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DNaseI sensitivity of the rat albumin and α-fetoprotein genes

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ABSTRACT

We have analyzed the DNaseI sensitivity of chromatin from the rat albumin and α-fetoprotein genes in the fetal liver (which synthesizes albumin and α-fetoprotein), adult liver (which synthesizes albumin), fetal yolk sac (which synthesizes α-fetoprotein), and adult kidney (which synthesizes neither). Active genes were much more sensitive than their kidney counterparts, and the adult liver α-fetoprotein and fetal yolk sac albumin genes showed intermediate levels of sensitivity. Sensitivity was analyzed as a function of the extent of DNaseI digestion. Rate constants were calculated for the degradation of individual DNA hybridization bands and normalized to the intrinsic rate constants of the same bands degraded in purified DNA. This enabled us to eliminate the inconsistencies that otherwise result from comparing chromatin sensitivity of different DNA sequences, or chromatin sensitivity in different nuclear environments.

INTRODUCTION

The differential expression of genes in higher animals is one of the central problems of molecular biology. Although the set of genes and encoded base sequences is virtually the same in all tissues, differential gene expression is associated with two modifications of gene structure. The cytosine methylation of active genes is altered from that of inactive genes (reviewed in Refs. 1,2). Active genes are also distinguished by their altered chromatin structure; one property of this altered chromatin is its increased sensitivity to nuclease digestion (reviewed in Ref. 3). Neither the chromatin nor the methylation changes are well understood. Clarification of the biological significance of these changes will require rigorous correlation with developmental modulation of expression of specific genes. Valuable correlations have been obtained for the globin (4), immunoglobin (5), and ovalbumin genes (6), among others, but many more are necessary.

A number of structural alterations have been described in the chromatin of active genes. It is more sensitive to a variety of nucleases and other cleaving agents including DNaseI (3,4,7,8), Micrococcal nuclease (7-9), S1

nuclease (10), restriction enzymes (11), and methidiumpropyl-EDTA·iron(II) (12). In chick erythrocytes, these alterations of chromatin structure include addition of the HMG proteins to active nucleosomes (13), and probably depletion of some histones (14). Such observations suggest that the increased sensitivity to nucleases results from a loosening of the association between DNA and histones, which ordinarily protect stretches of DNA from attack by nucleases. Of the cleaving agents, DNaseI is the best discriminator of active from inactive genes (3,4,7,8), while Micrococcal nuclease (7-9) and methidiumpropyl-EDTA·iron(II) (12) are more useful in probing the nucleosomal fine structure of genes. DNaseI can also identify special hypersensitive regions (reviewed in 15) prominent in active genes. These localized regions of hypersensitivity are distinct from the generalized nuclease sensitivity of active genes.

The albumin and α-fetoprotein (AFP) genes comprise an unusually easy-to-study set of genes that modulate their expression during development, and are thus an ideal system for studying the modifications of chromosome structure associated with regulation. Although these two genes diverged hundreds of millions of years ago (16), the proteins they encode retain significant structural homology (17,18). The genes are next to each other (at least in the mouse), comprising a chromosomal domain of about 60kb (19). Albumin and AFP behave as if coordinately controlled (20,21), and the expression of these genes changes dramatically during fetal development. Equally important, these serum proteins are synthesized in large amounts by the liver, which consists of 65% differentiated hepatocytes in the adult (23), and is an organ large enough to be easily manipulated during early stages of fetal development. From the analysis of albumin and AFP mRNA synthesis by our laboratory (22) and others (20,21), we have deduced four modes of expression for this gene pair: (1.) a fetal liver mode, in which both genes are expressed at high levels; (2) an adult liver mode, in which only albumin is synthesized; (3.) a yolk sac mode, in which AFP synthesis predominates; and (4.) an inactive mode, in tissues like adult kidney, in which neither gene is expressed. We have recently analyzed the DNA methylation of these genes in fetal, neonatal, and adult liver, yolk sac, and control tissues (22,24) and found that the albumin and AFP genes in inactive tissues have very high levels of DNA methylation. Fetal liver has an intermediate level of DNA methylation for both genes. By adulthood, the albumin gene becomes uniformly demethylated throughout its length, while the AFP gene, though inactive, has regions of marked demethylation in its

3'-half. In the present study, we have extended our analysis of these genes to their chromatin structure, as probed by DNaseI sensitivity. We used a method similar to that of Felber et al. (25), in which sensitivity is correlated with the amount of DNA digestion rather than the amount of nuclease added, and in which DNaseI sensitivity of a sequence in chromatin is related to its intrinsic sensitivity in purified DNA.

