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Chromatin structure of active and inactive human X chromosomes

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ABSTRACT

Nuclei from a variety of human cell lines and tissues were digested with gradually increasing levels of DNase I. The DNA was then purified, treated with restriction enzymes and subjected to Southern blot hybridization using a cloned cDNA probe to 3-phosphoglycerate kinase (PGK) a housekeeping enzyme. At relatively high levels of DNase I, a specific, slightly sensitive site in chromatin sequences encoding PGK was observed in all of the cell types examined. This slightly sensitive site resides on the active X-chromosome since cell lines with increased numbers of inactive X-chromosomes do not show an increase in the region of chromatin which is sensitive. Except for this restricted region of enhanced sensitivity on the active X-chromosome, the data suggest that, for PGK encoding sequences, chromatin configurations on the active and inactive X-chromosomes are similar.

INTRODUCTION

Mammalian females achieve a form of dosage compensation by inactivating, randomly, one of two X-chromosomes. Once either the paternal or maternal X-chromosome is inactivated, the inactive state of that chromosome is propagated in subsequent cell division (1-2). The inactive X-chromosome can be recognized cytologically as a heterochromatic Barr body and this heterochromatinization has been generally regarded as an alternative chromatin configuration which is directly or even causally related to inactivation of genes on this chromosome.

The enzyme, DNase I, has been established as an effective probe for detecting active vs. inactive configurations of chromatin. Several autosomal genes which are very actively transcribed (represented in mRNA on the order of 50,000 copies per cell) have been shown to be preferentially sensitive to DNase I, compared to inactive genes in the surrounding chromatin (3, 4, 5, 6). Many other active genes such as the heat shock loci in Drosophila (7), histone genes (8, 9), α actin (10) and others have DNase I hypersensitive sites. Hypersensitivity has been inferred either, because of a genomic restriction fragment which selectively disappears after low level DNase I treatment of

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nuclei, or because of selective DNase I-induced conversion of a restriction fragment to a well defined, shorter fragment or "sub-band."

Recently, Kerem et al. demonstrated preferential DNase I attack of the active X-chromosome in whole, fixed mitotic chromosomes (11). The latter study suggests some gross structural differences in chromatin comparing active and inactive X-chromosomes. However, since the chromosomes were fixed in a methanol-acetic acid mixture prior to nick translation in the presence of DNase, the relationship between preferred DNase I attack and native chromatin structure is difficult to interpret.

The present report describes a study of DNase I sensitivity of specific X-chromosome associated sequences encoding 3-phosphoglycerate kinase (PGK). Two main observations are made: (1) At high DNase I levels (about 0.7 times the amount of DNase I needed to digest bulk chromatin) we find one clearly defined, sensitive site specific to the active X-chromosome. (2) At lower DNase I levels normally used to observe so-called "hypersensitive" sites we find no detectable differences between active and inactive X-chromosomes at least in sequences encoding PGK. Thus, although there is a chromatin feature specific to the active X-chromosome, this slightly sensitive site is in a category separate from both, previously reported hypersensitive sites and DNase I sensitive domains.

MATERIALS AND METHODS

Cells and Cell Culture

Cultured cells were grown under conditions routinely used in this laboratory (12, 13). Briefly, the transformed human lymphoblast cell line, EBV-LCL (14) was grown in RPMI with 15% fetal calf serum. Human fibroblast cell lines were cultured in Eagles minimal medium with 10% fetal calf serum. In addition, all culture media contained 40 µg/ml gentamycin. Cells were propagated in a 5% CO_2 atmosphere. Cells from EBV-LCL were harvested during logarithmic growth. Fibroblasts were harvested during active growth or at slightly subconfluent stages with no detectable effects on the experimental results. Sex chromatin was determined on cells fixed in Carnoy's, hydrolyzed in 5N HCl at room temperature for 15 minutes and stained in tetrachrome (15).

Tissues and Tissue Preparation

Human fetal lung tissue was obtained from the Central Laboratory for Human Embryology, University of Washington. P. C. Anubis liver and lung samples were obtained from the Regional Primate Research Center at the University of Washington.

Tissues were homogenized with two-one second bursts of a motor-driven Tekmar Ultra Turax homogenizer set on 100. Tissue homogenates in cold phosphate buffered saline (PBS) were filtered twice through several layers of gauze. The cells were washed three to four times in PBS prior to isolation of nuclei.

Nuclear Isolation

Nuclei were isolated by a modification of previously reported procedures (16). Briefly, 50 ml centrifuge tubes containing washed cellular pellets were dried of adherent PBS using"Kimwipe" and forceps. The cellular pellets were brought up in 0.5% Nonidet P-40 (NP-40) in reticulocyte standard buffer (RSB) containing 10 mM Tris (pH 7.4) 10 mM NaCl and 5 mM $MgCl_2$. After disruption of clumps using a Pasteur pipette, the nuclei were pelleted at 1500 rpm in an IEC centrifuge for 2 min. The nuclei were subjected to three cycles of suspension and pelleting in the NP-40 containing buffer. The nuclear pellet was then suspended and pelleted in RSB without NP-40 and brought up in the same buffer prior to DNase I digestion.

