Volume 11 Number 11 1983 Nucleic Acids Research

Protection of expressed immunoglobulin genes against nuclease cleavage

Wolfgang O.Weischet, Boris O.Glotov[1] and Hans G.Zachau

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, München, FRG

Received 21 March 1983; Revised and Accepted 17 May 1983

ABSTRACT

Fragmentation of the actively transcribed kappa immunoglobulin gene in mouse myeloma nuclei with micrococcal nuclease and the restriction nuclease Bsp RI reveals a chromatin structure without the regularity of repeating nucleosomes found in bulk chromatin. Such regularity is restored about 2.2 kb 3' of the coding region. An only moderately increased micrococcal nuclease sensitivity and a 65% average protection of the Bsp RI sites indicates a DNA-protein interaction in the transcribed region which is not very different from that of an inactive gene. As determined by indirect endlabeling the frequency of Bsp RI cleavage both, after very mild and exhaustive digestion, varied moderately from site to site along the gene. In addition, it was not in each case the same at analogous sites on both alleles which are both transcribed. Thus, the experiments demonstrate differences between the chromatin structures of the genes which may be related to regulatory phenomena and thereby corroborate earlier findings made with DNAase I.

INTRODUCTION

There exists rather detailed knowledge on the structure of the bulk of the chromatin including its basic subunit, the nucleosome, and the various levels of higher order organization (for reviews cf. 3,4,5). In comparison little is known about the specific properties of that part of the chromatin which contains actively transcribed genes. Here much progress has been made by introducing DNAase I for probing for the enhanced sensitivity (6) and the hypersensitive sites (7,8) of active genes (4,5,9). Still, a considerable uncertainty remains when specific aspects of active genes are considered like, e.g., their organization in nucleosomes or their sensitivity towards other nucleases like micrococcal nuclease and restriction nucleases. This holds true even if one disregards multi-copy genes which, due to hetero-

geneous activity, may be prone to experimental artifacts. Part of the uncertainty results from the fact that, e.g., digestion studies using a single nuclease may not allow a discrimination among several alternative hypotheses (8). In addition, since different nucleases may recognize different features of a chromatin structure (10,11) there is a strong rationale for studying a model gene with several nucleases in parallel.

In a previous study from our laboratory (12) the restriction nuclease Bsp RI has been used which, like other restriction nucleases (13,14,15), has a strong preference for cleaving such recognition sites (GGCC) in bulk chromatin which appear in a nucleosomal spacer. Unlike micrococcal nuclease this enzyme was found to degrade a transcriptionally active gene only to a limited extent (12) leaving intact also fragments containing more than a dozen potential cleavage sites. Such obvious protection prompted us to quantitate the extent of cleavage achievable under conditions of exhaustive digestion and, by applying indirect endlabeling, to monitor the cleavage at the various sites independently.

The transcriptionally active gene studied is a single copy gene coding for an immunoglobulin kappa light chain. Cells which synthesize such proteins show a characteristic rearrangement of, at least, one allele of the corresponding DNA as compared to the germ line configuration (16,17). In case of the mouse myeloma studied here ("tumor T", (18,19)) both alleles have been rearranged in different ways such that their V-J segments (2) differ in length and sequence (cf.Fig.1). This provides the basis for the experimental distinction between the alleles which is a major subject of this report. Both alleles are transcribed in the tissue we use (unpublished results) but only one allele ("T1") is fully translated ("allelic exclusion"; review 20). The other allele ("T2") has undergone an aberrant rearrangement which causes a premature termination of translation (21).

The present study on the structure of the transcriptionally active immunoglobulin genes T1 and T2 complements reports on the inactive genes (12,22). In addition, we have recently shown that the two alleles in myeloma T are different with respect to their sensitivity towards DNAase I (23).