MATERIALS AND METHODS

Animals. Timed-pregnant female and other adult Sprague-Dawley rats were obtained from the Holtzman Company, Madison, WI. Gestational age of the rats was determined from data provided by the supplier and from developmental landmarks (26).

DNA clones. Rat albumin cDNA clones pRSA13, pRSA57, and pRSA510 (27,28) and AFP cDNA clones pRAF65 and pRAF87 (18) were kindly provided by Thomas Sargent (California Institute of Technology, Pasadena, CA). Hybridization of these clones to rat genomic MspI digests has been described by Kunnath and Locker (24).

Purification of nuclei. These methods were modified from Wu et al. (7). Fresh tissue was gently homogenized in 20 ml/g of homogenization buffer (60 mM KCl, 15mM NaCl, 0.5 mM spermidine, 15 mM Tris, pH 7.4, 0.5 mM dithiothreitol, 0.1mM phenylmethylsulfonylfloride) also containing 10 mM EDTA, 2.5 mM EGTA, and 0.5 M sucrose. When most nuclei were freed from cells, generally after about 10 motor-driven strokes, nuclei were pelleted for 10 min at 350 x g. Nuclei were thoroughly but gently resuspended in homogenization buffer (20 ml/g of tissue) containing 0.5 M sucrose, and applied to sucrose step gradients formed in homogenization buffer. The gradients were centrifuged in a Sorvall HB-4 swinging bucket rotor for varying times. Optimum gradients and centrifugation conditions for each tissue are as follows:

Adult liver - 10 ml nuclear suspension in 0.5 M sucrose, 10 ml 1.5 M sucrose, 5 ml 1.8 M sucrose; 11,000 rpm x 1 hr.

Fetal liver - 10 ml nuclear suspension in 0.5 M sucrose, 10 ml 1.8 M sucrose; 7000 rpm x 15 min.

Yolk sac - 10 ml nuclear suspension in 0.5 M sucrose, 10 ml 1.5 M sucrose, 5 ml 1.8 M sucrose; 10,000 rpm x 1 hr.

Adult kidney - 10 ml nuclear suspension in 0.5 M sucrose, 20 ml 1.5 M sucrose, 5 ml 1.8 M sucrose; 11,000 rpm x 1 hr.

After centrifugation, the sucrose layers were removed and the tube was carefully drained. The remaining nuclear pellets were difficult to see. The yield for 2 g of liver was about 10^9 nuclei.

Chromatin digestion with DNaseI. Nuclei were resuspended in digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris, pH 7.4, 0.5 mM dithiothreitol, 0.25 M sucrose, 0.05 mM $CaCl_2$, 3.0 mM $MgCl_2$). Digestion was carried out on aliquots of 1 x 10^8 nuclei, which yield 100 to 150 µg DNA. NP-40 was added to 0.2% and DNaseI was added from a 1 mg/ml stock solution in 50 mM Tris, pH 7.8, 1 mM EDTA, 50% glycerol. Samples were incubated for 3 min at 24°; digestion was terminated by the addition of EDTA to 20 mM and Sarkosyl to 0.5%. Deproteination and DNA purification are described in Kunnath and Locker (29).