DNase I Digestion

Nuclei were diluted in RSB to a concentration of 0.4 mg chromatin DNA per ml. Aliquots of the nuclear suspension were treated with increasing concentrations of DNase I, as indicated in the figure legends, all at 37°C for 10 min. Reactions were terminated by adding EDTA to 10 mM, SDS to 0.1% and proteinase K to 0.5 mg/ml. Proteinase K digestions were carried out for 1 hr. at 37°C.

DNA Purification and Restriction Enzyme Treatment

DNA was purified by 3 cycles of extraction in phenol-chloroform-isoamyl alcohol (1:1:1/48;v:v) and 2 extractions with chloroform-isoamyl alcohol (1:1/24;v:v) followed by ethanol precipitation. Dried DNA precipitates were brought up at 0.5 to 1 mg DNA/ml (determined by A_{260}) in 0.2 mM EDTA (pH 7.4).

Concentrated buffer solutions were added according to restriction enzyme manufacturer's specifications. Restriction enzymes were added at 3 to 8 units of enzyme per µg DNA and digestions carried out for 2 to 4 hr. During the last half hour of restriction enzyme digestion, 2 µg RNaseA (Worthington) per µg of DNA was added. Reactions were terminated by adding EDTA to 10 mM, SDS to 0.1%, glycerol to 5% and proteinase K to 0.5 mg/ml.

Agarose Gels and Southern Blotting

The proteinase K digested samples were loaded onto 0.7% agarose gels. HindIII digested λ DNA and HaeIII digested φX174 DNA served as molecular weight standards. DNA was blotted onto nitrocellulose (Schleicher and Schuell)

according to the method of Southern (18). Prehybridization mixtures contained the following final concentrations of components: 50% formamide, 0.3 M NaCl, 0.05 M Tris-HCl (pH 7.0 adjusted prior to formamide addition), 0.05 mg/ml salmon testes DNA (Sigma), 0.01 mg/ml polyguanylic acid and 0.01 mg/ml polycytidylic acid, 2.5 times concentrated Denhardt's solution and 0.05% SDS. Prehybridization was carried out at 42°C for 16 hr. Hybridizations were carried out in the same buffer with 10% dextran sulfate for 24 hr. at 42°C.

Hybridized filters were washed for 15 min. at 22°C in 2 x SSC with 0.1% SDS, followed by 1 hr. at 65°C in the same buffer. This rinsing was followed by two changes in 0.5 x SSC (PGK probe) with 0.1% SDS at 65°C for 2 hrs. The final wash was in 0.1 x SSC, 0.1% SDS at 22°C for 15 min.

DNA Probes and In Vitro Labeling

The cDNA to sequences encoding 3-phosphoglycerate kinase (PGK) was selected, by Singer-Sam et al. (17), using a synthetic oligonucleotide mixture from a human cDNA library. The cDNA insert of PGK-5 is 1.8 kb in length and includes about 35 bp 5' to the coding region and approximately 430 bp on the 3' side of the coding sequences. Other probes used in this report include PGK-6, PGK-7 and PGK-8. PGK-6 represents the internal HindIII fragment (595bp) of the coding region of PGK-5. PGK-7 includes the 5' 440 bp of the PGK coding region as well as 35 bp 5' to the initiation site. PGK-8 represents 210 bp of the 3' coding region and 440 bp of 3' noncoding sequence.

Probes to human PGK-encoding sequences were labeled by the method of second-strand synthesis (19). Unlabeled dCTP was used to supplement α-^{32}P labeled deoxycytidine triphosphate. The level of dCTP was calculated to insure sufficient molarity of dCTP for complete second-strand synthesis. After two hours of labeling using DNA polymerase I (New England Nuclear), the mixture was passed over a G-50 column to separate unincorporated nucleotides from the labeled probe. To the peak fractions, denatured, salmon testes DNA was added to 0.2 mg/ml and the mixture was treated with S1 nuclease to remove terminal hairpin loops (19). The method of second-strand synthesis yielded a much more efficient probe than the conventional nick translation (20) for PGK cDNA.

RESULTS

A DNase I Sensitive Site in PGK Encoding Chromatin

The male derived cell line, EBV-LCL, was used in an initial search for DNase I sensitive sites in PGK encoding chromatin. Pairs of DNA samples, from DNase I treated and control nuclei, were treated with a number of different

