DNase I sensitivity of the α2(I) collagen gene: correlation with its expression but not with its methylation pattern

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ABSTRACT

The chromatin structure of the chick α2(I) collagen gene was probed with DNase I. Because our previous work strongly suggested that the 5' end of this gene is not methylated whereas the rest of the gene is methylated whether or not the gene is expressed, we compared the relative DNase I sensitivity of the methylated and unmethylated segments. Both regions demonstrate similar relative DNase I sensitivities within a given tissue. In chromatin of chick embryo fibroblasts, we find a DNase I hypersensitive site which maps between 100 and 300 bp preceding the start of transcription. This site is not found in brain chromatin but is present in chick embryo fibroblasts transformed by Rous Sarcoma virus although the rate of transcription of the α2(I) collagen gene is greatly reduced in these cells. Hence, the mechanism responsible for the large decrease in α2(I) collagen gene expression in RSV transformed cells is different from the mechanism that is responsible for the presence of a DNase I hypersensitive site in the promoter. Furthermore, changes in the DNase I sensitivity of the chromatin of the α2(I) collagen promoter occur without changes in the methylation pattern of the gene.

INTRODUCTION

Transcriptionally active chromatin exhibits conformational modifications that are revealed by the increased susceptibility of its DNA to nuclease digestion (1,2). In addition to a general nuclease sensitivity throughout active genes, there are discrete DNA regions in actively transcribed chromatin which are hypersensitive to DNase I and are often located near the 5'end of genes (3,4) (for a review see 5). Also, the DNA of active genes is often less methylated than that of inactive genes (for a review see 6). This decrease or absence of methylation has therefore been correlated with enhanced DNase sensitivity. In the developing chick embryo, the appearance of a hypersensitive site in the chick β-globin gene occurs around the time that the gene becomes undermethylated and its expression is activated (7).

Our previous experiments have indicated that among seven sites that were examined for cytosine methylation in a segment of at least 1.2 kb preceding the start of transcription of the α2(I) collagen gene and 1 kb

following the start site, all seven sites were unmethlyated (8). This pattern was observed in different tissues whether or not the gene was expressed. On the other hand, the sites in the body of the gene that were examined were found to be methylated although two of these sites demonstrated some quantitative differences in the degree of methylation between expressing and nonexpressing tissues (8). These results suggested that a segment around the 5'end of the gene is unmethylated whereas the body of the gene is methylated in all tissues whether or not the gene is expressed. A class of similar unmethylated domains has recently been identified in chicken genomic DNA (9).

In this paper, we first asked whether the chromatin in the unmethylated segment and in the methylated segment of the α2(I) collagen gene have a similar or different DNase I sensitivity. We, further, examined the chromatin of this gene for the presence of a DNase I hypersensitive site in the promoter segment in both fibroblasts, which express the gene and brain, a tissue which does not. After having mapped such site in the promoter, we asked whether this hypersensitive site is present after transformation of chick embryo fibroblasts by Rous Sarcoma virus, a condition which results in a large and selective decrease in the expression of the α2(I) collagen gene (14-18).

MATERIALS and METHODS

Isolation of Nuclei

Nuclei were isolated from 3 sources: primary cultures of ten-day old chick embryo fibroblasts (CEF); CEF infected with Rous sarcoma virus (Schmidt-Ruppin) which exhibited a transformed phenotype (RSV-CEF); and brain tissue dissected from 13-day old chick embryos. Cells or tissues were washed, homogenized and nuclei isolated according to Wu et al., (3) except that nuclei were pelleted through a 1.5 M sucrose cushion at 100,000xg for 30 min. Sperm heads were isolated from rooster sperm (a gift from Dr. T. Sexton) by vortexing in the same nuclei isolation buffer with 0.25% NP40 to remove tails and centrifuged as above. Nuclei could be stored at -70°C in 50% glycerol for several months without detectable DNA degradation.