We observed that the rate of DNaseI digestion decreased rapidly as the enzyme became inactivated. Accordingly, we digested separate aliquots with different amounts of DNaseI, always for 3 min at 24°C, a procedure that allows much greater control. To compensate for variability of preparations and still provide a useful range of chromatin digests, we used the following procedure: A DNaseI concentration of 40 µg/ml is tested first. After addition of EDTA and Sarkosyl to this digest, the DNA should show an intermediate level of viscosity compared to a preparation of undigested nuclei. If no reduction in viscosity is apparent, this procedure is repeated with 50 and then with 60 µg/ml DNaseI. A range of 0.5 to 1.5 x the DNaseI concentration giving intermediate viscosity is then used to produce a series of digests. At the extremes of this range, the solutions should be as viscous as undigested chromatin and slightly viscous. After some practice, the viscosity levels are readily distinguished.

Analysis of extent of chromatin digestion. Following purification, DNA was digested with MspI and resolved on a 1% agarose gel (see Figure 1). The gels were stained with ethidium bromide and photographed under uv light. Mass average and number average molecular weight, $\bar{M}_w$ and $\bar{M}_n$ (30), respectively, were calculated as described in Kunnath and Locker (31). The different values obtained in this study compared to those in our earlier study (31) reflect more limited sampling of intervals in the present study, especially in the low molecular weight range. To compensate for slight differences between the center and edges of agarose gels, all scans were normalized to the position of two repetitive DNA bands (5.10 and 0.42 kb) found in MspI digests of rat DNA (31). $\bar{M}_w$ of MspI digests was utilized to determine the extent of DNA degradation. Since the number of MspI

sites/genome is constant, differences in $\bar{M}_w$ reflect the amount of DNaseI digestion (see Figure 1). Felber et al. (25) analyzed the DNA length distribution in the electron microscope to obtain a similar parameter.

For standard hybridization analysis, 3 or 4 chromatin digest preparations were chosen. An ideal set consisted of DNA's with $\bar{M}_w$ values of 5.4 (undigested chromatin), 4.0 and 3.0 kb.

DNA hybridization. DNA digests were blotted to nitrocellulose (32) and hybridized in dextran sulfate (37) according to a protocol described in Kunnath and Locker (29). The hybridization mixture contained 50 ng/ml ^{32}P-CDNA amounting to 1 to 1.5 x 10^6 Cerenkov counts/min.

Analysis of hybridization results. Autoradiograms were scanned with a densitometer. Only bright hybridization bands were quantitated, because faint ones give non-linear results. A baseline was graphically fitted to the scan, and bands were cut out and weighed. We found about 20% variation in the amount of DNA loaded in each lane, due to the difficulty of pipetting viscous solutions, and to contamination with small molecules that are detected at 260 nm. To compensate, all gels were photographed before blotting, and the amount of DNA in each lane was densitometrically quantitated, by comparison with standards run in the same gel. When such standards are included within the gel, it is not necessary to compensate for variable ethidium bromide staining.

RESULTS

Analysis of DNaseI sensitivity. Purified adult liver nuclei were divided into several groups, and digested for 3 min at 24°C with different amounts of DNaseI in the presence of NP-40 (7). After treatment, these preparations were immediately deproteinated and the DNA was purified. The purified DNA was then treated with MspI and resolved on a gel. $\bar{M}_w$ and $\bar{M}_n$ (30,31) were calculated, and a series of DNA's, with values of $\bar{M}_w$ ranging from about 3.0 to 5.4, was chosen for further analysis. Sets of MspI digests were resolved on agarose electrophoretic gels, blotted to nitrocellulose, and hybridized to ^{32}P-cDNA. Figure 1 shows the results for adult liver chromatin hybridized to AFP cDNA. Fading of bands is a function of the amount of DNaseI digestion and of the molecular weight of the band. In the illustrated experiment, chromatin digests produced with 0, 50, and 60 µg/ml of DNaseI resulted in MspI digests with $\bar{M}_w$ equal to 5.4, 3.8, and 3.2 kb, respectively. We chose to use MspI digests for our analysis, because that enzyme cuts the genes into fragments of an