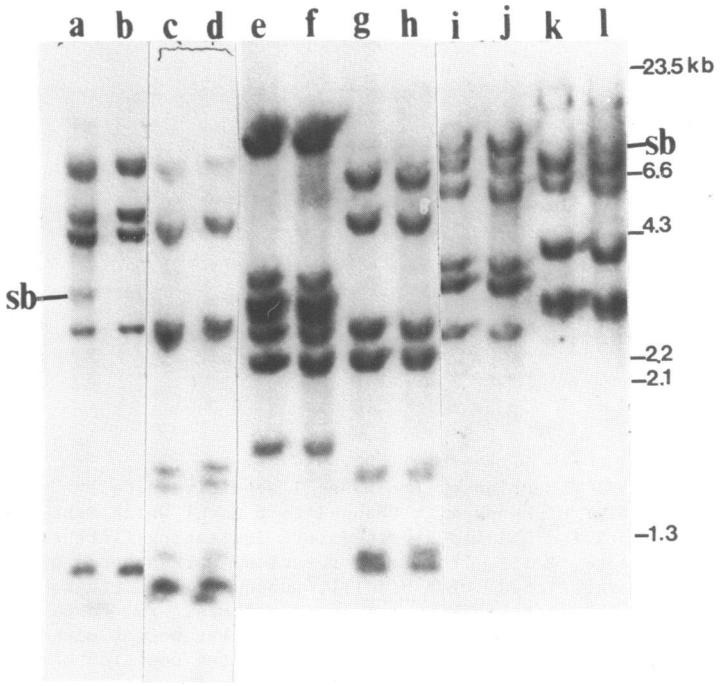


Figure 1. Nuclei from the transformed human lymphoblast cell line, EBV-LCL, were incubated with 7 µg/ml DNase I or with no DNase I under the conditions described in "Materials and Methods." The DNA was then purified and restricted with one of the following restriction enzymes: HindIII (lane a, DNase I; b, no DNase I); BglII-HindIII double digest (lane c, DNase I; d, no DNase I); EcoRI-BglIII double digest (lane e, no DNase I; f, DNase I); HindIII-EcoRI double digest (lane g, no DNase I; h, DNase I); EcoRI only (lane i, no DNase I; j, DNase I); BgIII only (lane k, no DNase I; l, DNase I). "sb" marks the position of the DNase I-induced sub-band in lanes a and l.

restriction enzymes, singly or in combination (Fig. 1). We observe a sub-band (a band present solely as a result of DNase I treatment of the nuclei) if the DNA is restricted with either HindIII or BglII. The sub-band is not obvious in many of the other restriction patterns, probably being either coincident with other bands or cut to a small size. An alternative explanation suggested by a reviewer is that the sub-band observed with HindIII or BglII might be an artefact of restriction enzyme digestion or Southern blotting. First, artefacts due to restriction enzyme digestion can be eliminated by the results of Fig. 2 where it is shown that the sub-band can be degraded by the exonuclease, Bal31 prior to any restriction enzyme digestion. Thus, the sub-band represents a double-stranded break put into chromosomal DNA prior to any use of

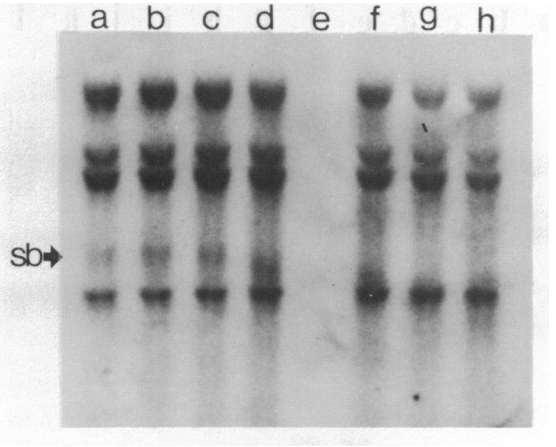


Figure 2. Terminal digestion of the DNAse I sensitive site by Bal31. DNA was purified from nuclei digested with DNase I to a level which maximizes sub-band material. The purified DNA was digested for increasing (left to right) times with the exonuclease, Bal31. The Bal31 reactions were stopped by adding EGTA. The DNA was then restricted with HindIII, subjected to Southern blotting and hybridization to the probe to PGK encoding sequences. a) No Bal31; b) 30 min Bal31 digestion; c) 60 min; d) 90 min; e) Molecular weight markers - not visible; f) 2 hr; g) 3 hr; h) 4 hr. "Sb" marks the position of the sub-band which is selectively attacked by Bal31.

restriction enzymes. Secondly, both the HindIII sub-band (shown extensively throughout this paper) and the BglII sub-band (Fig. 1) are very reproducibly observed although the intensity and breadth of the sub-bands varies from cell type to cell type. We believe the reason several restriction enzymes (Fig. 1 and also PstI, Bam HI, Hinf I and numerous pairwise combinations) do not show the sub-band is that within or near the coding region there is only one sensitive site which frequently gets obscured by other bands or cut to an undetectable size. The sub-band observed with HindIII can be unambiguously assigned to a parent fragment (see below). For this reason, we have explored differences at this site comparing active and inactive X-chromosomes.

The DNase I Sensitive Site is Specific to the Active X-Chromosome

In order to determine whether the sensitive site was located specifically on the active X-chromosome, we looked for the presence of this DNase I sensitive site in four, different human fibroblast cell lines which vary in the number of X-chromosomes per cell (Fig. 3). Two different male fibroblast cell lines (743 and 456) clearly show the HindIII sub-band although its appearance is somewhat broader, spanning about 200 bp, than in the case of the lymphoblast cell line (EBV-LCL, Fig. 1). The sub-band is also apparent in cell line

443, fibroblasts derived from a female with two X-chromosomes. A fibroblast line with three X-chromosomes (73-126) shows in the expected region similar sub-banding material, although in this case the sub-band is largely obscured by background material.

