdUrd shifted cells (12). The α1(I) mRNA, which codes for the other type of chain present in the type I collagen molecule is, by contrast, present in the cytoplasm of untreated chondrocytes at less than 3% of its level in BrdUrd-shifted cells. Subsequent nuclear run-on experiments have shown that the untreated chondrocytes transcribe the α2(I) gene at rates similar to the BrdUrd-treated cells (13). Because of the unusual situation of the apparent synthesis of the α2(I) mRNA but the absence of synthesis of the α2(I) polypeptide, we wished to study further the transcription of the α2(I) gene in untreated chondrocytes. A DNase I hypersensitive site has been reported to be present at 200 bp 5′ to the transcription start site of the α2(I) gene in transcriptionally active chromatin but not in transcriptionally inactive chromatin (14,15). Therefore, we undertook to examine the chromatin structure of the α2(I) gene in chondrocytes.

* To whom correspondence should be addressed

In contrast to all the previously observed cell types that actively transcribe this gene (14, 15), chondrocyte chromatin was found to be lacking the hypersensitive site at −200 bp. This chromatin did contain, however, a novel major hypersensitive site in intron 2 (exons and introns are numbered throughout this paper according to the fibroblast α2(I) gene). Since these major changes in chromatin structure seemed likely to be related to the recently reported observation (16, 17) that chondrocytes initiate transcription of the α2(I) gene at a different start site than fibroblasts, located in intron 2, we showed by RNase protection assays that these chondrocytes also use this new start site. The observation that BrdUrd causes chondrocytes to switch initiation of transcription from the chondrocyte start site to the fibroblast start site provided a way to show that the chondrocyte-unique start site and hypersensitive site are coordinately regulated, since the BrdUrd-treated chondrocytes were found to have lost the hypersensitive site located in intron 2.

The use of a 5′ probe allowed the resolution of the −200 bp hypersensitive site, present in the chromatin of fibroblasts and BrdUrd-treated chondrocytes, into two sites and the detection of a new site in fibroblast chromatin at −1,000 bp.

MATERIALS AND METHODS

Isolation of Cells and Nuclei

Chondrocytes were isolated from the sterna of 14-day old chick embryos (purchased from Spafas, Norwich, CT) by the floater selection method (18), except that the cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum (Gibco) and 10% CO_2. The chondrocytes were induced to shift to a fibroblastic phenotype by culture in the presence of 6.5×10^{-5} M bromodeoxyuridine (Boehringer Mannheim). Chick embryo fibroblasts (CEF) were isolated from decapitated 10-day old embryos by a standard procedure (19) and incubated on tissue culture plates under the same conditions as chondrocytes for 2−4 hours, prior to harvesting of the attached cells. The brain cells from 14-day embryos were treated as cultured cells after dissociation of the tissue by mincing with a spatula. The erythrocytes were obtained from the blood of 14-day old chick embryos. Nuclei were prepared from cells chilled to 0°C and were stored in 400−800 μl aliquots at −80°C until needed according to the procedure of Konieczny and Emerson (20). The chick intestinal epithelial nuclei were a generous gift from Mark Mooseker. The pelleted nuclei were resuspended in nuclear storage buffer (20) and stored at −80°C.

DNase Treatment of Nuclei and Isolation of DNA

The nuclei were thawed on ice and washed once in 10.0 ml of DNase I digestion buffer (21) and pelleted by centrifugation for 5 min at 1000×g. The subsequent suspension in digestion buffer and incubation with 5−100 μg/ml of DNase I (Boehringer Mannheim) for 10 min at 30°C followed exactly the procedure of Babiss et al. (21). The digestion was stopped and proteinase K added as described (21) and the sample was incubated for 1−3 hours at 50°C. The DNA was then isolated by phenol:chloroform extraction followed by ethanol precipitation in the presence of ammonium acetate (22). The DNA (25 μg) was digested to completion with 100 units of *Eco*RI overnight at 37°C and was recovered by ethanol precipitation as above. It was resuspended in 50 μl of TE (22) and stored at −80°C. Deproteinized chick genomic DNA was prepared from the livers of 14-day chick embryos by a standard procedure (see p 9.17−9.19, ref. 22).

Identification of α2(I) DNase I Hypersensitive Sites

10 μg of DNA after the DNase I and *Eco*RI digestions were fractionated on a 1.2% agarose gel in tris-borate-EDTA buffer by electrophoresis overnight at approximately 3 volts/cm (22). The DNA fragments were then transferred by capillary transfer (22) to nitrocellulose filters (Schleicher and Schuell). The filters were prehybridized and, after the addition of probes (see below), were hybridized overnight at 42°C in 50% formamide by a standard procedure (see p. 9.52−9.53, ref. 22). The filters were washed as described (see p. 2.116, ref. 22) but after the room temperature washes the next wash was with 0.1×SSC and 0.5% SDS for two hours at 68°C and the final wash was with 1×SSC in 1% SDS for 30 min at 68°C.

The probes were ^{32}P-labeled by the random primer method (23). They were separated from unincorporated nucleotides by Nu-Clean D-50 Spun Columns (IBI) and were further purified by filtration through a 0.45 μm cellulose acetate filter (Nalgene). The 5′ probe consisted of the 400 bp *Eco*RI-*Bam*HI fragment from the 5′ end of pCg5.7, a pBR322 plasmid clone generously provided by Dr. Helga Boedtker (24, 25) that contains a chick α2(I) genomic insert which extends from 1.6 kb upstream of the fibroblast transcription start site to 4.1 kb downstream of this site (see Fig. 4). This 400 bp fragment was subcloned into the *Eco*RI and *Bam*HI sites of pUC18. The 3′ probe consisted of the 360 bp *Hind* III−*Eco*RI fragment from the 3′ end of pCg5.7 (see Fig. 4). This fragment was subcloned into the same restriction sites in the plasmid pBluescribe (pBS, Stratagene). The α2(I) inserts were isolated from the vectors prior to labeling. The probes were present in the hybridization reaction at a concentration of 10 ng/ml and a specific activity of approximately 10^9 cpm/μg.