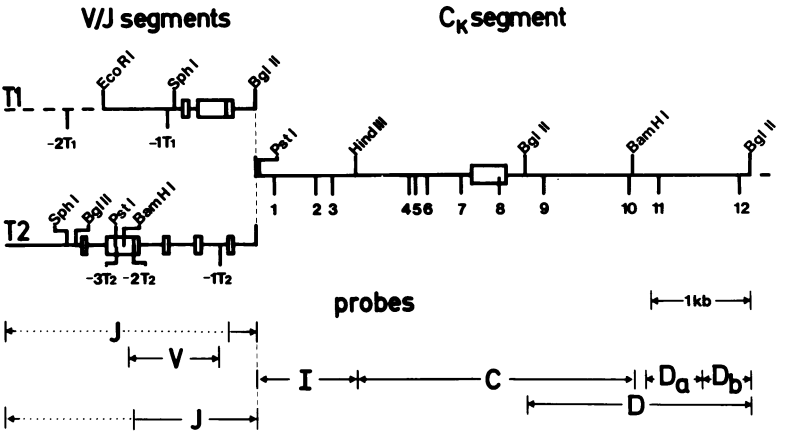


Figure 1: Map of the kappa light chain genes in myeloma T, as compiled from refs. 19, 21, 24, 25. Sequenced regions are marked by the continuous horizontal lines; at the left end the DNA sequence differs between both alleles, T1 and T2. The boxes indicate (from left to right) the coding sequences for leaders, joint V+J segments (2) plus additional J segments in T2, and C region. The numbers refer to recognition sites of the restriction enzyme Bsp RI, the other restriction sites shown are relevant for the indirect endlabeling experiments reported here. The DNA probes J, I etc. are described in Materials and Methods. The bottom scheme indicates their hybridizing and non-hybridizing parts (full and dotted lines) relative to the maps of T1 and T2 above.

MATERIALS AND METHODS

Myeloma T (19), continuously propagated in BALB/c mice by subcutaneous implantation, was removed after 3 weeks of growth. Nuclei were isolated from homogeneous tissue as described previously (23). 70-100 A_{260} units of nuclei were digested in buffer A (26) containing 0.34 M sucrose and 0.5 mM PMSF with Bsp RI (50-40 000 u/ml, 30 min at 37°C; +7 mM $MgCl_2$,+1 mM EGTA) or micrococcal nuclease (Worthington; 2-320 u/ml, 5 min, 37°C; +1 mM $CaCl_2$). Controls with purified DNA were digested in the same buffers. Conditions for indirect endlabeling (27,28), gel electrophoresis, Southern transfer (29), nick-translation, hybridization and autoradiography have been summarized before (23). All DNA probes are described elsewhere (23,25) except for D_a and D_b which are Taq I-Taq I and Taq I-Bgl II subclones, respectively, of D, cloned in pBR 322. (Previous nomenclature (23) for J,V,I,C: J1/1, L6/2,

L1/1, C1, respectively.) Note that probe V (kindly provided by H. Schnell) contains the leader and part of the V gene segment (2) of allele T1 (30) and therefore hybridizes also with the non-rearranged allele of this V segment but not with T2. Probe J contains a 2 kb insert of the J region in the germ-line configuration (23). It hybridizes therefore only with its 3' end to a 0.3 kb or 1.2 kb region on T1 or T2, respectively. Marker fragments for gel electrophoresis consist of a mixture of restriction fragments (kindly provided by J. Höchtl) containing fragments and ligation products of pBR 322.

RESULTS

Lack of a regular nucleosomal repeating pattern on the transcriptionally active kappa immunoglobulin genes. In order to assay for a nucleosomal repeat we treated myeloma nuclei with various concentrations of micrococcal nuclease and probed among the DNA fragments with cloned DNA from parts of the C gene region (probes J, I, C; Fig.1). In no case did we find a clear nucleosomal ladder on autoradiographs like with the transcriptionally inactive gene in liver nuclei (22) although the total ethidium bromide stained material shows identical repeats in both tissues (data not shown). Instead, the hybridizing fragments had a continuous size distribution which shifted to smaller sizes with increasing extent of digestion (Fig.2A). With probes I and C even the fragments after extensive digestion had an almost homogeneous size distribution with only a faint indication of a few superimposed bands which apparently did not reflect a nucleosomal repeat. At present we do not know whether or not they correspond to some more stable particulate intermediates in digestion. Only with probe J did we find a faint nucleosomal ladder in samples after extended micrococcal cleavage (not shown). However, this probe hybridizes not only with the two transcribed alleles of the kappa light chain gene but also with the fT-fragment (31,32). Since this fT-fragment need not exist in a typical transcriptionally competent structure along with the genes themselves (cf. 33) it may give rise to a nucleosomal ladder like any inactive chromatin domain.