A comparison of the active and inactive states of the X-chromosome is facilitated by the fact that human male cells contain only the active X configuration. Female cells each contain one active and one inactive copy. Thus, the contribution of the inactive X can be examined not only by making male-female comparisons, but by looking at cell lines with multiple inactive X-chromosomes with only one active X-chromosome per cell (eg. 73-126-below). These comparisons depend, at least, on semi-quantitative comparisons. We examined the hybridization Southern blotting procedure used throughout this paper for quantitativeness and find (Fig. 4) that the method, in our hands, is quantitative with the following restrictions:

(1) As shown in Figure 4, bands migrating between 10 kb and 3.0 kb can be transferred and detected with signals directly proportional to the actual amount of DNA loaded. The proportionality is reliable if the densitometer (see legend); is equiped with a laser source having a beam width wider than the lanes being scanned. Densitometers with pin-point beam widths (Joyce Loebl) are much less reliable for this purpose. Very large or small fragments outside the 3 to 10 kb range may depart from proportionality.

(2) Only conditions of highest possible stringency, determined experimentally, were used.

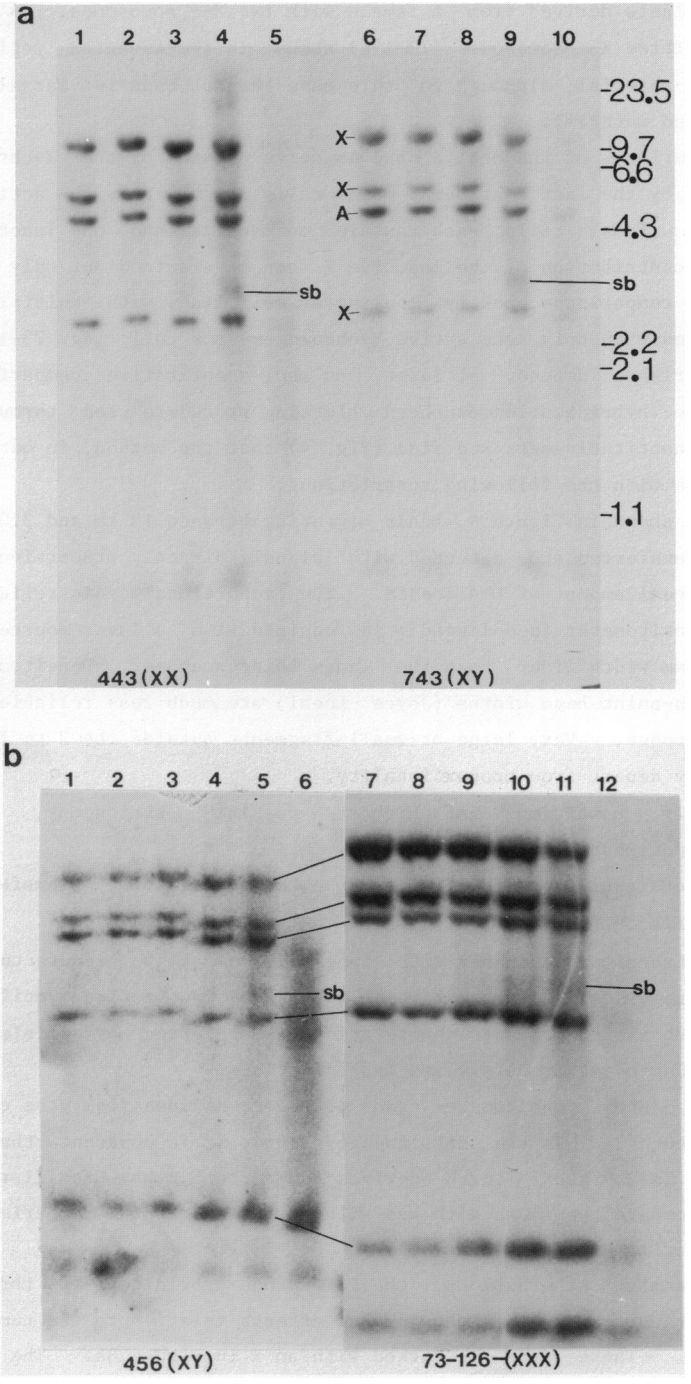
(3) Blots exhibit edge effects, so that quantitative transfer is only reliable for the middle 70% of the gel.

(4) Nitrocellulose paper with imperfections at the manufacturing level were discarded if the initial wetting was not entirely uniform.

(5) Each statement as to quantitation is supported by a minimum of two independent transfers and hybridizations.

Figure 5 shows densitometer scans of selected lanes from the experiments shown in Figure 3. In the densitometry scans, it is apparent that the sub-band which is 3.9 kb in length derives from the 5.6 kb HindIII restriction fragment (compare the scans with and without DNase I in the experiments using EBV-LCL DNA; Fig. 5, scans a and b).