Nuclease Digestions

All nuclei samples were washed in DNase I digestion buffer (3) and diluted to a DNA concentration of approximately 200 µg/ml. Since genomic DNA is extremely viscous, the DNA concentration was determined by lysing an aliquot of the nuclei sample in 1% SDS and mixing with an equal volume of

1 ug/ml ethidium bromide. The intensity of the "dot" fluorescence under UV light was compared to that of known DNA standards. Samples were digested with DNase I (Boehringer Mannheim) as indicated for 5 min. at 23°C. DNA was then isolated according to Wu et al., (3).

Transfer and Hybridization

DNA was cleaved with EcoR I and fractionated by electrophoresis on an agarose gel in Tris-Acetate Buffer (40mM TRIS-HCL [pH 7.5], 5mM Sodium Acetate and 1mM EDTA) at 25 volts overnight. The DNA on the gels was transferred to nitrocellulose filters (Schleicher & Schuell) according to Southern (10). For the dot blots, 5ug of DNA in 2M ammonium acetate was loaded on to each dot of the nitrocellulose filter (11). Probes specific for the α2(I) collagen gene were isolated from the genomic clones as follows: a 5.7 kb EcoR I fragment containing the 5' end of the gene from λcol-323 (12); a 3.4 kb EcoR I fragment containing the central portion of the gene from λcol-271 (12); and a 1.7 kb Hind III fragment containing the 3' portion of the gene from λcol-204 (13). The probes used to map the DNase I hypersensitive site are segments of the 5.7 kb EcoR I fragment from λcol-323 as indicated in Figure 3. The probes were labelled with P^{32} deoxynucleotides by using a nick translation kit (Amersham). Hybridization and washing procedures are described elsewhere (8).

RESULTS

Comparison of the DNase I Sensitivity in Methylated and Unmethylated Regions of the α2(I) Collagen Gene

We have compared the relative DNase I sensitivity of chromatin containing the different segments of the α2(I) collagen gene using probes for three regions of the gene. A region consisting of at least 1.2 kb upstream and 1 kb downstream from the start site is believed to be unmethylated in all tissues studied (8). The central and 3' regions of the gene are highly methylated in all tissues examined whether or not the gene is expressed (8). The question we asked was whether the methylated and unmethylated segments have a different DNase I sensitivity. We examined four different tissues with varied levels of type I collagen synthesis: cultured chick embryo fibroblasts (CEF) that synthesize 1-3 percent of their total protein as type I collagen; CEF transformed by Rous sarcoma virus (RSV) which show a 5-10 fold reduction in collagen synthesis (14, 15) and collagen RNA levels (16, 17, 18); 13-day old chick embryo brain in which only the glial cells, a minor proportion of all the cells, make small amounts of type I collagen (Vonder Mark, K., personal commu-