By using DNA probes from the 3' side of the C gene segment

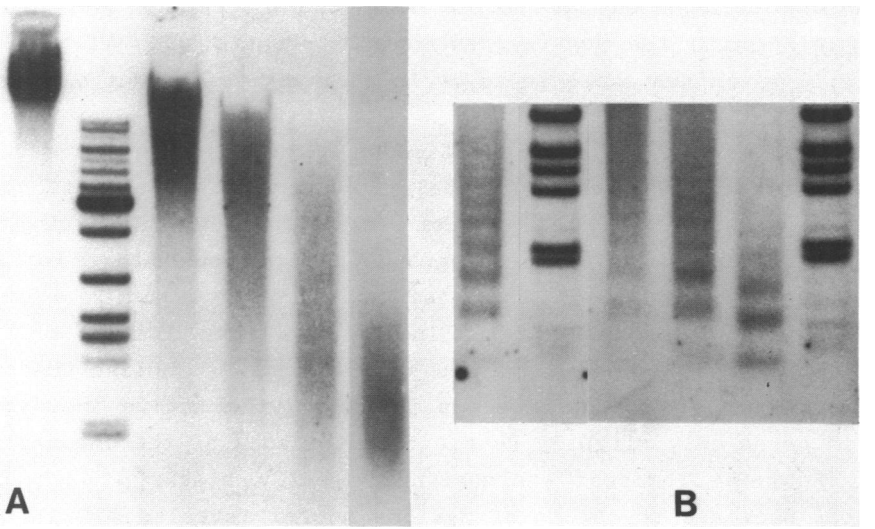


Figure 2: Fragmentation by micrococcal nuclease of the kappa light chain gene in nuclei of myeloma T. **A**: Autoradiograph after hybridization with probe C. Digestion with 1.6, 4.5, 10, 26, 60 u nuclease/ml. **B**: Hybridization with probe D_b; (enlarged lower end of an autoradiograph). Two nuclear preparations are shown after digestion with 20, and 8, 20, 100 u nuclease/ml (from left to right). Sizes of the marker (M) restriction fragments (from bottom to top): 0.62, 0.68, 1.13, 1.34, 1.57, 2.12, 3.24, 4.36 (very strong), 5.2, 6.5, 7.8, 9.1, 12.2, 15, 20 kb in A; in B sizes from 0.62 to 2.12 kb as above.

we searched for the appearance of the normal nucleosomal repeating pattern. Probe D produced a continuum of fragment sizes with faint superimposed bands after extended digestion but its subclone D_b (cf.Fig.1) yielded a clear nucleosomal ladder (Fig.2B) with a somewhat higher background than expected from totally inactive chromatin. This ladder is most likely not due to an experimental artifact because probe D_b showed only a weak tendency to cross-hybridization yielding more than 80% of the total signal in the two expected bands when hybridized to a Bsp digest of myeloma DNA. Subclone D_a (Fig.1) revealed only traces of nucleosomal material (not shown). Evidently there is a transition to a regular nucleosomal array at the very end of the range covered by probe D, i.e. some 2.2 kb downstream of the immunoglobulin gene.

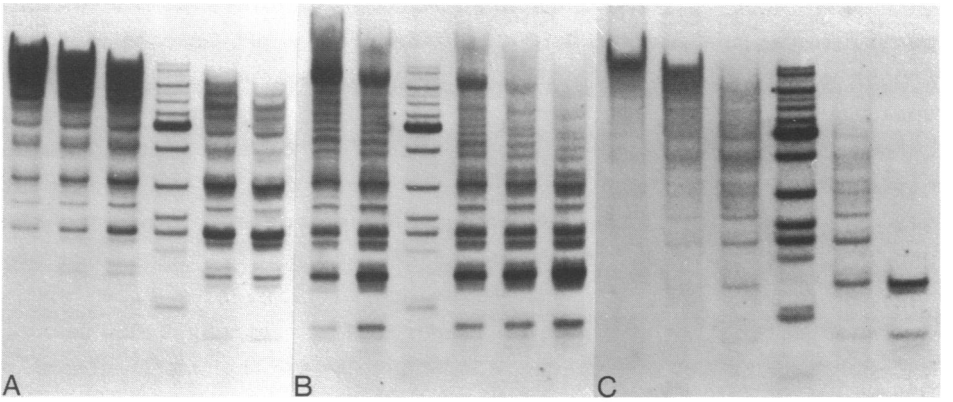
A similar search at the other ends of both alleles is not

feasible because any DNA probe from the 5' flanks must cross-hybridize with the non-rearranged counterparts of V-T1 or V-T2 (Fig.1) which are expected to be in an inactive chromatin structure (33).

Despite the apparent continuous size distribution of the fragments produced by micrococcal nuclease the transcriptionally active genes are not cleaved like protein-free DNA. Comparing the fragment patterns on autoradiographs (at various stages of digestion of the nuclei) to those produced by ethidium bromide staining of the same material indicates a very similar distribution of mass between large and small fragments. Unlike DNAase I (23) micrococcal nuclease seems to cleave the active immunoglobulin genes only slightly faster than the bulk of the chromatin.

Applying indirect endlabeling to early stages of a micrococcal nuclease digestion of myeloma nuclei revealed a multitude of preferred cleavage sites; they were mostly identical with the recognition sequences for this enzyme (34) and therefore were also found in digests of free DNA (23). Possible differences in their accessibility between chromatin and DNA which may bear on structural aspects were not evaluated because of the intrinsic complexity of such analyses (35,36) and their interpretations (37,38).