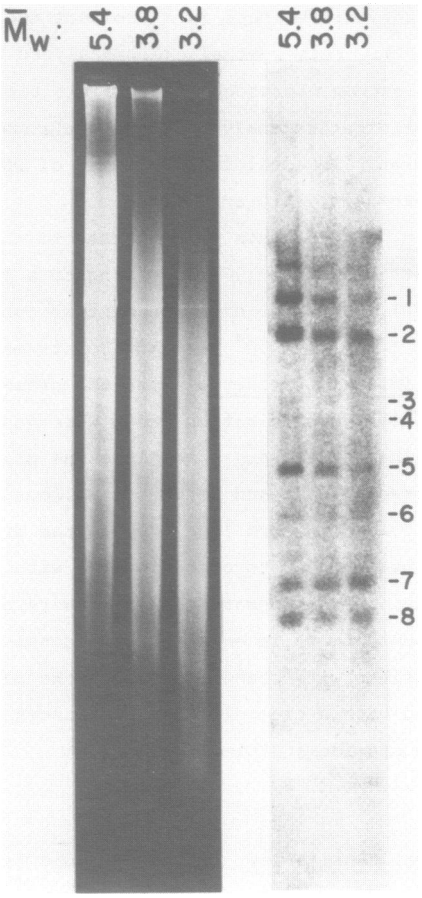

Figure 1. Method for analysis of DNaseI sensitivity. Hybridization with AFP gene probes to adult liver DNA digests is illustrated. The left panel show an ethidium-stained gel of MspI digests of DNA purified from chromatin. The digests were blotted and hybridized to a mixture of ^{32}P-labled pRAF65 and pRAF87 DNA (18) (shown on the right). DNA of $\bar{M}_w$ = 5.4 is from native chromatin, while the digests of $\bar{M}_w$ = 3.8 and 3.2 are from chromatin treated with DNaseI. Fading of bands is generally proportional to the $\bar{M}_w$ of the digest. The numbers on the right indicate the specific AFP hybridization bands (see below).

intermediate size (0.5 to 3 kb) that allows some fine structure mapping. We previously analyzed DNA methylation at HpaII/MspI sites (24).

A rate constant, k (defined as the slope of the plot of relative hybridization intensity, I/I_o, versus $\bar{M}_w$), was calculated for each band. Initial experiments used larger numbers of chromatin digests and we

observed that I/I_o varied linearly with $\bar{M}_w$, while log (I/I_o) varied linearly with $\bar{M}_n$. We prefer $\bar{M}_w$, since it is measured more accurately than $\bar{M}_n$ (31), and can be approximated by examination of the stained gel.

The linear relationship was enhanced when we decreased the DNaseI digestion time to 3 min, and when we corrected for the DNA concentration in each gel lane. Under our conditions, the relationships appear linear for hybridization bands from 0.5 to 3.0 kb in DNA from chromatin digests with $\bar{M}_w$ from 2.0 to 5.4. Since tissues contain more than one kind of cell, we expected to see curved plots, resulting from the simultaneous digestion of active and inactive chromatin. The simple linear behavior we generally observed can probably be accounted for by our preparative methods, which greatly enrich for large nuclei from easily broken cells. This was confirmed by microscopic inspection of nuclear preparations. The smaller stromal and hematopoietic cells remain largely unbroken and will not sediment through 1.8 M sucrose. It should be emphasized that linear behavior is not required for comparing chromatin sensitivity in different tissues.

Once we determined these conditions, we chose sets of 3 digests, to allow a large number of tissues to be analyzed. We established the reproducibility of this approach by comparing the hybridization of multiple sets of chromatin digests from different animals. A detailed description of the data has been presented by Kunnath (34).

Digests of purified DNA. To define the level of sensitivity of chromatin digests from different tissues, and also to investigate the intrinsic DNaseI sensitivity of each hybridization band, we digested purified high-molecular-weight DNA with DNaseI. This required about 1% of the DNaseI required for chromatin preparations to achieve similar digestion. When hybridization to digests of purified DNA was analyzed by densitometry, we found a linear relationship between hybridization intensity of each band and $\bar{M}_w$. Typical results, for four bands hybridizing to albumin cDNA, are shown in Figure 2. We consider a rate constant determined for purified DNA to be an intrinsic rate constant, k_d, which can be used as a value for chromatin analysis.