A 100 ng sample of *Bst*EII-digested lambda DNA was included in each gel as a size-marker and, after localization of the α2(I) fragments on the nitrocellulose filter, the filter was hybridized with ^{32}P-labeled lambda DNA to locate the *Bst*EII fragments. In all cases, the radioactive bands were detected by exposure of the filters to Kodak X-Omat X-ray film for 1−10 days at −80°C, followed by development in a Kodak M35A X-Omat automatic processor.

S1 Nuclease Digestion

The digestion of supercoiled pCg5.7 or pBR322 DNA with S1 nuclease and the subsequent purification of the DNA were as described (24). After digestion with *Eco*RI as described above, S1-treated or untreated DNA samples (0.2 ng) were mixed prior to electrophoresis with approximately 10 μg of chondrocyte DNA obtained from chondrocyte nuclei by the usual procedure except omitting the addition of DNase I.

RNase Protection Assay

RNA was isolated as described previously (12). The antisense RNA probes were transcribed *in vitro* with T3 RNA polymerase after inserting the desired α2(I) DNA sequences into the plasmid vector pBS which contains a bacteriophage T3 promoter. The template for the antisense riboprobe that began in exon 6 at nt +375 from the transcription start site and extended through nt +9 in exon 1 (see Fig. 6A) was made by ligating a *Sau*3A-*Sma*I fragment from an α2(I) cDNA clone pMF21 (25), generously provided by Louis Gerstenfeld, into the *Bam*HI and *Sma*I sites of pBS. Prior to transcription with T3 polymerase, this plasmid was linearized by digestion at its *Sma*I site. An identical template,

but linearized at the *Bst*UI site, yielded an antisense riboprobe that extended from nt +375 in exon 6 through nt +155 in exon 1 (Fig. 6B). The template for the riboprobe (Fig. 6C) that began at nt +11 of exon 3 and contained the adjoining 470 nt from the 3′ end of intron 2, was prepared by ligating the corresponding genomic *Pst*I fragment, isolated from the α2(I) genomic clone, pCg5.7, into the *Pst*I site of pBS. This plasmid was linearized at its *Sma*I site. RNA probes were purified by electrophoresis in 6% polyacrylamide sequencing-type gels. The transcription reaction in the presence of [α-^{32}P] UTP used the Stratagene Transcription kit according to the manufacturer's directions. Hybridization at 45°C overnight and subsequent digestion with RNase A and TI were according to Melton *et al.* (26), except that these enzymes were reduced to one-tenth the recommended levels, 4μg/ml and 70 units/ml, respectively, since these amounts reduced degradation of the protected fragment and gave cleaner autoradiograms. The termination of the RNase digestion, recovery of protected hybrids and electrophoresis on 6% polyacrylamide sequencing gels were as described (26).

RESULTS

Chondrocyte Chromatin Has a Unique Major DNase I Hypersensitive Site in Intron 2 of the α2(I) Collagen Gene

The DNase hypersensitive sites present in the chromatin of the α2(I) gene in different cell types were mapped by the indirect end-labeling method (27) within a 5.7 kb *Eco*RI genomic fragment spanning the region from 1.6 kb upstream to 4.1 kb downstream of the fibroblastic transcription start site (24, 25). The probes used for mapping were a 400 bp *Eco*RI−*Bam*HI fragment from the extreme 5′ end of this 5.7 kb region and a 360 bp *Hind* III−*Eco*RI fragment from the extreme 3′ end (see Fig. 1).

When the 5′ probe was used to locate DNase I hypersensitive sites, a major band of 4.0 kb was found in the chromatin of these sternal chondrocytes (Fig. 1). This hypersensitive site maps to approximately 250 bp downstream from the 5′ end of intron 2, which is the region that contains the reported chondrocyte-specific transcription start site (17). This hypersensitive site is present at very low levels in the chromatin from chick embryo fibroblasts (Fig. 1, CEF lanes) or from several other cell types that have been examined (see below). The location of this novel hypersensitive site in chondrocyte chromatin was confirmed by mapping with the 3′ probe, which detected a major band resulting from DNase cleavage at 1.7 kb from the 3′ end of this 5.7 kb genomic fragment (Fig. 2, Chond. lanes). Although this site was also detectable in fibroblast chromatin with the 3′ probe (Fig. 2, CEF lanes), its concentration relative to that in chondrocyte chromatin is very low.

Chondrocyte chromatin was also found to contain a second minor DNase hypersensitive site not present in fibroblast chromatin. This site maps to 1.8 kb from the 5′ end of the 5.7 kb α2(I) genomic fragment (Fig. 1), which would place it in the region of the junction between exon 1 and intron 1. The remaining three sites in chondrocyte chromatin (Fig. 1) are shared by fibroblast chromatin and are discussed below.

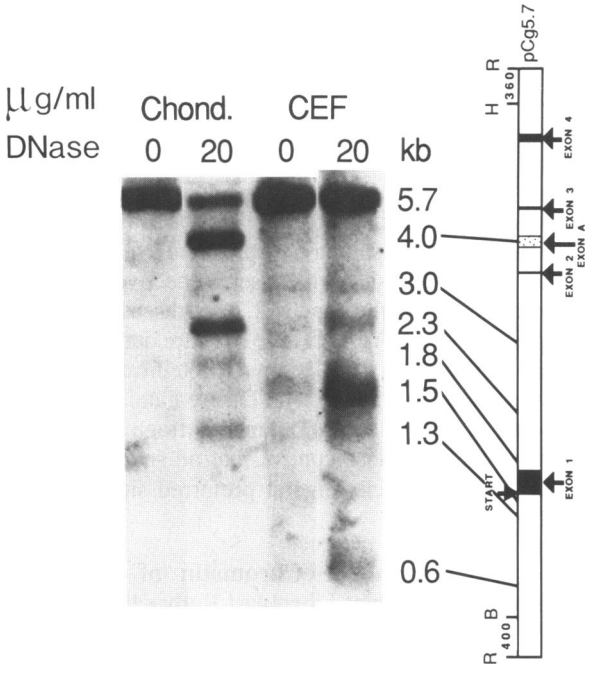

Fig. 1. DNase I hypersensitive sites in the 5′ region of the collagen α2(I) gene in chondrocytes and fibroblasts mapped with the 5′ probe. The sites of DNase cleavage within a 5.7 kb *Eco*RI fragment of the α2(I) gene were mapped by hybridization of Southern blots to a ^{32}P-labeled 400 bp sequence from the 5′ end of this region (see map at right). The sizes of the fragments smaller than the intact 5.7 kb fragment correspond to the distances of these sites from the 5′ end of this 5.7 kb genomic region and allow placement of these sites on the gene as shown on the far right. pCg5.7 refers to the plasmid clone of this *Eco*RI genomic fragment (24,25). R, B, and H symbolize *Eco*RI, *Bam*H and *Hind*III sites, respectively.