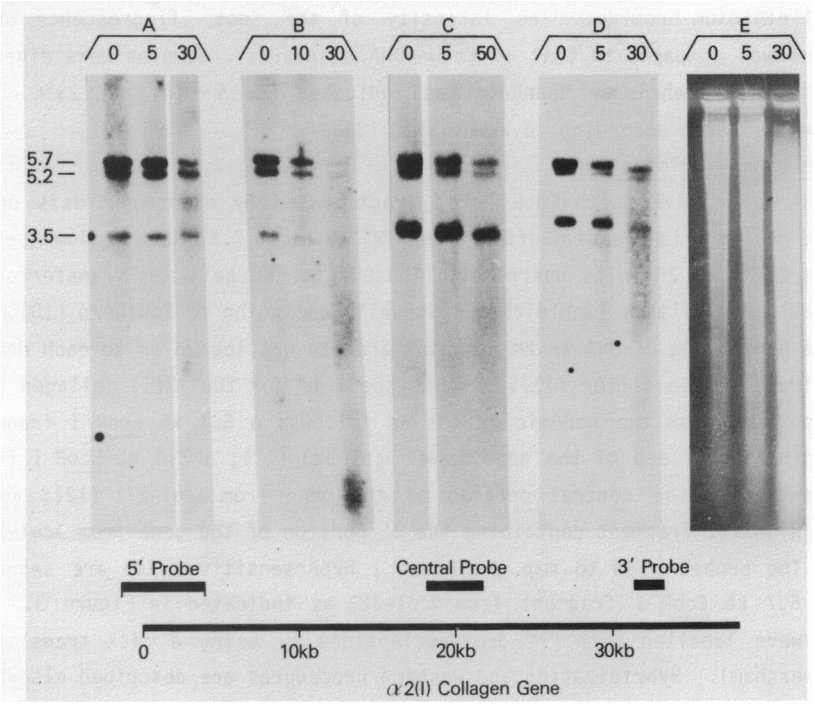

Fig. 1: DNase I sensitivity of three different segments of the α2(I) collagen gene. Nuclei from A) CEF B) RSV-CEF C) Brain and D) Sperm were digested at 23°C for 5 min. with DNase I at concentrations of 5-50 U/ml as indicated above and DNA was isolated as described. The DNA was cleaved with EcoR I and electrophoresed on a 0.8% agarose gel. A typical ethidium bromide stained digestion of sperm DNA is shown in E. The DNA on these gels was transferred according to Southern (10) and hybridized with a mixture of nick translated probes: a 5.7 kb EcoR I fragment (from λcol-323) which hybridizes to a 5.7 kb segment at the 5' end of the gene; a 3.4 kb EcoR I fragment (from λcol-271) which hybridizes to a 5.2 kb segment near the middle of the gene; and a 1.7 kb Hind III fragment (from λcol-204) which hybridizes to a 3.5 kb segment near the 3'end of the gene (12,19) as diagrammed below. The appearance of a band at 4.3 kb can be seen in A at a DNase concentration of 30 U/ml.

nication); and sperm in which no collagen synthesis can be detected. Three DNA probes were used. The first detects a 5.7 kb EcoR I fragment in the 5' region of the α2(I) collagen gene. It contains 1.7 kb preceding and 4.0 kb following the unique start site for transcription (19). The second probe hybridizes to a 5.2 kb EcoR I fragment located in the middle of the gene. The third probe hybridizes to a 3.5 kb fragment located toward the 3' end of the gene (see Fig. 1, lower part).

Two different assays were used. In the first (see Fig. 1), the DNase

Table I

α2 Collagen Gene is Equally Sensitive to DNase I in Three Different Areas of the Gene

Bands (kb)	Tissue											
	CEF			RSV-CEF			Brain			Sperm		
	DNase I U/ml			DNase I U/ml			DNase I U/ml			DNase I U/ml		
	0	5	30	0	10	30	0	5	50	0	5	30
5.7	17.7	20.0	5.0	14.7	4.6	1.0	5.2	2.0	1.2	10.8	4.4	2.8
5.2	10.2	12.8	5.0	8.8	3.2	0.7	2.3	1.4	0.4	3.7	1.9	1.6
3.5	3.6	4.2	2.5	2.3	0.7	0.3	8.8	7.2	2.3	5.9	4.1	1.7
5.7/5.2	1.7	1.6	1.0	1.7	1.4	1.4	2.3	1.4	3.0	2.9	2.3	1.8
5.2/3.5	2.8	3.0	2.0	3.8	4.5	2.3	0.26	0.19	0.17	0.63	0.46	0.94

Different exposures of the autoradiographs shown in Figure 1 were examined by microdensitometry. The numbers correspond to the peak heights of the bands. The 5.7 kb band corresponds to an EcoRI fragment at the 5'end of the gene, the 5.2 kb band to a fragment in the middle of the gene and the 3.5 kb band to a fragment at the 3'end of the gene.