Direct analysis of the Bsp RI fragment patterns from myeloma nuclei. The course of fragmentation of the kappa chain genes by varied amounts of Bsp RI provides some clues on the chromatin structure of these genes in an active state. A comparison reveals obvious differences between the patterns from myeloma nuclei and those from the corresponding protein free DNA or the transcriptionally inactive genes in liver nuclei (Fig.3A-C; cf.also ref. 12; analogous observations were made with probe I). The band patterns after exhaustive digestion of the two types of nuclei reveal more mass in the large bands (> 2 kb) from liver than from myeloma indicating a higher average cleaving efficiency at the Bsp sites in the tumor gene. Still, stable fragments up to about 9 kb (extending across about 15 Bsp sites) are found in myeloma digests also. Therefore, the cutting efficiency can differ only moderately from that in liver in which a regular nucleosomal cover of the immunoglobulin gene was found (12,22).



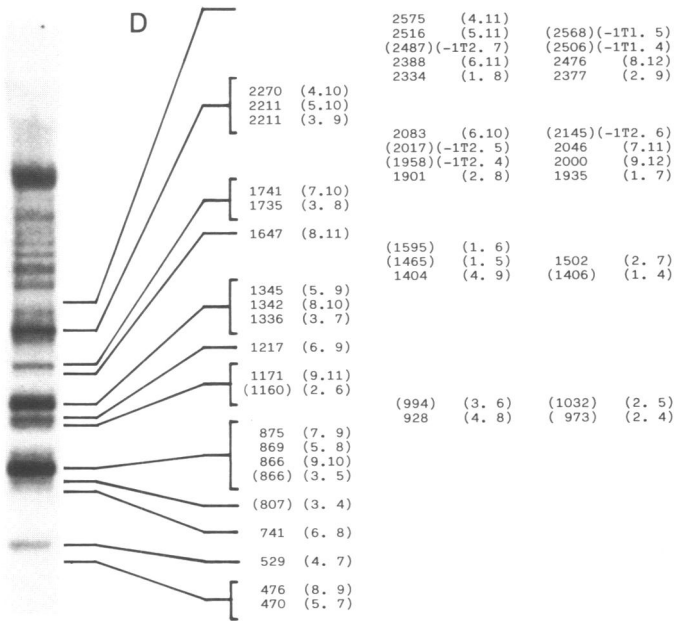


Figure 3: Band patterns after digestions of liver nuclei (A), myeloma nuclei (B), and myeloma DNA (C) with increasing amounts of Bsp RI. Hybridization with probe C. For marker sizes cf. Fig. 2. Nuclei were digested under standard conditions with 500, 1500, 5000, 15 000, 50 000 u Bsp RI/ml (**A**) and 50, 250, 1250, 6200, 40 000 u Bsp RI/ml (**B**). (**C**): 50 µg DNA digested in 40 µl with 1, 1.8, 3.6, 5.6, 14.8 u Bsp RI, resp.; 10 min, 37°C. **D**: Enlarged center track from B with listing of the contributing fragments; given are the fragment length (in bp) and the Bsp sites at both ends (designations as in Fig.1). The left column contains the identifiable main contributor; minor components are listed in the two columns at the right side. Fragments which do not fully hybridize with probe C are given in parentheses.

Among the myeloma fragments the smaller sizes dominate; in particular, the composite 0.87 kb band (Fig.3D) is a major component at all stages of digestion and, to a lesser extent, this applies to the 0.53 kb fragment, too. This is a crucial finding because fragments of such lengths cannot be cleaved from a chromatin region containing normal nucleosomes with a 200 bp repeat (22). Accordingly, these bands are under-represented among the Bsp fragments produced from the transcriptionally inactive immunoglobulin gene segments from liver nuclei (Fig.3A). Vice versa, as reasoned in the accompanying paper (22), a strong 1.35 kb band is expected to be produced predominantely if the immunoglobulin gene is packed into nucleosomes. Thus, these observed differences in band intensities indicate that the transcriptionally active immunoglobulin genes are free of, at least, a nucleosomal structure with a regularity like in bulk chromatin (see Discussion).