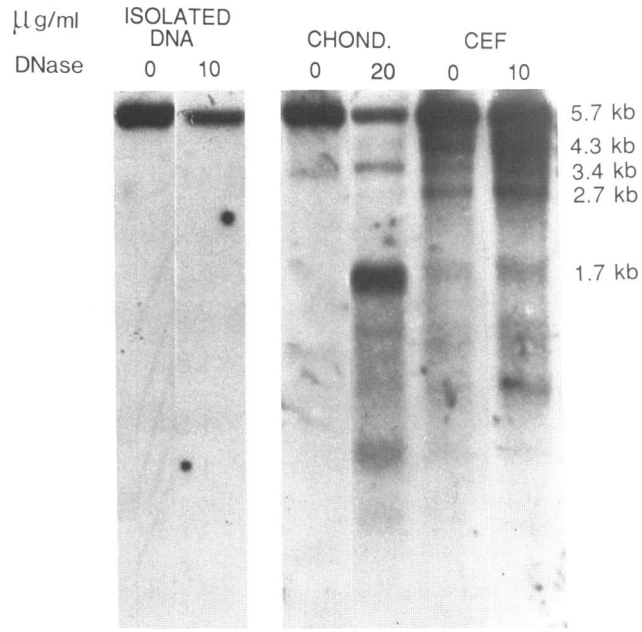

Fig. 2. DNase I hypersensitive sites in the same region of α2(I) DNA as in Fig. 1, but mapped with the 3′ probe. The ^{32}P-labeled DNA probe used here was a 360 bp fragment from the 3′ end of the 5.7 kb genomic α2(I) *Eco*RI fragment (see map at right in Fig. 1). The sizes of the fragments correspond to the distance of the cleavage sites from the 3′ end of this 5.7 kb fragment. The two lanes on the left contained chick DNA which was isolated and deproteinized to destroy chromatin structure prior to treatment with DNase and subsequent isolation by the usual procedure.

The α2(I) Gene in the Chromatin of Chick Embryo Fibroblasts Contains Two Major Hypersensitive Sites that Appear to be Associated with Transcriptional Activity

Previous mapping of the DNase hypersensitive sites in the α2(I) gene in the chromatin of chick embryo fibroblasts identified a major hypersensitive site at approximately 200 bp upstream of the transcription start site, that was present only in transcriptionally active chromatin (14, 15). These workers detected this site as a 4.3 kb band with the same 3′ probe used in the present studies. We obtained identical results with fibroblast chromatin (Fig. 2, CEF lanes), but this site was barely detectable in chondrocyte chromatin (Fig. 2, Chond. lanes). When mapped with the 5′ probe, this site in fibroblast chromatin was resolved into two sites, a major site at 1.5 kb and a minor site at 1.3 kb from the 5′ end of the 5.7 kb fragment (Fig. 1). While the DNase sensitivity of the minor site at 1.3 kb appears to be unchanged by whether transcription begins at the chondrocyte or fibroblast start site, the major site at 1.5 kb is present only in fibroblast chromatin (Fig.1). The site at 1.5 kb is therefore apparently associated with transcription of the α2(I) gene from its fibroblast start site.

The same region of DNA that contains the DNase I hypersensitive site at 4.3 kb when mapped with the 3′ probe has been found to contain an S1 nuclease-sensitive site when the isolated DNA is present in a supercoiled plasmid (24, 28). The resolution of the 4.3 kb site into two sites at approximately 300 and 100 bp 5′ to the fibroblast transcription start site raised the question whether the S1 site was in the more sensitive region closer to the start site or in the more 5′ region. When the electrophoretic mobilities of the 1.3 and 1.5 kb fragments from DNase cleavage were compared with that of the S1-released fragment from the 5.7 kb region present in the supercoiled plasmid pCg 5.7 (24, 25), the 1.5 kb DNase site was found to be in the same DNA region as the S1 site, whereas the 1.3 kb site was further upstream of the transcription start site (data not shown). Since this S1 site has been mapped at nucleotide resolution to several phosphodiester bonds at around −190 bp (24), the 1.5 kb DNase hypersensitive region probably extends 5′ to at least −200 bp.

In addition to the 1.5 kb site, a second major hypersensitive site was present in fibroblast, but not chondrocyte, chromatin at 600 bp from the 5′ end of the 5.7 kb α2(I) genomic fragment (Fig. 1). This previously unreported site is approximately 1000 bp upstream of the fibroblast transcription start site. This site may be associated with the high transcription rate of the α2(I) gene in these fibroblasts, since it is almost absent from the transcriptionally less active BrdUrd-treated chondrocytes (Fig. 3) which also utilize the fibroblast transcription start site, as shown below. Although this site was always highly visible with the 5′ probe, it was not detected with the 3′ probe. This result was expected, however, since sites that were at a large distance from where the probe hybridized were consistently found to be difficult to detect, presumably because of the increased occurrence of nuclease cleavages between these sites and the region recognized by the probe.

The presence of identical DNA fragments in the samples from nuclei incubated with or without added DNase I, especially in the case of the chromatin from fibroblasts (Figs. 1 and 2) and from BrdUrd-shifted chondrocytes (Fig. 3), is due to endogenous DNase activity, which has been noted in other cases (see for example references 29, 30). These endogenous fragments are clearly given by DNase hypersensitive sites, since they have repeatedly been seen to increase in concentration with increasing levels of added DNase. Also the 4.3 and 3.4 kb bands detected in fibroblast chromatin with the 3′ probe (Fig. 2, CEF lanes) have been previously shown to represent DNase hypersensitive sites (14, 15); these sites give rise, respectively, to the bands at 1.3 and 1.5 kb and the band at 2.3 kb (discussed below) when the 5′ probe is used (Fig.1). These sites are due to the chromatin structure of the gene, since when deproteinized chick genomic DNA was similarly digested with DNase I and then with *Eco*RI, the amount of the 5.7 kb α2(I) genomic fragment decreased, as compared to a no-DNase control, but no smaller fragments of discrete sizes due to cleavage at preferred sites were detected (Fig. 2).