In genomic Southern blots, the PGK probe hybridizes to three HindIII fragments which reproducibly show dosage effects relative to the number of X-chromosomes. These bands are marked with an X in Figure 3a. The PGK probe


a
1 2 3 4 5 6 7 8 9 10
-23.5
-9.7
-6.6
-4.3
-2.2
-2.1
-1.1
X
X
A
X
sb
sb
443(XX)
743(XY)
b
1 2 3 4 5 6 7 8 9 10 11 12
sb
sb
456(XY)
73-126-(XXX)

also hybridizes to one HindIII fragment which does not show X-related dosage effects. This band represents autosomal sequences (A) related to the PGK probe. Autosomal PGK sequences have been documented in a number of mammalian systems (21-24). The autosomal PGK sequences are not expressed in tissues other than testes and therefore serve as an internal standard for non-expressed genes. The 5.6 kb HindIII fragment which gives rise to the 3.9 kb sub-band reproducibly shows dosage effects. We conclude that the DNase I sensitive site, which gives rise to the sub-band, is situated on an X-chromosome as opposed to an autosomal locus.

Furthermore, it appears that the sensitive site is specific to the active state of the X-chromosome. This is because the amount of the sub-band in identical experiments does not increase in proportion to the number of X-chromosomes. For example, in the 743 cell line (XY) the sub-band maximally represents 11.6% of the total hybridizing material as determined by densitometric scanning (Fig. 5). The sub-band of the 443 cell line (XX, one Barr body observable) represents 8.7% of the total. Finally, in the 3X cell line (73-126, two Barr bodies) the sub-band represents only 5.3% of the total.

If both active and inactive X chromosomes were contributing to the sub-band, there should be no change in the proportion of the sub-band relative to the other hybridizing material. Instead, we see a clear reduction in the sub-band, relative to other hybridizing fragments as the number of inactive X-chromosomes per cell is increased.

A similar conclusion was reached by examining the 5.6 kb fragments. Due to selective reduction in intensity in male nuclei, the 5.6 kb fragment is assigned as the parent fragment of the 3.9 kb sub-band. By measuring either

Figure 3. DNase I digestion of nuclei from four human fibroblast cell lines varying in the number of X-chromosomes per cell.

a. Nuclei from cell line 443 (2X chromosomes, lanes 1-5) or 743 (XY, lanes 6-10) were digested with increasing concentrations of DNase I. The DNase level increases from left to right. Lanes 1 and 6, no DNase; 2 and 7, 0.2 µg/ml DNase I; 3 and 8, 0.8 µg/ml; 4 and 9; 4 µg/ml; 5 and 10, 10 µg/ml. The DNA was purified, restricted with HindIII and blot hybridized to the PGK probe as in Figure 1. "X" marks the position of bands reproducibly showing dosage effects relative to the number of X-chromosomes. "A" marks a band which clearly does not show dosage effects in repeated experiments. Unlabeled, lower molecular weight bands have not been characterized with respect to dosage.

b. An experiment similar to that of part a. except lanes 1 through 6 represent DNA from the cell line 456 (XY) and lanes 7-12 are from 73-126 (3X chromosomes).

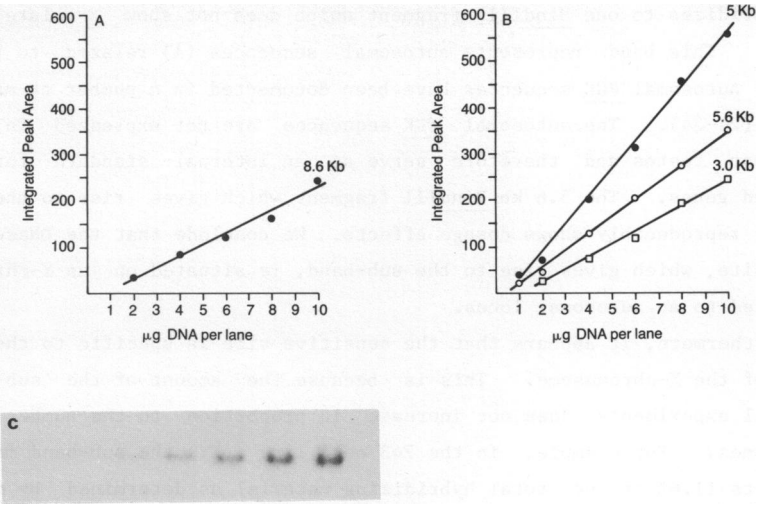


Figure 4. Proportionality of probe detection signals. Various amounts of HindIII digested human genomic DNA were electrophoresed on a 0.7% agarose gel, subjected to Southern transfer, and blot and hybridization with the PGK-5 cDNA probe. Lanes were scanned with a 525 nm laser source with a beam-width 30% larger than the width of the lanes being scanned. Integrated peak areas were plotted vs. the amount of total genomic DNA loaded. Plots of each fragment shown were taken from a single film. An exception is the 8.6 kb fragment which was analyzed on a separate exposure because of grain saturation in the original exposure. (A) Peak area under the 8.6 kb fragment vs. amount of total genomic DNA loaded. (B) Area vs. amount loaded for the 3.0, 5.0 and 5.6 kb fragments. (C) Portion of autoradiogram showing the 5.0 kb fragment with increasing (left to right) amounts of total genomic DNA loaded.

peak heights or areas, the reduction in the 5.6 kb band (at maximal sub-band intensity) is clearly greatest for male nuclei (typically 50% the intensity of the no DNase I control, less (80%) in nuclei with one inactive X (e.g. 443) and the difference is barely detectable in the cell line with three X chromosomes (Fig. 5). We conclude that these data indicate that, in sequences hybridizing to PGK, there is a slightly DNase I sensitive site that is specific to the active X-chromosome.