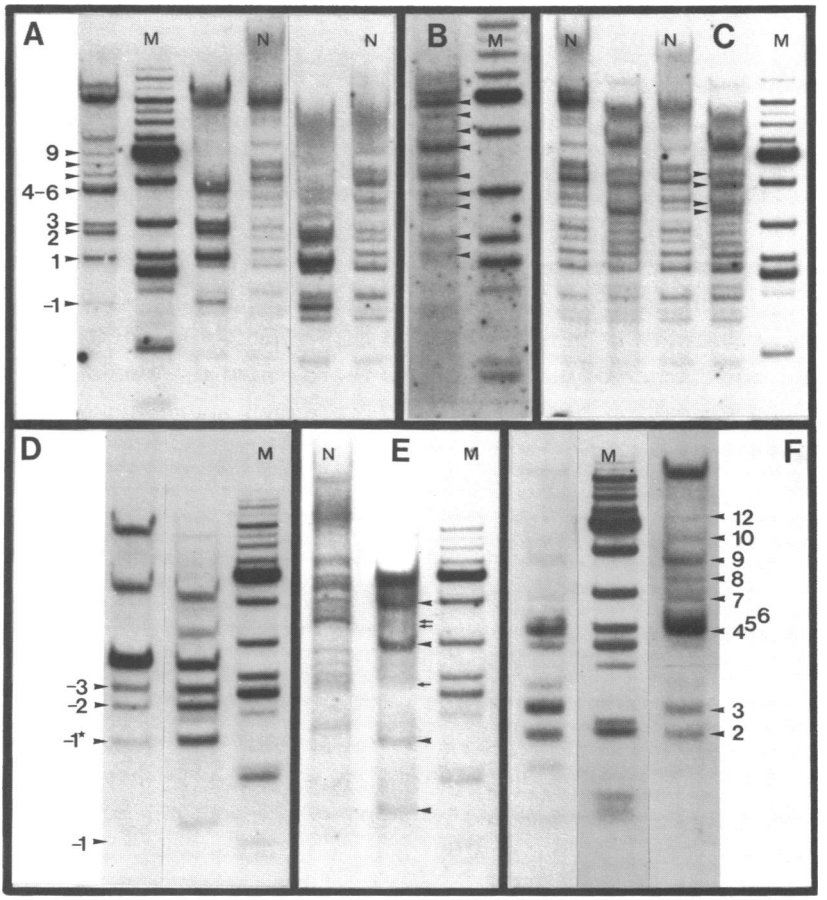
A comparison between the digests of myeloma nuclei and those of the corresponding free DNA shows that the band patterns in the first case do not simply resemble intermediate stages of the DNA digests as would be expected if the active genes were evenly covered with proteins. The differences are related to an uneven cleavage observed at the various Bsp sites by indirect endlabeling (see below). For instance, the strong bands around 1.17, 1.22 and 2.2 kb (Fig.3B) are produced by cuts at some preferred chromatin cleavage sites. It should be noted that the relative dominance of these bands exists from early through late stages of the digestion which means that an increased susceptibility of these sites to initial cuts leads to a higher total cleavage after exhaustive digestion. This may argue against a protein redistribution during the course of digestion with Bsp RI.

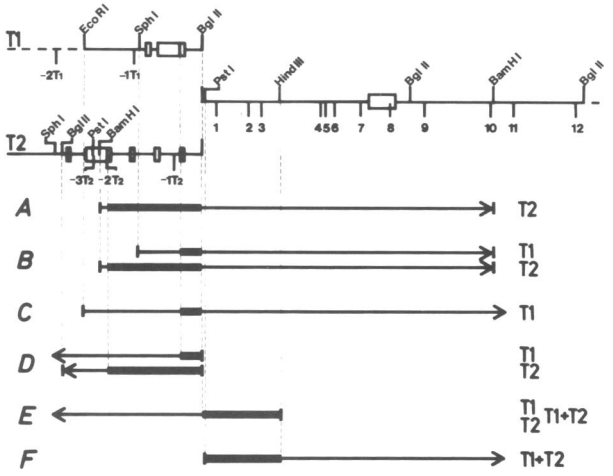
Cleavage of Bsp RI sites in myeloma nuclei as monitored by indirect endlabeling. Instead of using micrococcal nuclease like in many earlier reports we evaluated the cleavage of the strictly defined recognition sites for the restriction nuclease Bsp RI within the two alleles of the kappa chain genes. This has several advantages: For one, under rigorous conditions micrococcal nuclease will entirely degrade the chromatin region under investigation (Fig.2) while even an exhaustive treatment with the restriction nuclease Bsp RI was shown to cleave each site only in a

small fraction of the nuclei at a time (Fig.3B). This allows a quantitation of the residual protection at well defined locations within the genome (see below). Secondly, with the small number of already well mapped potential cleavage sites present the endlabeling data can be directly analyzed with respect to the intensity of cleavage. Even bands from sites within the DNA region which hybridizes to the probe will not much complicate the analysis. Therefore, one can afford choosing rather long probes which yield a strong signal on autoradiographs and thus allow a monitoring of "single cutting events". This enables us to distinguish between the "accessibility" of a site to initial cleavage and its extent of digestion or "cleavage efficiency" shown after exhaustive digestion.

We have previously reported on significant differences with respect to the chromatin structure of the alleles (23). Therefore, we particularly designed indirect endlabeling experiments which allow monitoring the cleavage of the Bsp sites on both alleles separately. There are three different ways of doing so: First, by using a specific probe, like probe V (Fig.1), which hybridizes with allele T1 only; this has been done here in one case (cf. Fig.5). Secondly, by employing for the endlabeling a restriction nuclease which cleaves at widely separated locations near the 5' ends of the physical maps of T1 and T2, respectively. We used Bam HI, Eco RI, and Sph I for this purpose. For example, Bam HI cleaves within the V segment of T2 (Fig.4, bottom panel) generating a 5.6 kb fragment which contains the C gene segment also. On allele T1 the corresponding upstream Bam site is located about 5 kb outside the left end of the map in Fig.1. Accordingly, if the endlabeling is performed with Bam HI and probe J (Fig.4A) only such Bam-Bsp fragments from T1 will show up on autoradiographs which are larger than 8 kb (terminating at the upstream site) or larger than 4 kb (extending between any Bsp site and the Bam site 3' of the C segment). All endlabeled bands smaller than 4 kb must thus originate from allele T2.