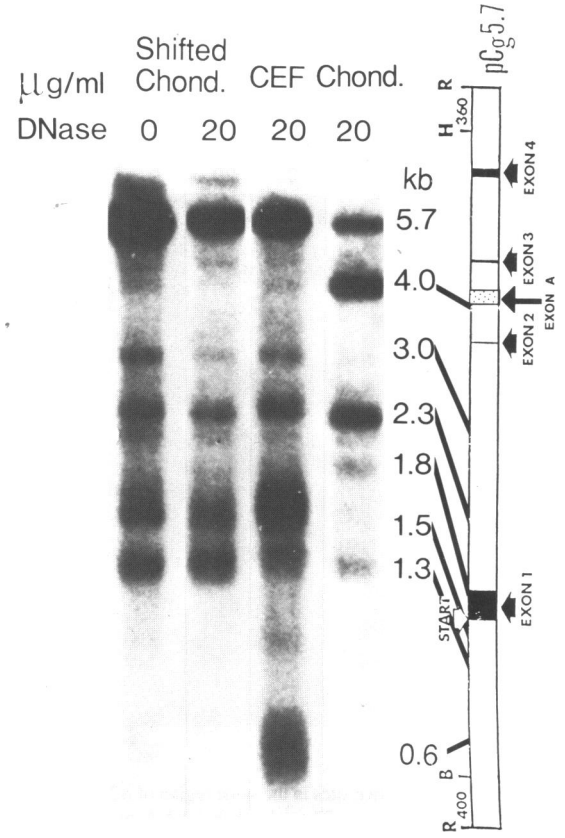

Fig. 3. DNase I hypersensitive sites in the collagen α2(I) gene in BrdUrd-shifted chondrocytes mapped with the 5′ probe and compared with the sites in chondrocytes and fibroblasts. The experimental procedure was the same as that described in Fig. 1.

The α2(I) Gene in the Chromatin of BrdUrd-Shifted Chondrocytes Resembles Fibroblast Rather than Chondrocyte Chromatin

Since BrdUrd-shifted chondrocytes have a fibroblastic phenotype and synthesize type I collagen, it seemed likely that the α2(I) chromatin structure in these cells might have shifted from the chondrocyte to the fibroblast type. This expectation was fulfilled, since the chromatin of shifted chondrocytes had lost the major site at 4.0 kb characteristic of untreated chondrocytes and had gained the major site at 1.5 kb which is associated with the transcriptionally active form of this gene in fibroblasts (Fig. 3). The major site at 600 bp which is present in fibroblast chromatin was, however, barely detectable in the chromatin of the BrdUrd-

shifted chondrocytes, although both types of cells synthesize α2(I) mRNA. The chromatin of BrdUrd-shifted chondrocytes also resembled the chromatin of fibroblasts rather than of chondrocytes in its pattern of minor DNase I hypersensitive sites (Fig. 3).

Three hypersensitive sites detected by the 5′ probe appeared to be present in the chromatin of all three cell types (Fig. 3). One is the minor site at 1.3 kb from the 5′ end of the 5.7 kb genomic fragment discussed above. The second is a major site at 2.3 kb, which places it approximately 450 bp into intron 1. The third is the minor site at 3.0 kb, located near the middle of intron 1. This site was much weaker in chondrocytes. Both the 2.3 and 3.0 kb sites were also detected with the 3′ probe as bands of, respectively, 3.4 and 2.7 kb (Fig. 2).

Chromatin Structure of the α2(I) Gene in Chick Intestinal Epithelial Cells (CIE), Brain Cells and Erythrocytes

In order to get more evidence for which of the hypersensitive sites in the chromatin of fibroblasts and chondrocytes were correlated with the transcriptional activity of this gene, the DNase hypersensitive sites were mapped in the chromatin of intestinal cells, brain cells and erythrocytes, since these three cell types were expected to contain the α2(I) gene in a transcriptionally inactive state (Fig. 5). Although each of these three types of cells appeared to have a unique pattern of hypersensitive sites, all were alike in containing very low levels of the sites associated with transcription from either the fibroblast or chondrocyte transcription start sites. These hypersensitive sites were undetectable with the two exceptions of a faint very diffuse 1.5 kb band in brain chromatin, which has been reported to make low levels of type I collagen due to the presence of glial cells (14), and a faint band at 4.0 kb in the chromatin of intestinal epithelial cells.

The 1.3 kb site at approximately 300 bp upstream from the fibroblast transcription start site shows unique behavior in that it is missing from the chromatin of brain, intestinal and red blood cells (Fig 5), yet is present in the chromatin of both chondrocytes and fibroblasts, including the fibroblast-like BrdUrd-treated chondrocytes. It may, therefore, be associated with transcription of the α2(I) gene, regardless of whether this transcription utilizes the fibroblast or chondrocyte start site.

The sites at 2.3 and 3.0 kb appear to be constitutive since they were found in brain, intestinal and red blood cells (although the 2.3 kb band is barely visible in the erythrocyte sample in Fig. 5, it was clearer in other autoradiograms), as well as in chondrocytes, fibroblasts and BrdUrd-treated chondrocytes. The 2.3 kb band has also been previously suggested to be constitutive (14).

To summarize these DNase I hypersensitive studies (see Fig. 4), chondrocyte chromatin has two hypersensitive sites not present in the chromatin of fibroblasts or BrdUrd-shifted chondrocytes: a major site at 4.0 kb and a minor one at 1.8 kb from the 5′ end of the 5.7 kb α2(I) genomic fragment. Fibroblast chromatin has two major sites not present in chondrocyte chromatin: the newly discovered site at 600 bp and the site at 1.5 kb which had previously been mapped to 4.3 kb with the 3′ probe (14, 15). BrdUrd-shifted chondrocytes closely resemble fibroblasts in their chromatin structure, except that the site at 600 bp is barely detectable and the site at 1.5 kb is less pronounced. All three cell types share the site at 1.3 kb which is not present in the transcriptionally inactive gene. The major site at 2.3 kb and the minor site at 3.0 kb appear to be constitutive sites that are present in both the transcriptionally inactive and active forms of the gene.