As described above, the most critical step in these experiments is the treatment of isolated nuclei with DNase I. To show that the heterochromatinization of the inactive X-chromosome was not affected by the manipulations involved in isolating nuclei, we examined nuclei for the presence of Barr bodies (Fig. 6). Supporting the validity of the approach, heterochromatic Barr bodies remain afer purification of nuclei from cells with multiple X-chromosomes.

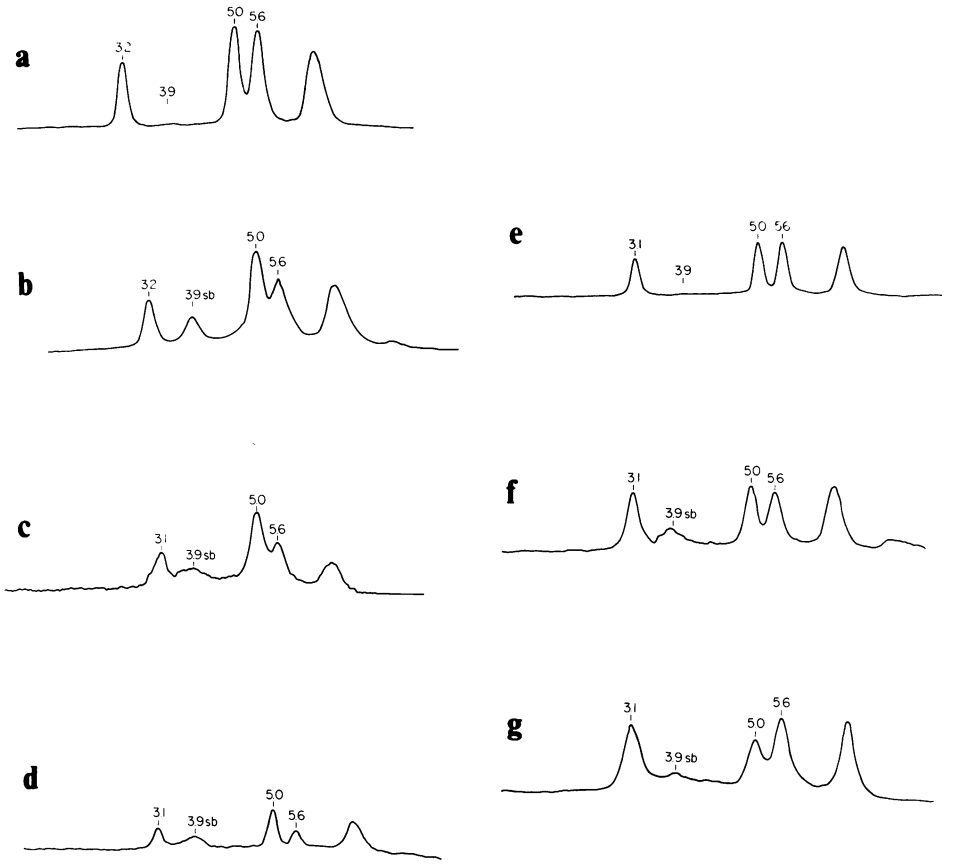


Figure 5. Densitometer scans of HindIII-digested DNA hybridizing to PGK. Shown for each cell line are scans of lanes showing maximal intensity of DNase I-induced sub-band material and some no DNase I controls.

a. Transformed human lymphoblast, EBV-LCL. No DNase I digestion of the nuclei.
b. Transformed human lymphoblast, EBV-LCL. DNase I digestion of the nuclei. The lower case "sb" at 3.9 kb refers to sub-banding DNA.
c. Human fibroblast 456 (XY). DNase I-digested nuclei.
d. Human fibroblast 743 (XY). DNase I-digested nuclei.
e. Human fibroblast 443 (XX). No DNase I-digestion.
f. Human fibroblast 443 (XX). DNase I-digested nuclei.
g. Human fibroblast 73-126 (XXX). DNase I-digested nuclei.

Controls not shown here (456, 743, 73-126) can be examined in Figure 3.

Position of DNase I Sensitive Sites Relative to the PGK Transcription Unit

The precise genomic map of PGK encoding sequences is not yet determined. However, using fragments of the cDNA probe to PGK, we have determined that the

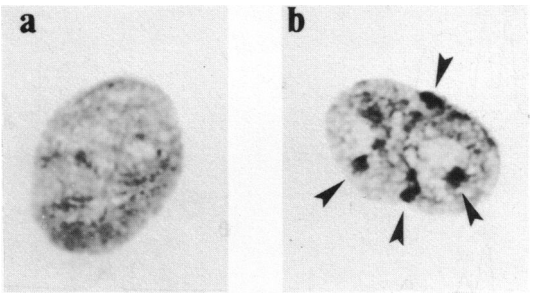


Figure 6. Barr body analysis performed on isolated nuclei. Nuclei were prepared by the same procedure used throughout this report, and subjected to the Barr body analysis described in "Materials and Methods."

a. Nuclear preparation from a male (456-XY) fibroblast cell line.
b. Nuclear preparation from a fibroblast cell line (73-221) containing 5X chromosomes per cell. Arrows indicate densely stained inactive copies of X-chromosome.