The third method of monitoring the Bsp sites on both alleles separately utilizes their different coordinates in the translocated parts of the genes. For instance, after endlabeling with Bgl II and probe J one should be able to identify the Bsp sites -1 to


A
M
N
N
9
4-6
3
2
1
-1
B
M
N
N
C
M
D
M
-3
-2
-1*
-1
N
E
M
M
F
12
10
9
8
7
4 5 6
3
2


T1
Eco RI
Sph I
Bgl II
-2T1
-1T1
Pst I
Hind III
Bgl II
BamH I
Bgl II
1
2 3
4 5 6
7
8
9
10
11
12
T2
Sph I
Bgl II
Pst I
BamH I
-3T2
-2T2
-1T2
A
T2
B
T1
T2
C
T1
D
T1
T2
E
T1
T2
T1+T2
F
T1+T2

-3(T2) and -1 and -2(T1) by their different distances from the reference restriction site (Fig.4D).

In addition to these types of experiments we performed one kind of indirect endlabeling which doesnot yield allele-specific information (using Pst I and probe I; Fig.4F).

Representative autoradiographs of several experiments are shown in Fig.4; the strategies of endlabeling are specified in the correspondingly labeled sections of the bottom panel.

In the following we will comment on additional details of the experiments: A specific monitoring of the sites on allele T1 was possible by Eco RI restriction and hybridization with probe J (Fig.4C). Here the same argument applies as mentioned before for Bam HI because the Eco RI site on T2 is removed from its counterpart on T1 by about 5 kb (to the left in Fig.1). As long as fragments smaller than 7.7 kb are regarded they are either Bsp-Bsp fragments (from either allele) or Eco RI-Bsp fragments from T1 which hybridize rather weakly with this probe. Both kinds of fragments can be distinguished from each other by use of a non-redigested control. An independent evaluation of each allele was also possible by a combined Bam HI +Sph I redigestion and hybridization with probe I (Fig.4B). In this experiment Bam HI works as the reference restriction site on allele T2 and SphI on T1. This way, even Bsp sites located in the region of the common sequence

Figure 4: Indirect endlabeling of Bsp RI digests of myeloma nuclei. Panels A-F: Autoradiographs after digestion with the following Bsp RI concentrations (in u/ml, from left to right, resp.): A:50, 1250, 40 000; B:50; C:1250, 6300; D:50, 40 000; E:50; F: 6000, 50. Not redigested controls are marked by N; all other tracks contain endlabeled material as specified below. For sizes of the marker fragments (M) cf. Fig.2. Endlabeled fragments are marked by arrow-heads and, space allowing, by the Bsp site at which they originate (designations as in Fig.1). Corresponding sites in panels B, C, E are listed below, proceeding from bottom to top. **A**: Bam HI/J, T2 spec.; **B**: SphI plus Bam HI/I; sites: 2(T1), 3(T1), 2(T2), 3(T2), 4+5+6(T1), 4+5+6(T2), 8(T1)+7 (T2), 9(T1)+8(T2), 9(T2); **C**: Eco RI/J, T1 spec.; sites: 2, 3, 4+5, 6+7; **D**: Bgl II/J; sites: -1*: -1(T1), other sites on T2; **E**: Hind III/I; sites: 2, 1,-1(T1),-2(T1); nondetectable sites (small arrows): -1(T2),-2(T2),-3(T2); **F**: PstI/I. Bottom panel: Schematic representation of the endlabeling conditions of experiments A-F above: Location of reference restriction site (bar) and overlap with the probe used (heavy line); allele-specificity of the detectable Bsp sites (allele T1 or T2), indiscriminatory results: T1+ T2.