The effect of DNA sequence on the rate of degradation is substantial. We observed that k_d decreased with decreasing molecular weight, with considerable scatter around a simple linear relationship (analysis not illustrated). The scatter is presumed to represent intrinsic differences in sensitivity to DNaseI due to differences in DNA sequence. There was

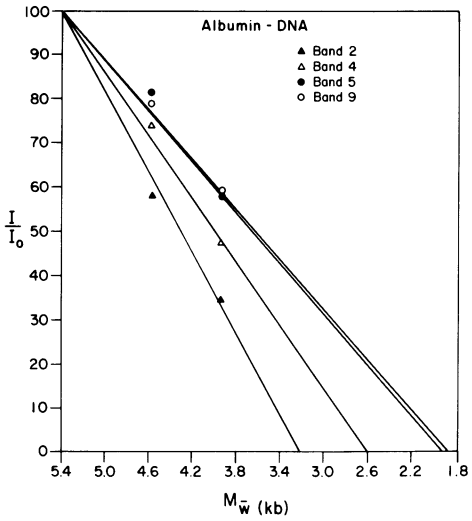

Figure 2. DNaseI degradation of albumin gene bands in purified DNA. Autoradiograms of hybridization of albumin cDNA to MspI digests of native and DNaseI degraded DNA were scanned with a densitometer. I is the intensity of a hybridization band, and I_o is the intensity of the same hybridization band in undergraded DNA. The raw values were corrected for the DNA concentration in each gel lane, plotted against M_w and fitted to a straight line by linear regression analysis. The figure illustrates analysis of 4 albumin hybridization bands (see Figure 3). A rate constant for each band, k, was measured as the slope of the line. Note that bands 5 (2.15 kb) and 9 (1.0 kb) have almost identical rate constants.

much more scatter for small DNA fragments, indicating that larger DNA segments sample more sequences and thus have more average rates of digestion. We calculated that the distribution of k_d for 1 kb fragments would have a standard deviation of ±32%, while for 3 kb fragments the standard deviation would be ±11%. These observations strongly indicate that the widespread practice of controlling for the hybridization of one DNA band with the hybridization of another, whether of the same or different size, may yield meaningless experimental results.

Chromatin DNaseI sensitivity. We analyzed albumin and AFP gene chromatin from adult liver, which synthesizes albumin but not AFP; from 18-day fetal liver, which synthesizes both; from 18-day yolk sac, which synthesizes AFP and low levels of albumin; and from kidney, which synthesizes neither. Figure 3 illustrates comparative hybridizations of albumin cDNA to adult liver, purified DNA, and adult kidney preparations. At various levels of digestion, band fading is much greater in the liver

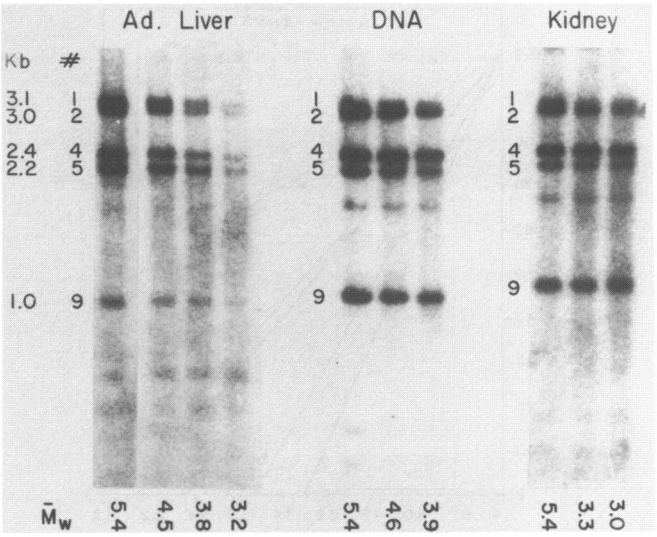

Figure 3. Albumin gene DNaseI sensitivity. DNaseI digests of chromatin from adult liver (left-hand panel) and kidney (right), and digests of purified DNA (middle), were blotted and hybridized as in Figure 1, to ^{32}P-DNA from a mixture of 3 albumin cDNA clones pRSA510, pRSA57, and pRSA13 (30,31). (The relative proportions of these plasmids were different in the liver digest hybridization than in the other two). DNA of $\bar{M}_w$ = 5.4 is from native chromatin. Judging from the amount of fading at comparable $\bar{M}_w$ values, the albumin gene has high sensitivity in adult liver chromatin, intermediate sensitivity in purified DNA, and low sensitivity in kidney chromatin.