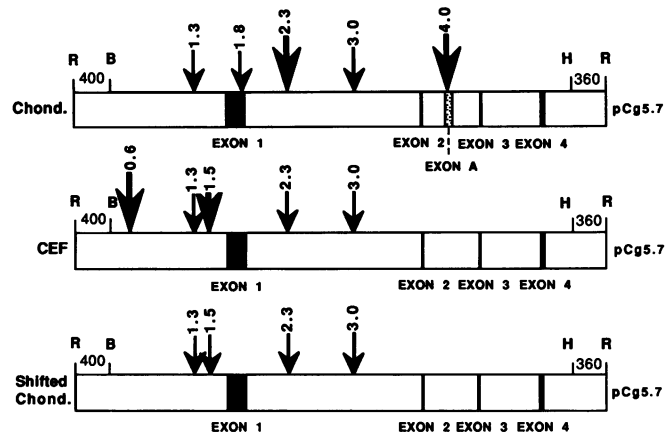

Fig. 4. Comparison of the DNase I hypersensitive sites in the collagen α2(I) gene in the chromatin of chondrocytes, fibroblasts and BrdUrd-treated chondrocytes. Major sites that give rise to markedly darker bands on the autoradiograms are indicated by the larger arrow heads. The distance of the sites from the 5′ end of the 5.7 kb genomic fragment is shown in kb above the arrows. Exons 1 and 2 are shown in the chondrocyte gene to facilitate comparision, but they are absent from chondrocyte transcripts, being replaced by exon A.

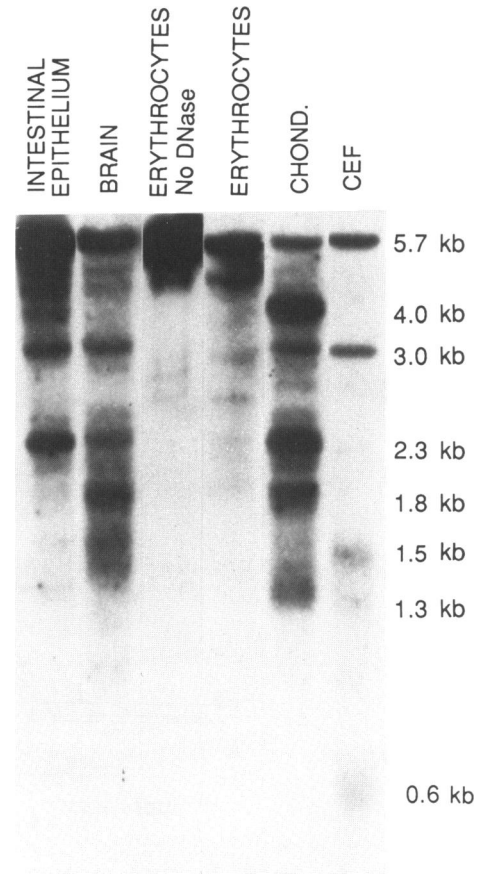

Fig. 5. DNase I hypersensitive sites in the chromatin of cells that contain a transcriptionally inactive collagen α2(I) gene. The sites were mapped with the 5′ probe by the usual method. The chondrocyte and fibroblast samples were included to facilitate comparison with the sites in the inactive chromatin samples. The erythrocyte lanes are from a 3-day exposure and the other lanes from a 10-day exposure of the autoradiograms. The nuclei from the erythrocytes and intestinal cells were digested with 50 μg/ml of DNase and the nuclei from brain with 40 μg/ml.

Sternal Chondrocytes Contain α2(I) RNA with an Altered 5′ End

To investigate whether the differences described above between the α2(I) chromatin structure in chondrocytes and fibroblasts is related to the presence in these chondrocytes of the recently reported α2(I) mRNA with an altered 5′ end (16, 17), the α2(I) mRNA in these chondrocytes was examined by RNase protection experiments. Although a primer extension experiment had previously indicated that this altered α2(I) RNA was present in the total RNA isolated from embryonic chick sterna (16), its presence in our cultured chondrocytes needed to be confirmed since its level might vary with the development stage of the sterna used as a source of the chondrocytes (7, 12) and with the culture conditions (16). The probe previously used in RNase protection assays gave only indirect evidence for the presence of the altered RNA, which was inferred from the absence of any protected probe fragment (16). The antisense probes that we used were chosen so that the presence of unique fragments protected by the chondrocyte-type but not the fibroblast-type of α2(I) mRNA would give direct evidence for the presence of this altered species.

When the RNA probe contained 367 nucleotides complementary to mature spliced fibroblast-type α2(I) mRNA from position +9 of exon 1 to +23 of exon 6 (Fig. 6A), all of these sequences were protected by hybridization to the RNA isolated from embryonic calvaria, a rich source of the fibroblast-type of α2(I) RNA (Calvaria lane). When, however, chondrocyte RNA was assayed (Chond. lane), only trace amounts of the 367 nt piece protected by fibroblasts were present, and a new fragment was obtained consistent with the predicted size of 163 nt for the fragment protected by chondrocyte-specific α2(I) RNA (17). This result suggests that these sternal chondrocytes also contain the recently reported chondrocyte-specific form of α2(I) RNA (16, 17) that is missing the first two fibroblastic exons (the absence of these exons would be expected to result in a protected fragment of 161 nt, but two nt, AG, are fortuitously the same whether exon A or exon 2 is spliced to exon 3). Since the caudal two-thirds of the sternum, which comprises permanent hyaline cartilage, differs by day 17 of development in its pattern of collagen synthesis from the cephalic one-third which comprises presumptive calcification cartilage (31), chondrocytes isolated from these regions of 14-day sterna were analyzed separately and were found in both cases to contain the cartilage-specific type of α2(I) RNA (Fig. 6A). In additional RNase protection experiments (data not shown) the altered α2(I) RNA in chondrocytes was found to be in the poly(A)-containing RNA fraction, a finding consistent with our previous evidence that the α2(I) RNA in chondrocytes is polyadenylated (12). The presence of small amounts of the fibroblastic α2(I) mRNA in these chondrocyte RNA samples, as shown by the 367 nt protected fragment, is probably due to fibroblast contamination of these cultures, rather than representing an authentic chondrocyte product. The relative concentration of this RNA is much lower in some chondrocyte samples than in others (Fig. 6A, Cephalic and Caudal Chond. lanes versus Chond. lane), presumably due to more complete removal, during preparation of the chondrocyte cultures, of the fibroblast-containing perichondrium that surrounds the sternum.