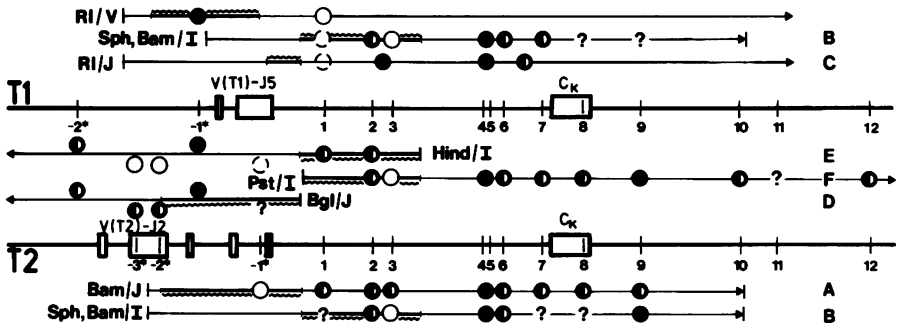


Figure 5: Results of the indirect endlabeling experiments. The center part shows maps of alleles T1 and T2 and the locations of the Bsp sites (numbers) and gene segments (cf. Fig.1). Horizontal lines above and below represent individual experiments by their reference restriction site (vertical bars) and direction of probing (arrow-heads) as defined by the DNA probe used (wavy lines). Labels A-F refer to panels A-F in Fig.4. Full and open circles indicate locations and average cutting frequencies of Bsp cleavage as determined from the observed intensities of endlabeled bands, corrected for imperfect hybridization, if neccessary (●,◐, o,◌,?: strong, normal, weak, very weak or undetectable cleavage, undecisive result, resp.). The top three and the two bottom experiments provide specific information on alleles T1 and T2, respectively. The three experiments in the center yield specific information for T1 and T2 (symbols above and underneath line) or indiscriminatory information with respect to the allele involved (symbols on line).

downstream of the J segments yield fragments of different lenghts from T1 and T2; the T1 specific ones are shorter by about 450 bp than their counterparts from T2. Since probe I hybridizes equally well with the fragments from T1 and T2 this experiment provides a direct comparison between both alleles with respect to the accessibilities at corresponding sites.

Analyzing early stages of the nuclear Bsp digests ("accessibility") yielded the results summarized in Fig.5 and Table 1. Clearly, the sites are not all identically susceptible to an initial nuclease attack. On both alleles sites (4+5) are particularly well accessible and site 3 is less accessible than most of the other sites. In none of the experiments presented a clear separate information was gained about sites 4 and 5. The most likely interpretation from the broad but inhomogeneous joint band in the Pst/I experiment (Fig.4F) indicates an increased accessi-

D N A				CHROMATIN		
L	MYELOMA T1	T2	SITE	L	MYELOMA T1	T2
++	+	+	-1*	++	++	-
+	-		1	+	--	+
+	+		2	+	+	+
-	-		3	-	-	-
+	+		4	++	++	++
+	+		5	++	+	+
+	+		6	-	+	+
+	+		7	+	+	+
+	+		8	+	+	+
+	+		9	+	+	++
+	+		10	++	+	+
+	?		11	-	?	?
+	+		12	+	+	+
			13	+		

Table 1: Comparison of the accessibility of the various Bsp sites as determined in active and inactive chromatin (myeloma and liver (L) nuclei, resp.) and in the corresponding DNAs. Site designations as in Figs. 1 and 5. Site -1 assumes different locations on either allele T1 or allele T2 and liver genome. Data were compiled from Fig.5 and the corresponding table in the accompanying paper (22). **Symbols:** ++,+, -,--,?: very strong, strong, weak, very weak cleavage, ambiguous data.

bility at site 4 and a normal cleavage at site 5. This is also consistent with the results of the digestions with Bsp alone as mentioned above.

The differences in the accessibility of the Bsp sites may have several sources. In order to exclude effects which reside in the DNA sequence we ran control experiments with purified DNA. It was isolated from myeloma nuclei, treated with Bsp to various extents, and subjected to the indirect endlabeling like material from chromatin digests (Fig.6). The results are also summarized in Table 1. As with liver DNA (22) all sites are almost evenly attacked by Bsp RI; only site 3 and to a lesser extent site 1 show a reduced accessibility.

A comparison with respect to the Bsp accessibility between the chromatin and the DNA digestion data reveals a few changes only which may be caused by the chromatin structure. In nuclei site 4 (on both alleles) and site -1(T1) are major sites of nuclease attack. In contrast, site -1(T2) appears particularly protected.

In addition, we found some interesting differences between the alleles in the regions of the identical DNA sequences which must be caused by differences in the chromatin structure. For instance, sites 1 and 9 are considerably more accessible on allele T2 than on T1. Note that these are local alterations only because the similar intensities of the corresponding bands in the (Bam HI