digested chromatin complex was cleaved with EcoR I after deproteinization and the resulting fragments fractionated by electrophoresis, blotted and hybridized with the three probes mixed together. This experiment allows one to compare the relative disappearance rate of three segments of the gene at a given DNase I concentration within a single DNA sample. The disappearance of a restriction fragment can, of course, result from a single DNase I cleavage within this fragment. For each of the four cell types, CEF (A), RSV-CEF (B), brain (C) and sperm (D), three lanes are shown; the first was not digested with DNase I and the next two lanes were digested with increasing concentrations of DNase I. As the DNase I concentration is increased, the three bands representing the three regions of the collagen gene are digested. By scanning the autoradiograph with a Joyce-Loebl densitometer, the relative intensities of the bands can be compared to each other within a single lane (20). The peak heights for each of the lanes shown in Figure 1 have been tabulated in Table I. Note that the absolute values listed cannot, however, be compared between tissues, because the data for each tissue, as presented in both Figure 1 and Table I, correspond to different experiments and the specific activity of the three probes varies between experiments. Furthermore the amount of DNA loaded in each lane varies somewhat between experiments and finally different exposures of the autoradiographs were scanned in order to be in the linear range of the film. If the three regions of the gene are equally sensitive to DNase I the ratio between the 5.7 kb band and the 5.2 kb band and the 3.5 kb band should remain approximately constant for a given tissue. The results presented in Table 1 show that the ratios for a given tissue do not differ by more than 2-fold. In CEF, there is a DNase I hypersensitive site (discussed in detail below) within the 5.7 kb that generates a band at 4.3 kb as seen in Fig. 1A. This band accounts for a small percentage of the total digestion of the 5.7 kb by DNase I. It is possible that the middle and 3' end segments of the gene also contain discrete sites with higher sensitivities to DNase I.

In a second assay we used dot blot hybridization to measure the sensitivity of the three different segments of this gene to DNase I. CEF nuclei were treated with increasing amounts of DNase and three identical dot blots were made. Each dot blot was hybridized with one of the three collagen gene probes. As can be seen in Figure 2, the digestion of each segment of the gene occurs at the same rate. By this assay we measure the digestion rate of these fragments to DNA lengths that are unable to bind to the nitrocellulose filter probably under 200 bp. The two assays measure very different

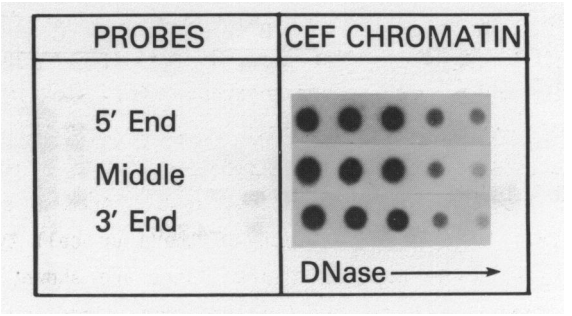

Fig. 2: DNase I sensitivity of three segments of the α2(I) collagen gene. CEF nuclei were digested with 0,5,10,20 and 30 U/ml DNase I at 23°C for 5 min. After DNA isolation and digestion with EcoR I, three identical dot blots were made and hybridized with an equivalent number of counts of each of the three probes depicted in figure 1.

stages in DNase I sensitivity. Whereas the first assay examines the first or first few double stranded DNA breaks, the second measures a much more extensive digestion process. Both sets of data indicate that the less methylated region of the gene has a similar DNase sensitivity in chromatin as the methylated regions.