Since the shorter probe fragment protected by chondrocyte RNA compared to fibroblast RNA could be due to the absence from chondrocyte α2(I) RNA of sequences present at either the 5′ end or 3′ end of this region of the fibroblastic α2(I) mRNA,

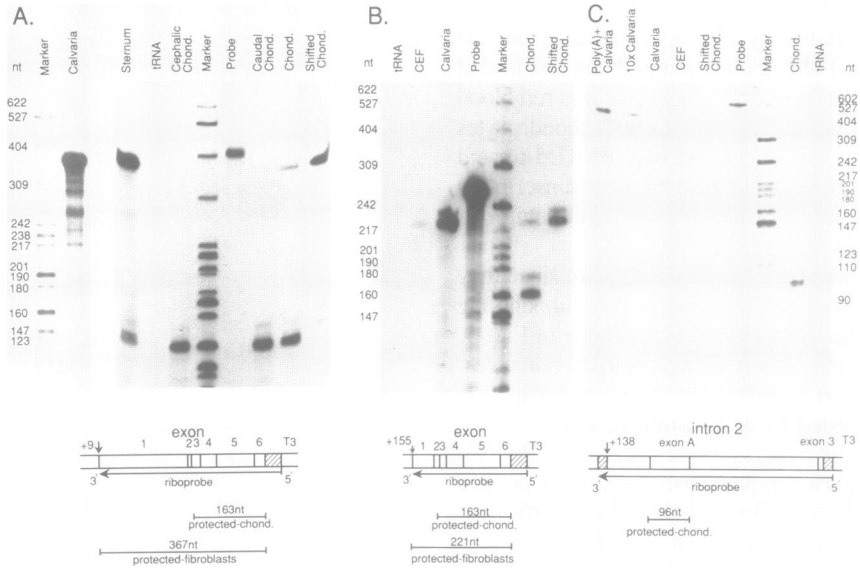

Fig. 6. Comparison of the 5′ regions of α2(I) RNA in fibroblasts, sternal chondrocytes and BrdUrd-treated chondrocytes by the RNase protection assay. RNA from the sources shown above the lanes was hybridized with the ^{32}P-labeled antisense α2(I) riboprobes that are shown, along with the DNA templates, below each panel. Transcribed pBS vector sequences are indicated by hatched boxes. The Marker lanes contained 10,000 cpm of ^{32}P-labeled *Msp*I-digested pBR322, and the Probe lanes contained 1000 cpm (10,000 cpm in panel B) of the un-hybridized ^{32}P-labeled antisense RNA. The following amounts of RNA in μg were hybridized with 50,000 cpm of riboprobe: calvaria, 0.2 except 0.83 in panel A and 2.0 in panel C, 10×sample); sternum, 2; tRNA, 10; chondrocyte, including cephalic and caudal, 20; BrdUrd-shifted chondrocyte, 2; poly (A)$^+$ calvaria, 2; CEF, 0.02 in panel B and 0.2 in panel C. (A) The riboprobe of 405 nt contained 38 nt of pBS sequence and 367 nt of α2(I) mRNA sequence, starting at nt +23 in exon 6 and extending to nt +9 in exon 1. The two left lanes are from a different autoradiogram than the other lanes; in the marker sample run with the right-hand lanes, the 404 bp fragment is running just ahead of the undigested probe, and the 160 bp fragment is running with the RNA fragment protected by chondrocyte RNA. (B) The same riboprobe was present as in A, but truncated so as to extend only through nt +155 of exon 1; in addition to the 38 nt of pBS sequence it contained 221 nt of α2(I) mRNA sequence. (C) The riboprobe of 520 nt contained 39 nt of pBS sequence and 481 nt of α2(I) genomic sequence (11 nt of exon 3 and 470 nt of intron 2).

another probe was constructed that had the same 5′ end, in exon 6, as the previously used probe, but which, at its 3′ end, contained only 49 nt complementary to exon 1 mRNA sequences (Fig. 6B), rather than the 195 nt complementary to exon 1 in the probe used in Fig. 6A. If the chondrocyte form of α2(I) RNA is missing the fibroblastic exons 1 and 2 as reported (16, 17), deletion of these exon 1 sequences should not change the length of the probe sequence that is protected by chondrocyte RNA. In accord with this prediction, this deletion had no effect on the size of the probe protected by chondrocyte RNA, which was still 163 nt (Fig. 6B, Chond. lane). As expected, the size of the fragment protected by fibroblast-type α2(I) mRNA was shorter, being 221 nt (Calvaria and CEF lanes). The fibroblast α2(I) sequences that are missing from the chondrocyte α2(I) RNA must therefore be from the 5′ end of the fibroblast mRNA, a finding consistent with the conclusions from the previous reports (16, 17).

To detect the presence of exon A, which is a 96 bp region including bp +234 through bp + 329 of intron 2 (17), an antisense RNA probe that began at nucleotide 11 of exon 3 and extended through position 138 from the 5′ end of intron 2 was synthesized from a genomic fragment as template (Fig. 6C). Mature spliced fibroblast RNA lacks introns and would not be expected under our assay conditions to protect any fragment of this probe, since it contains only 13 complementary nucleotides, 11 nt of exon 3 plus the last two nucleotides of exon 2 (AG) which fortuitously match the last two nucleotides of intron 2. If chondrocyte RNA, however, contains 96 nt of intron 2 ('exon A') one would expect it to protect a 96 nt probe fragment. In agreement with this prediction chondrocyte RNA did protect a fragment of this probe of approximately this size (Fig. 6C, Chond. lane). Calvaria and CEF RNA, which contain the fibroblast-type of α2(I) RNA, were unable to protect this probe (Fig. 6C, Calvaria and CEF lanes) when present at levels that give strong protected bands from α2(I) exon-containing probes. When 2 μg, 10 times the usual amount of calvaria RNA was assayed, or 2 μg of calvaria poly (A)$^+$ RNA, a protected species appeared of approximately 481 nt, the predicted size if unspliced fibroblastic α2(I) RNA was present (Fig. 6C, 10x Calvaria and poly (A)$^+$ Calvaria lanes). Even at these levels, however, no protected product of 96 nt corresponding to the presence of exon A of chondrocyte-type α2(I) RNA was seen. The results of these assays with all three RNA probes show therefore that the previously reported altered form of α2(I) mRNA (17) is present in these sternal chondrocytes.