preparations than in the others; yet all preparations were digested to similar average sizes. In liver chromatin, the unprotected albumin gene is cut much more than average. In kidney chromatin, the albumin gene is cut much less than average, since it is in an inactive, protected state. In purified DNA, no genes are protected, and thus all are degraded at the same average rate. Thus, rate constants are intermediate in purified DNA. Figures 4 and 5 illustrate some of these comparisons for the albumin and AFP genes. Figures 6 and 7 summarize the relative sensitivity of the albumin and AFP genes in different tissues. In these figures, the data are presented as sensitivity, that is, the ratio of the rate constant, k, of each band in chromatin to the rate constant, k_d, of the same band in purified DNA. The sensitivities are plotted in order across the HpaII/MspI restriction enzyme map of the gene. For most of the data points, determinations of sensitivity were carried out twice on different chromatin

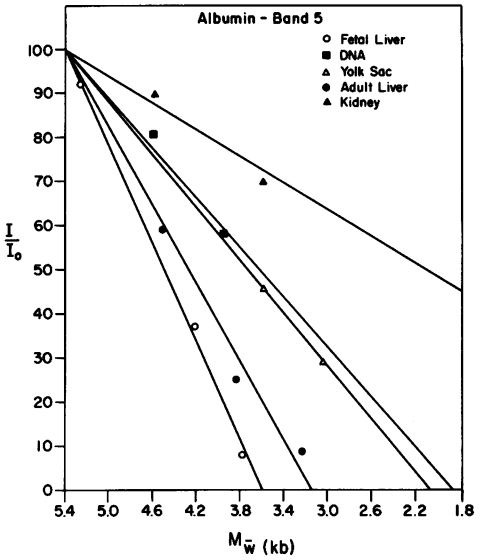

Figure 4. Representative albumin (band 5) chromatin degradation. Fitted linear plots of data sets for adult liver, 18-day fetal liver, 18-day yolk sac, adult kidney, and purified DNA are illustrated. Determination was carried out as described for Figure 2.

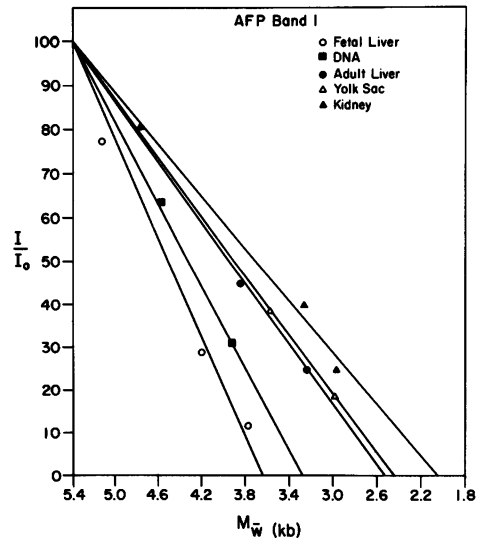

Figure 5. Representative AFP (band 1) chromatin degradation. Fitted linear plots of data sets for a typical AFP band are illustrated. Determination was carried out as described for Figure 2.