Mapping the DNase I Hypersensitive Site

As already noted above, there is a short region at the 5' end of the collagen gene in CEF chromatin which is hypersensitive to DNase I. Such a site is detected by digestion of CEF chromatin with small amounts of DNase I. After DNase I digestion the DNA is extracted, cleaved by EcoR I and blot hybridized. A small 360 bp Hind III - EcoR I fragment that maps at the 3'end of the 5.7 kb EcoR I fragment was used as probe to map the hypersensitive site by indirect "end-labeling" (4,21) (Fig 3, lower part). After DNase I digestion this probe hybridizes to the 5.7 kb EcoR I fragment and to an additional band of around 4.3 kb (Fig. 3A). No band at 4.3 kb was observed when the same probe was hybridized to naked DNA which was digested with DNase I (fig. 4B). By measuring a distance of 4.3 kb from the 3' end of the 5.7 kb fragment, the hypersensitive site is localized around 100-300 bp upstream from the start of transcription. A probe that maps at the 5'end of the 5.7 kb EcoR I fragment was also used, but no discrete additional band was observed after DNase I digestion, although there was a smear around 1.3-1.6 kb. The hypersensitive site could be relatively broad therefore not creating a discrete fragment when mapped from the 5' end. Two additional probes which flank the start site of transcription were hybridized to the same southern blot used in Fig. 3A to confirm the localization of this hypersensitive site in the

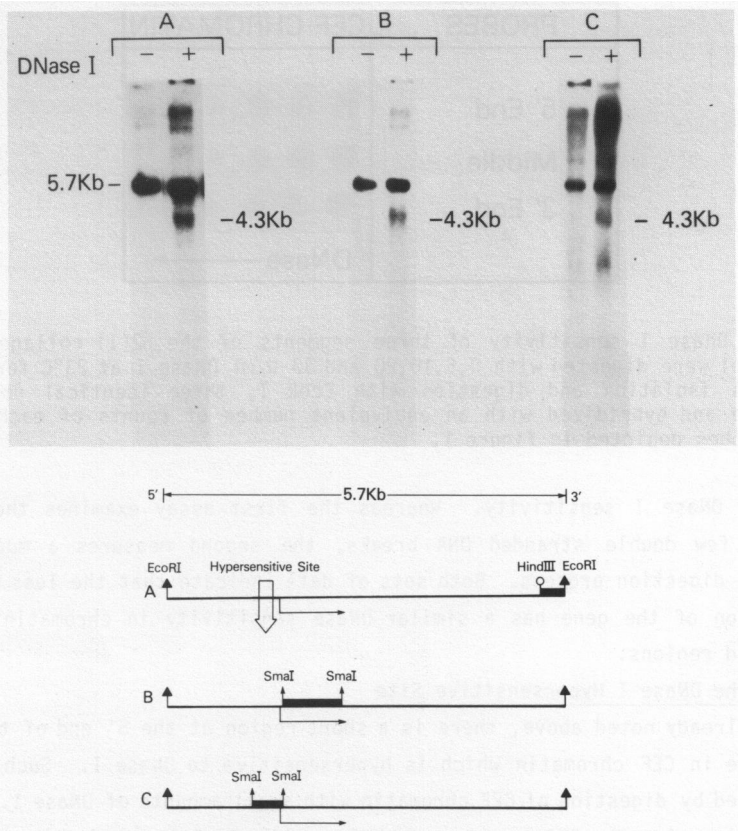

Fig. 3: A DNase I hypersensitive site maps near the 5'end of the gene. CEF nuclei were digested with 30 U/ml DNase I. DNA was isolated, digested with EcoR I and electrophoresed on a 1.2% agarose gel. The gel was blotted and the same filter was hybridized sequentially with: (A) a 360 bp Hind III-EcoR I fragment from the 3'end of the 5.7 kb fragment (B) an 880 bp Sma I fragment and (C) a 420 bp Sma I fragment as indicated in the map at the bottom of the figure. The horizontal arrow marks the start site of transcription. The lighter bands above 5.7 kb in A and B indicate a small fraction of incomplete EcoR I digestion. The smear above 5.7 kb in C is probably due to the G/C richness of the probe.