Treatment of Chondrocytes with BrdUrd Causes the Disappearance of the Chondrocyte-Specific α2(I) RNA and the Appearance of the Fibroblast-Type α2(I) mRNA

The BrdUrd-induced shift in collagen synthesis from type II to type I was shown above to be accompanied by the loss of the major DNase hypersensitive site in the region of exon A and the acquisition of the hypersensitive site characteristic of fibroblasts, at 1.5 kb from the 5′ end of the 5.7 kb genomic fragment. If the conclusion is correct that the presence of each of these hypersensitive sites is associated with the use of the neighboring transcription start site, one would predict that the BrdUrd-shifted chondrocytes would no longer use the chondrocyte start site but would initiate transcription at the fibroblast start site. This prediction is fulfilled, as shown by the RNase protection assays. With the antisense RNA probe used in Fig. 6A, the RNA from BrdUrd-shifted cells protected the 367 nt piece characteristic of fibroblast type α2(I) RNA (Fig. 6A, Shifted Chond. lane) rather than the 163 nt piece that is protected by chondrocyte RNA. The antisense probe used in Fig. 6B also showed that the α2(I) RNA in BrdUrd-shifted chondrocytes had the 5′ end characteristic of the α2(I) mRNA in fibroblasts (Shifted Chond. lane). The probe used in Fig. 6C that is specific for the exon A of chondrocyte-type α2(I) RNA was not protected at all by the RNA from the BrdUrd-shifted chondrocytes (Fig. 6C, Shifted Chond. lane), in agreement with the behavior of the RNA from Calvaria or CEF (Fig. 6C). BrdUrd-treated chondrocytes therefore simultaneously lose the transcription start site in intron 2 of the α2(I) gene and the DNase hypersensitive site in this region, and they simultaneously acquire the fibroblast-type transcription start site and the DNase hypersensitive site approximately 100 bp upstream of this site.

DISCUSSION

This paper reports major differences between the chromatin structure of the α2(I) gene in chondrocytes and fibroblasts, both of which actively transcribe this gene. We also show here that these differences are associated with the different transcription start sites used by these cells. When chondrocytes, which initiate transcription of the α2(I) gene in intron 2 (16, 17 and this paper), are induced to switch to the usage of the fibroblast start site by BrdUrd treatment, they lose the unique hypersensitive site in intron 2 and acquire the hypersensitive site at −100 bp that is characteristic of fibroblasts.

The BrdUrd-induced loss of both the chondrocyte-specific transcription start site and the nearby hypersensitive site suggests that the presence of this hypersensitive site is associated with the activity of the chondrocyte-specific transcription start site. Hypersensitive sites associated with the activity of transcription start sites have been shown in a number of cases to be nucleosome-free regions of DNA that are bound by transcription factors (27). An analysis of the transcription factors bound by the chondrocyte-specific hypersensitive site, currently underway in our laboratory, is likely to help in understanding the mechanism of this switch in transcription start site. Motifs similar to a TATA box and a CCAAT box are present 5′ to the chondrocyte start site in intron 2 (17).

When the hypersensitive site at −200 bp in fibroblast chromatin (14, 15) was resolved into two sites, the major site at approximately −100 bp was found to be associated with transcriptional activity of the gene from the fibroblast start site, whereas the minor site at approximately −300 bp was associated with the transcriptional activity of the gene, regardless of whether the fibroblast or chondrocyte start site was used. The more sensitive site at −100 bp is likely to correspond to the DNase I hypersensitive site found to be present near the transcription start site of the mouse α2(I) gene in fibroblast chromatin but absent from the chromatin of a myeloma cell line, which does not transcribe this gene (32). This site was found to extend from approximately −240 bp to +110 bp by its accessibility to restriction enzymes (32). Since these regions of the chick and mouse α2(I) promoters contain blocks of conserved sequences at similar locations (33), it is likely that the chick hypersensitive site spans a similar region. The lower DNase sensitivity of the 1.3 kb site in the chick, mapping to −300 bp, is in agreement with the observation that mouse α2(I) fibroblast chromatin is less sensitive to nucleases in this region than in the −240 to +110 bp region (32).

Within the major hypersensitive site associated with the transcriptional activity of the α2(I) gene in fibroblasts is a site that has been shown to be sensitive to S1 nuclease cleavage *in vitro*, when present in a supercoiled form in plasmids. In the chick promoter, this S1 nuclease site lies at around −190 bp in a stretch of 42 pyrimidines interrupted by a single G (24, 34), and in the mouse promoter a similar S1 nuclease site lies in a partially conserved pyrimidine-rich sequence (28). The significance of this site for the regulation of transcription is, however, unknown.

No direct evidence is available concerning the identity of the transcription factors that are bound to the DNase hypersensitive sites in the chick α2(I) gene in fibroblast chromatin. From studies of the mouse α2(I) gene, reviewed in refs. 35 and 36, three sites that bind factors present in fibroblast nuclei and that are required for maximal promoter activity have been identified in the 5′-flanking region, and an enhancer region has been localized to within intron 1. The most upstream of these binding sites at −307 bp, which binds nuclear factor 1 (NF-1), has not been located in the chick promoter. The factor that binds to the next site in the mouse promoter at −250 bp has not yet been identified, but an homologous factor is likely to bind to the chick promoter, which contains an identical 8 bp site, beginning at −282 bp. It is not clear whether this site lies within the region most sensitive to nucleases, mapped to −100 bp or within the less nuclease-sensitive region, mapped to −300 bp. The third binding site in the mouse promoter at −84 bp, contains an inverted CCAAT box in an 8 bp region that is conserved in the chick promoter in approximately the same location. It is probable, therefore, that a factor homologous to that identified in mouse fibroblast nuclei also binds to the chick promoter in this region, which would be within the major fibroblast DNase I hypersensitive site. Additional blocks of conserved sequences in the chick and mouse promoters suggest that further unidentified regulatory factors may bind to these promoters (32). No homology to the region containing the mouse enhancer could be found in intron 1 of the chick gene (37).