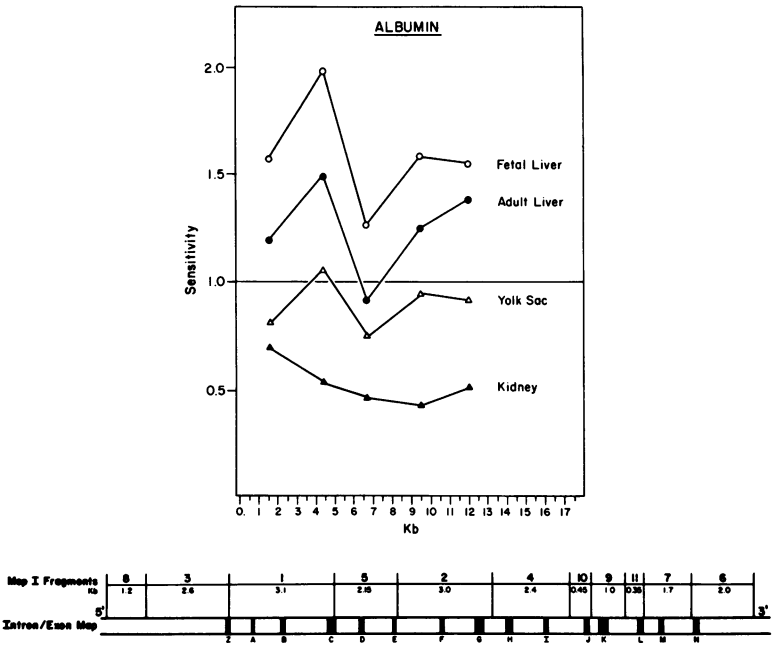

Figure 6. Albumin gene chromatin sensitivity. Sensitivity, k/k_d, is plotted for 5 hybridization bands within the albumin gene. Purified DNA has a defined sensitivity of 1.0. The points are plotted as the midpoints of the bands illustrated in the gene map (24) at the bottom of the figure. Determination for liver and purified DNA were carried out in duplicate on different digest sets, and averaged. Detailed data analysis is presented by Kunnath (34).

digest sets. The rate constants were found to be highly reproducible (34). The differences in sensitivity among tissues and bands are much greater than the variability of these measurements.

DISCUSSION

An Absolute Scale of Sensitivity. We defined sensitivity as the ratio k/k_d. Thus, the sensitivities of all purified DNA bands will be 1.0. In purified DNA, with correction for sequence effects, all fragments have an average sensitivity. In chromatin, the average sensitivity of all regions is probably not 1, since the percentage of sensitive genes is certainly much less than 50, and the amount of protection afforded by different chromatin structures may not be strictly proportional. Nevertheless, the sensitivity scale provides an absolute frame of reference. Average values of 0.6 or less seem to indicate inactive genes and values of 0.9 or greater

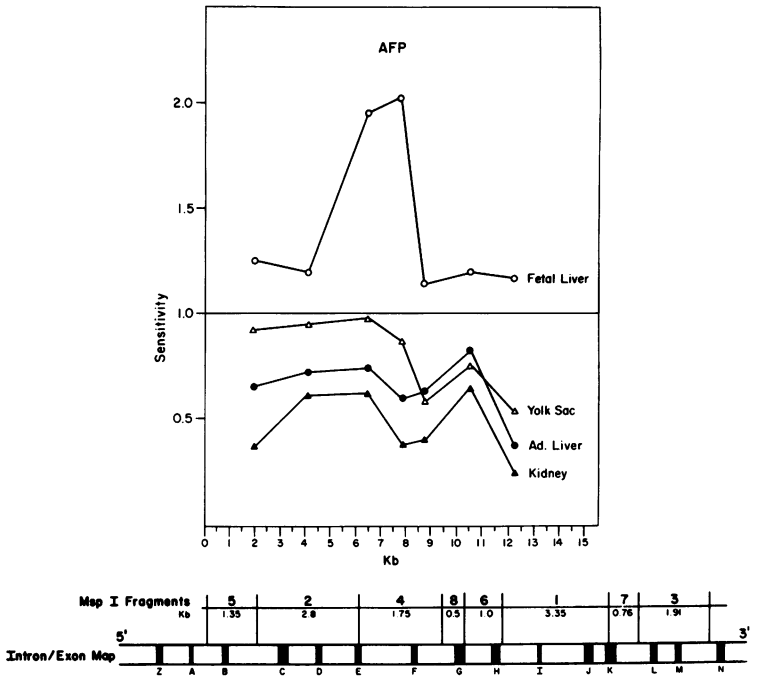

Figure 7. AFP gene chromatin sensitivity. AFP gene chromatin sensitivity is plotted as in Figure 6. The map of the HpaII/MspI sites with approximate exon locations is discussed in Kunnath and Locker (24). Determinations for liver were carried out in duplicate on different digest sets and averaged. Detailed data analysis is presented by Kunnath (34).