promoter. When a 880 bp Sma I fragment that contains most of the first exon and part of the first intron was used, it similarly hybridizes to the same 4.3 kb band when CEF chromatin is digested by DNase I (Fig. 3B). When another probe that maps between +8 and -410 (+1 corresponds to the start of transcription), was hybridized to the same blot, it also revealed the 4.3 kb band although a higher background was observed (Fig. 3C). A second minor species of 3.5 kb was detected with this probe (Fig. 3C). This species has

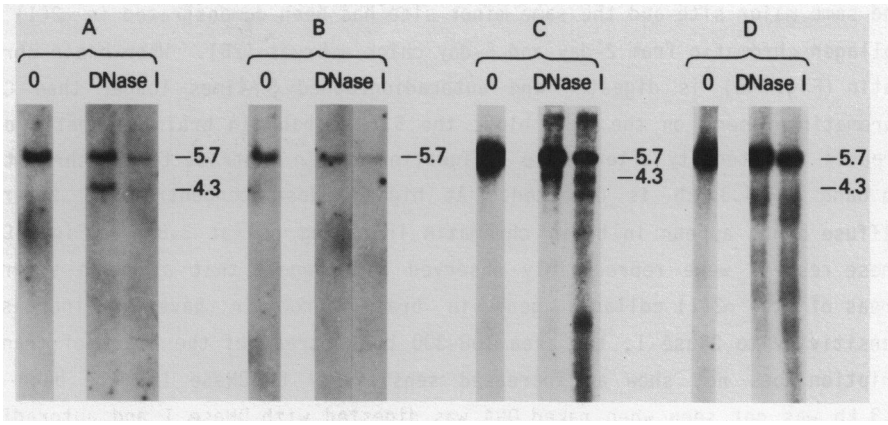

Fig. 4: Probing for a DNase I hypersensitive site in different tissues. Nuclei were isolated and digested with increasing concentrations of DNase I from A) CEF (0, 20, 30 U/ml) C) RSV-CEF (0, 20, 30 U/ml) and D) brain, (0, 30, 50, U/ml). B) Naked chicken DNA was digested with 0,1,2 U/ml DNase I. DNA was cleaved by EcoR I and electrophoresed on a 1.2% agarose gel. The filters were hybridized with the 360 bp Hind III - EcoR I probe from the 3' end of the 5.7 kb fragment.

not yet been mapped. This higher background could be caused by the high G/C content of this probe which probably hybridizes to other G/C rich DNA segments present in the chick genome. This experiment indicates that the hypersensitive site detected by the presence of the 4.3 kb species must lie upstream of +8. We have not yet determined the exact boundaries of the DNase I sensitive site, but the location of the hypersensitive site is similar to the location of analogous sites in the chick β-globin gene (22), SV40 (23, 24), and polyoma virus (25) where the hypersensitive region is estimated to be approximately 200 bp in length.

The Hypersensitive Site is Tissue Specific

Because transcription of the type I collagen genes is greatly reduced in Rous Sarcoma virus transformed CEF, we first asked whether the same hypersensitive site is present in these cells. We also examined chromatin from brain, a tissue which does not synthesize type I collagen. The DNase hypersensitive site is best visualized in CEF chromatin by the appearance of a 4.3 kb fragment using the 360 bp Hind III-EcoR I probe described above (Fig.3A and 4A). When chromatin from Rous Sarcoma Virus transformed CEF was digested with DNase I and hybridized with the probe the same 4.3 kb species is observed (Fig. 4C). After increased digestion several other bands appear including a prominent band at 3.8 kb. This less sensitive site maps in the the first intron of the collagen gene. A similar pattern of DNase I sensitivity with both