3.9 kb DNase I sub-band, which is characteristic of all of the cell types examined, as well as the 5.6 kb band which is the most likely parent of the sub-band based on densitometry experiments, both hybridize only to the extreme 3' region (Fig. 7) of the cDNA. The corollary is also true. That is, probes to the 5' and middle of the coding region repeatedly fail to label either the 5.6 kb band or the sub-band. We conclude that the DNase sensitive site is on the 3' end of the transcription unit and within the 5.6 kb band. Due to the 3' position of this sensitive site, its role in regulating transcription is uncertain. However, the constancy of this site in the various cell types examined (Figs. 1, 3, 8) and its observed specificity to the active X-chromosome suggests a relevance of this site to gene activation.

The DNase I sub-band we have described is in fact the only obvious difference we can detect comparing chromatin of active and inactive X-chromosomes using the cDNA to PGK encoding sequences. Since this cDNA includes both 5' and 3' non-coding regions, any other sensitive sites relevant to PGK activation would have to be more distant from the coding regions than is the case for most other reported DNase I sensitive sites (3-10).

Presence of the DNase I Sensitive Site in Human Fetal Lung Tissue

We have also found a DNase I sensitive site, similar to that described above, in nuclei from human fetal lung tissue (Fig. 8). This finding indicates that the sensitive site is not dependent on the growth of cells in culture. It is of some interest that various cell types including EBV-LCL, a lymphoblast line, a variety of independent fibroblast lines, and human fetal

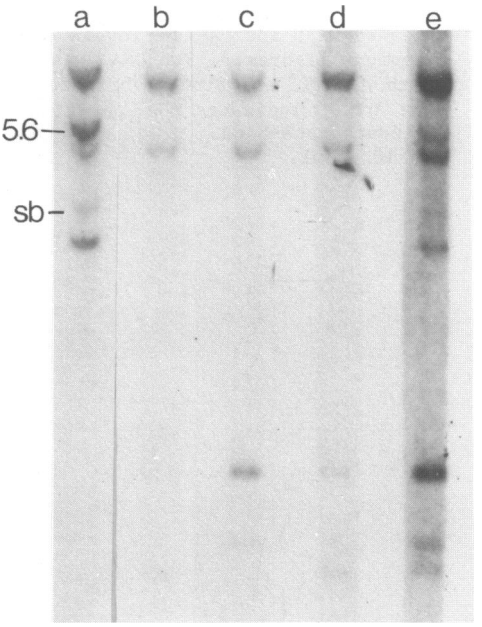


Figure 7. Hybridization of HindIII digested genomic fragments with probes to various regions of the PGK cDNA. EBV-LCL nuclei were digested with DNase I to an extent maximinizing the DNase I induced sub-band. The DNA was purified, HindIII digested, and equivalent amounts run in multiple lanes of a 0.7% agarose gel. The resulting blot was cut into identical strips for hybridization to four separate probes.

a Strip hybridized to PGK-8 representing 210 bp of the 3' coding region and a portion of the 3' non-coding region (Methods).
b Strip hybridized to PGK-7 (the 5' 440 bp of the PGK coding sequence, as well 35 bp on the 5' side of the initiation site).
c Strip hybridized to PGK-6, the internal 595 bp of the coding region.
d Same as (a).
e Strip hybridized to PGK-5, the complete cDNA insert.

lung cells all have this sensitive site. Within the precision of our measurements it always occurs at the same position along the chromatin DNA. Cell types do vary somewhat in the "sharpness" of the sub-band possibility indicating enhanced susceptibility to DNase I - "end-nibbling" in chromatin from some cell types. The characteristic nature of this site possibly reflects the constant or so-called "housekeeping" function of PGK.

Comparison of Chromatin DNA with Naked DNA

The possibility existed that the HindIII sub-band was produced by a DNA sequence slightly more sensitive to DNase I than the neighboring sequences. To test the possibility that the HindIII sub-band might not be dependent on

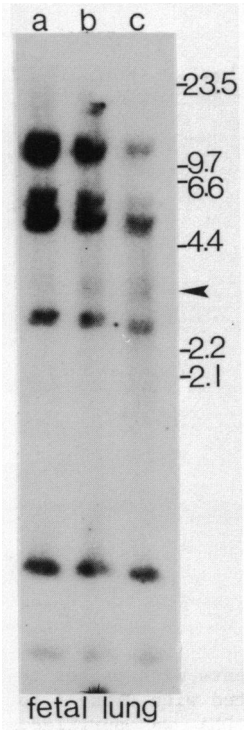


Figure 8. Nuclei from human fetal lung tissue were treated with increasing levels of DNase I as in previous figures. The DNA was purified, restricted with HindIII and blot hybridized to the PGK probe. The arrow indicates the position of the 3.9 kb DNase I-induced sub-band. a, 2 µg/ml DNase I; b, 5 µg/ml; c, 10 µg/ml.

the presence of chromatin proteins, we digested purified DNA from EBV-LCL cells with increasing amounts of DNase I. Since Ca^{2+} may affect the specificity of DNase I cutting (Razin, personal comunication) this experiment was performed with either Ca^{2+} or Mg^{2+} as the major cation. In these experiments we observed no sub-bands, and no selective sensitivity of any of the bands hybridizing to PGK (Fig. 9). We repeated these experiments using very gradual increases of DNase I between 3 and 10 ng/ml and were unable to detect sub-banding. Thus, the specific DNase I cutting which produces the HindIII sub-band requires the presence of chromatin proteins.