seem to indicate active genes. The range of values for active genes, from 0.9 to 2.0 may represent differing levels of activity, different proportions of sensitive genes in individual cells, or different numbers of active cells in various tissues. The intermediate range is difficult to interpret; it may indicate intermediate gene states, such as the yolk sac albumin gene and the adult liver AFP gene. In yolk sac, we have found a low but detectable level of albumin mRNA synthesis, about 1/80th of the level found in liver (22). In adult liver, the AFP gene is essentially inactive (22). However, the AFP gene is active in fetal liver, and some activity of this gene remains inducible in adult liver.

Fine structure of the Albumin and AFP genes. We measured sensitivity of 7 different DNA fragments in the AFP gene and 5 in the albumin gene. Both genes show a pattern of varying sensitivity of subregions, with a region of relatively lower sensitivity in the middle. The albumin genes of

all tissues except kidney show the same pattern but not the same level of sensitivity. The AFP genes of all tissues except fetal liver are similar to one another in their arrangement of higher and lower sensitivity regions.

The patterns of DNaseI sensitivity presumably represent features of chromatin organization in these two genes. In kidney, both genes show the lowest levels of chromatin sensitivity and the highest levels of DNA methylation, throughout their length. However, for the tissues in which expression of the genes is regulated, liver and yolk sac, the correlation between DNA methylation and chromatin sensitivity is unclear; e.g., fetal liver has higher chromatin sensitivity and DNA methylation of both genes than adult liver. There does, however, appear to be a further correlation with the pattern of DNA methylation. In adult liver, the inactivated AFP gene shows two regions of marked demethylation, at the MspI sites separating bands 8 and 6, and 7 and 3. Bands 6 (1.0 kb) and 7 (0.8 kb) have lower DNaseI sensitivity in all tissues that we analyzed, and band 8 (0.5 kb) shows a marked drop in methylation from fetal to adult liver, corresponding to a marked reduction in methylation during the same interval. Reduced sensitivity implies a chromatin structure that protects the DNA from nucleases better than in other parts of the gene. We have proposed (24) that demethylation results from a similar mechanism, where newly replicated DNA is protected from the action of methylases by bulky or tightly bound proteins.

Varying sensitivity along the length of these genes contrasts with uniform sensitivity along the length of the chicken β-globin gene, observed by Wood and Felsenfeld (35). However, the rat albumin and AFP genes are much larger than the 6 kb region surveyed by these investigators; the phenomena we analyzed may organize larger regions of chromatin. In their study, Wood and Felsenfeld found that a single fragment containing a hypersensitive region was about twice as sensitive as the rest of the gene. We did not localize any regions that fulfil the criteria for hypersensitivity, i.e., cleavage to produce new bands. This phenomenon requires that a localized region have significantly greater sensitivity than its surroundings. The albumin and AFP genes have "very" sensitive regions that are about as sensitive as hypersensitive regions, but more extended. Perhaps true hypersensitive regions are present as well, but not detected by our analysis.

Conclusions. The rate of chromatin digestion by DNaseI clearly

distinguishes transcriptionally active and inactive states of the albumin and AFP genes. By measuring sensitivity as a function of the amount of DNA degradation rather than the duration of enzyme digestion or the amount of enzyme added, and by normalizing rate constants to the intrinsic rate constant of each individual DNA band, we were able to distinguish intermediate states between activity and inactivity, and to construct detailed maps of regions of varying sensitivity along the lengths of genes. The regions of varying chromatin structure that we discerned are probably related to the ways in which these genes are controlled.

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