the same major site and the same minor site has been demonstrated in α2(I) collagen chromatin from 2-day and 5-day chick embryos (26). When brain chromatin (Fig. 4D) is digested and autoradiographed 5 times longer than CEF chromatin present on the same blot, the 5.7 kb band in brain chromatin decreases in intensity after DNase I digestion but in contrast to CEF chromatin no band at 4.3 kb is detected. At higher DNase concentrations several diffuse bands appear in brain chromatin including one at 3.8 kb (Fig. 4C). These results were reproducibly observed and suggest that although several areas of the α2(I) collagen gene in brain chromatin have an increased sensitivity to DNase I, the area 100-300 bp upstream of the start of transcription does not show an increased sensitivity to DNase I. The band at 4.3 kb was not seen when naked DNA was digested with DNase I and autoradiographed on the same blot as CEF chromatin (Fig. 4B) indicating that the DNase I sensitivity in this region is a result of the chromatin structure not the DNA sequence.

In summary, a major hypersensitive site is located around 100-300 bp upstream of the start site of transcription. This site is tissue specific because it is found in chromatin from tissues known to synthesize collagen, but not in a tissue that does not. The minor site which maps in the first intron seems to be present in all three tissues; however, it might be more prominent in cells that synthesize low levels of collagen such as RSV-CEF and early chick embryos.

DISCUSSION

The results presented here indicate that the chromatin of the α2(I) collagen gene has the same relative DNase I sensitivity in the segments which are methylated as in those which appear to be unmethylated. Our analysis was performed on three segments of this 38 kb gene, one at the 5' end, one in the middle, and one towards the 3'end of the gene. Together, these segments cover over one third of the gene. In addition, in chromatin from CEF, the unmethylated region at the 5'end of the gene contains a site which is hypersensitive to DNase I digestion. The DNase hypersensitive site is located near the start site of transcription and is only found in cells where the gene is expressed. Hence, there is no correlation between the DNase I sensitivity or hypersensitivity of this gene in chromatin and its methylation pattern. A similar uncoupling between methylation pattern and general DNase I sensitivity was demonstrated in the rRNA genes of *Xenopus borealis* (27). These two examples do not support a model in which methylation of DNA would prevent binding of HMG proteins and thereby produce a region

of DNase I insensitivity(28). However, there is a correlation between the presence of a DNase I hypersensitive site in the promoter of this gene and the expression of the $\alpha 2$(I) collagen gene. An analogous result has been reported for the mouse adult α and β_{Major}-globin genes in erythroleukemia cells where the induction of globin synthesis is accompanied by the appearance of a DNase I hypersensitive site without changes in the methylation patterns of these two genes (29).

In chick fibroblasts transformed by RSV there is at least a 10-fold decrease in type I collagen synthesis, although, low levels of collagen synthesis are detected (14, 15). This selective reduction in collagen synthesis is due to the presence in the cell of an active p60^{src} protein and is mediated by a transcriptional control mechanism (16, 17, 18, 30). The severe decrease in the expression of the gene occurs without a change in the methylation pattern (8) or a change in the DNase hypersensitivity of a discrete site near the start of transcription. Therefore the mechanism which is responsible for the p60^{src} dependent reduction of $\alpha 2$(I) collagen gene transcription must be different from the mechanism which is responsible for the presence of a DNase I hypersensitive site in the promoter.

There is an increasing body of evidence suggesting that the DNase I hypersensitivity demonstrated in chromatin may be caused by the absence of nucleosomes in this segment (24,22,31). The region which is hypersensitive to DNase in the $\alpha 2$(I) collagen promoter has several additional interesting features. When the DNA of this region in either the chicken or the mouse $\alpha 2$(I) collagen gene is present in a supercoiled plasmid it contains a discrete site that is also sensitive to S_1 nuclease (32). Comparison of the sequence in this region shows that it is highly conserved in the two species and is composed almost exclusively of pyrimidines on one strand which contains two direct repeats with a short palindrome in each repeat (32). The pyrimidine-rich nature of this sequence around the S_1 sensitive site might favor the dissociation of one or a few nucleosomes (33,34) and hence cause an increased sensitivity to DNase I.

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