DISCUSSION

This report describes a DNase I sensitive site specific to the active X-chromosome of a number of different cell types (lymphoblasts, four independent fibroblast lines and human fetal lung cells) which vary in the number of inactive X-chromosomes. At the outset of this study, we expected to find extensive differences in DNase I susceptibility of PGK encoding sequences on the active and inactive X-chromosomes. For example, the region of the active

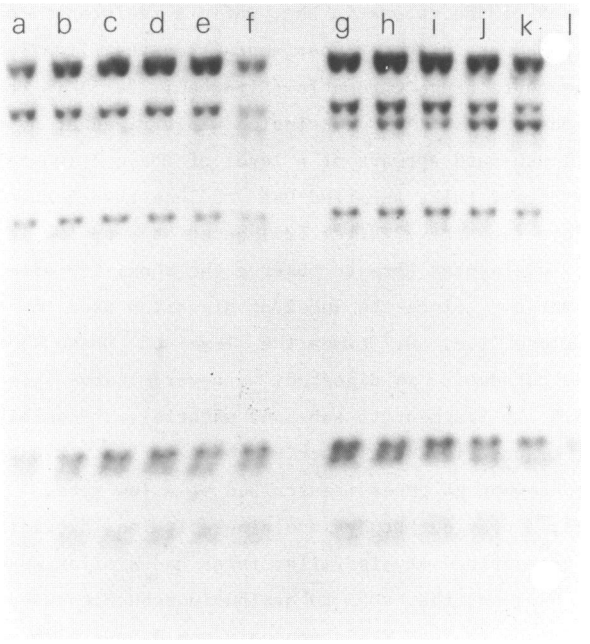


Figure 9. Purified DNA from the transformed human lymphoblast cell line, EBV-LCL, was suspended in buffer similar to RSB ("Materials and Methods") except that in lanes a-f Ca^{2+} was substituted for Mg^{2+}. The DNA was treated with increasing concentrations of DNase I. Lanes a-f, no DNase I, 0.1 ng, 0.3 ng, 1 ng, 3 ng, 10 ng DNase I/ml, respectively. Lanes g-l, no DNase I, 0.1 ng, 0.3 ng, 1 ng, 3 ng, 10 ng DNase I/ml, respectively. The DNA was then purified, restricted with HindIII and blot hybridized to the PGK probe. Note: for unknown reasons the third HindIII band (5.0 kb-autosomal) does not show up well after exposure to Ca^{2+}.

X-chromosome we have explored does not appear to be part of a large sensitive domain of the kind described by other investigators (25,26,27). Furthermore, our results seem somewhat in contrast to the study involving DNase I-induced nick translation of fixed, whole mitotic chromosomes (11). The latter study seemed to suggest extensive structural differences between active and inactive X-chromosomes. Possibly, the apparent differences between the results of the two methodologies could be explained by the contribution of very active genes in the latter study, although there is no evidence for very active genes on the X-chromosome (28). Alternatively, fixing in methanol-acetic acid may in some way selectively fix the chromatin of the inactive X-chromosome making it more resistant than other chromosomes. In fact, we have found that the fixing method employed in that study induces greater sensitivity (by a few orders of

magnitude) of much of the chromatin DNA, while a resistant component remains.

It became apparent in these experiments that the slightly sensitive site in PGK sequence containing chromatin (Figures 1, 2, 5) is considerably more protected than naked DNA. This conclusion was arrived at by the observation that the HindIII sub-band appears at a level of DNase I 100-fold greater than that needed to digest bulk, purified DNA to less than 5 kb. Also, at least some reported "hypersensitive" sites (25) occur at very low levels of DNase I compared to the levels used here to observe the sensitive site in PGK sequence containing chromatin. Since the level of digestion used to maximize the PGK sub-band is so close (i.e. 0.7 times the level of DNase I) to the level at which all of the chromatin is digested, we never observe complete conversion of the parent 5.6 kb fragment to sub-band material. Possibly, the relative resistance of the sensitive site described here reflects a category of chromatin architecture common to genes transcribed at a low rate.

In contrast, a previous study involving solution hybridization suggested that sequences transcribed at dissimilar rates had similar susceptibilities to DNase I (29). However, the cDNA to ovalbumin sequences used as the rapidly transcribed prototype in that study probably would have also detected the so-called "X" and "Y" genes. The latter are related to ovalbumin but transcribed at a lower rate (30), thus complicating the study by solution hybridization. Further experiments will be required to resolve the relationship between accessibility to DNase I and gene activity.

To summarize, in sequences encoding PGK, we find a specific difference in chromatin comparing active and inactive X-chromosomes. Rather than spanning a large region of the PGK encoding chromatin, this difference is confined to a narrow region (about 200 bp) near the 3' end of the transcription unit.

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