Although studies of the mouse gene to date have not implicated any regulatory region as far upstream from the transcription start site as −1000 bp (33), the presence of a strong DNase hypersensitive site in this region in the chromatin of chick embryo fibroblasts, as reported here, suggests this region may play a regulatory role. The absence of this site from the other cell types examined, including BrdUrd-shifted chondrocytes that transcribe this gene from the fibroblast start site, suggests that this site might contribute to the higher rate of transcription of this gene in fibroblasts.

ACKNOWLEDGEMENTS

We thank Martin Dieck, Burke Mamlin, and Geoffrey Parsons for technical assistance. We thank John Wareham for preparing the figures and Sylvia Fazzino for typing the manuscript. We are indebted to Helga Boedtker for giving us the chick genomic α2(I) clone, pCg5.7, and to Louis Gerstenfeld for the cDNA clone, pMF21. We thank Mark Mooseker for the nuclei from chicken intestinal epithelial cells. This work was supported by the United States Department of Agriculture grant 87-CRCR-1-2483.

REFERENCES

1. Mayne, R., and Burgeson, R.E. (eds.) (1987) Structure and function of collagen types. Academic Press, London, U.K.
2. Miller, E. J. and Gay, S. (1987) *Methods in Enzymol.* **144**, 3–41.
3. Abbot, J. and Holtzer, H. (1968). *Proc. Natl. Acad. Sci. USA* **59**, 1144–1151.
4. Mayne, R., Vail, M.S. and Miller, E.J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4511–4515.
5. Pawlowski, P.J., Brierley, G.T. and Lukens, L.N. (1981) *J. Biol. Chem.* **256**, 7695–7698.
6. von der Mark, K., (1980) *Curr. Top. Dev. Biol.* **14**, 199–225.
7. Finer, M.H., Gerstenfeld, L.C., Young, D., Doty, P. and Boedtker, H. (1985) *Mol. Cell. Biol.* **5**, 1415–1424.
8. Pacifici, M. and Holtzer, H. (1977) *Am. J. Anat.* **150**, 207–212.
9. Adams, S.L., Boettiger, D, Focht, R. J., Holtzer, H. and Pacifici, M. (1982). *Cell* **30**, 373–384.
10. Allebach, E., Boettiger, D., Pacifici, M. and Adams, S. L. (1985) *Mol. Cell. Biol.* **5**, 1002–1008.
11. Pacifici, M., Boettiger, D., Roby, K. and Holtzer, H. (1977) *Cell* **11**, 891–899.
12. Saxe, S.A., Lukens, L.N. and Pawlowski, P. J. (1985) *J. Biol. Chem.* **260**, 3812–3819.
13. Askew, G. R., Wang, S. and Lukens, L. N. *J. Biol. Chem.*, in press.
14. McKeon, C., Pastan, .I. and deCrombrugghe, B. (1984) *Nucleic Acids Res.* **12**:. 3491–3502.
15. Merlino, G.T., McKeon, C., deCrombrugghe, B. and Pastan, I. (1983) *J. Biol. Chem.* **258**, 10041–10048.
16. Bennett, V.D., Weiss, I. M. and Adams, S.L. (1989) *J. Biol. Chem.* **264**, 8402–8409.
17. Bennett, V.D. and Adams, S.L. (1990). *J. Biol. Chem.* **265**, 2223–2230.
18. Schiltz, J.R., Mayne, R. and Holtzer, H. (1973) *Differentation* **1**, 97–108.
19. Solomon, J.J. (1975) Preparation of avian cell cultures, p. 7–11. In Evans, V. J., Perry V. P. and Vincent, M. M. (eds.). *Tissue Culture Assoc. Manual*, Vol. 1. Tissue Culture Assoc., Rockville, MD.
20. Konieczny, S.F. and Emerson, C.P. (1985) *Mol. Cell. Biol.* **5**, 2423–2432.
21. Babiss, L.E., Bennett, A., Friedman, J. M. and Darnell, J. E. (1986) *Proc. Natl. Acad. Sci. USA* **83** 6504–6508.
22. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbor, N.Y.
23. Feinberg, A.P. and Vogelstein, R. (1983) *Anal. Biochem.* **132**, 6–13.
24. Finer, M.H., Fodor, E.J. B., Boedtker, H. and Doty, P (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1659–1663.
25. Tate, V.E., Finer, M.H., Boedtker, H. and Doty, P. (1983) *Nucleic Acids Res.* **11**. 91–104.
26. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
27. Gross, D.S., and Garrard, W.T. (1988) *Annu. Rev. Biochem.* **57**, 159–197.
28. McKeon, C., Schmidt, A. and deCrombrugghe, B. (1984) *J. Biol. Chem.* **259**, 6636–6640.
29. Ayres, K., Neuman, W., Rowekamp, W. G. and Chung, S (1987) *Mol. Cell. Biol.* **7**, 1823–1829.
30. Pavlovic, J., Banz, E. and Parish, R. (1986) *Nucleic Acids Res.* **14**, 8703–8722.
31. Reginato, A.M., Lash, J.W. and Jimenez, S.A. (1986) *J. Biol Chem.* **261**, 2897–2904.
32. Liau, G., Szapary, D., Setoyama, C. and deCrombrugghe., B. (1986) *J. Biol. Chem.* **261**, 11362–11368.
33. Schmidt, A., Yamada, Y. and deCrombrugghe, B. (1984) *J. Biol. Chem.* **259**, 7411–7415.
34. Boedtker, H., Finer, M. and Aho. S. (1985) *Ann. N.Y. Acad. Sci.* **460**, 85–116.
35. deCrombrugghe, B., Karsenty, G., Maity, S., Vuorio, T., Rossi, P. Ruteshouser, E.C., McKinney, S.H. and Lozano, G. (1990) *Ann. N.Y. Acad. Sci.* **580**, 88–96.
36. Vuorio, E. and deCrombrugghe, B. (1990) *Annu. Rev. Biochem.* **59**: 837–372.
37. Rossi, P. and deCrombrugghe, B. (1987) *Proc. Natl. Acad. Sci. USA.* **84**, 5590–5594.