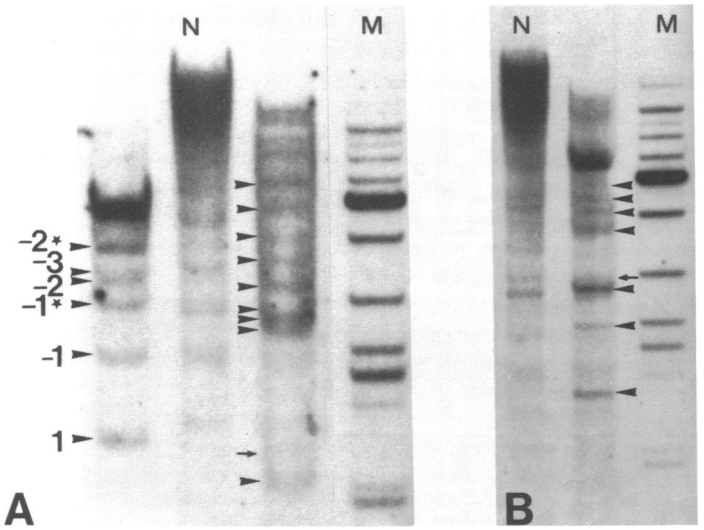


Figure 6: Indirect endlabeling applied to Bsp digests of protein-free DNA from myeloma; lettering as in Fig.4. **A**: Digestion: 150 µg DNA in 240 µl; 1.8 u Bsp RI; 10 min, 37°C. Experiment: track 1 (left of N): Hind III/I; (*): sites on allele T1, other sites on T2; track 3: Pst I/I: sites 2, 4 to 9, 10+11, 12; site 3 not detectable (small arrow). **B**: Digestion : 200 µg DNA in 120 µl; 2.5 u Bsp RI; 10 min, 37°C. Experiment: Bam HI/J (T2 spec.); sites: -1, 1, 2, 4+5, 6, 7, 8; site 3 not detected (small arrow).

+Sph I)/I experiment (Fig.4B) prove a similar accessibility of both alleles in general. As another local peculiarity allele T1 was found much more accessible at site -1 than allele T2 at any site in the V-J region (Fig.4E).

A determination of the cleavage frequencies from the band intensities after indirect endlabeling is straight-forward only in cases of initial cleavage. However, as an approximation it may also be applicable to any stage of a substantially incomplete digestion, like late stages of the nuclear chromatin digests (see Discussion in the accompanying paper). In order to estimate the maximum achievable cleavage at any Bsp site in myeloma nuclei ("cleavage efficiency") we subjected material from exhaustive Bsp digests to the endlabeling procedure. In the Bam/J experiments endlabeled fragments still stretching across 5 Bsp sites (to site 4) were resolved (Fig.4A). From the decrease in band intensity to about 1/10 from fragment (Bam- Bsp(-1)) to fragment (Bam- Bsp(4))

one estimates a minimal average protection at the intervening sites of more than 50% (cf. Discussion in the following paper (22)). Analogously, from the Pst I/I experiment (endlabeled bands using sites 2 to 7; Fig.4F) we calculated an average protection of at least 65-70%. The actual protection must be somewhat higher than suggested by these figures because this estimate basing on the remaining intensities of the endlabeled bands disregards the small fraction of material included in the Bsp-Bsp bands. Although crudely estimated such a high protection is compatible with the results of the Bsp band patterns after exhaustive digestion. Moreover, strong cleavage sites (-1(T1), 4) which, at an average, may be cleaved twice as often as normal sites do not cut off the patterns of endlabeled fragments (Fig.4F). Thus, even they must be protected to some extent (e.g. 30-40%, as resulting from the calculation above). Regions behaving like free DNA could not be detected around the kappa light chain gene.

DISCUSSION

The lack of bands after fragmentation of the transcribed kappa chain gene by micrococcal nuclease suggests the absence of a normal nucleosomal organization on the gene itself and its immediate surroundings. But the DNA is not free of protein as clearly indicated by the rate of the degradation by micrococcal nuclease which is similar to that of bulk chromatin. More important the Bsp RI restriction sites within the genes appeared well protected even against a high excess of enzyme added. From the indirect endlabeling experiments we estimated that under exhaustive conditions these sites can be cleaved to 35%, at an average. This is also consistent with the apparent stability of very long ($\gg$ 2 kb) fragments in nuclear Bsp digests (Fig.3B). The low extent of cleavage is not fundamentally different from that of the inactive gene as determined in liver nuclei (35% vs. 15-20% (22); cf. also Fig.3B vs.3A). Therefore, it appears inappropriate characterizing the two states of the gene in terms of "open" and "tightly packed".

There exists no indication that the observed high resistance of the immunoglobulin genes in myeloma towards Bsp RI might be caused by an experimental artifact like a heterogeneous nuclear

preparation containing a low amount of transcribed and highly sensitive genes while the major part is not transcribed and as resistant as, e.g., the genes in liver nuclei. For one, restriction analysis confirms the typical gene rearrangement in virtually 100% of the nuclei. Moreover, stable genes should contribute nucleosomal bands after micrococcal nuclease digestion and major amounts of very long ($\geq$ 4kb) Bsp fragments (cf. Fig.3A) both of which were not observed (Fig.2A, 3B).

The other conclusion from the Bsp digests of myeloma nuclei, besides the remarkable protection, concerns the absence of a nucleosomal regularity on the active immunoglobulin gene. The even intensity distribution of the Bsp fragments from myeloma (Fig.3B), as opposed to the more structured patterns of liver with its nucleosomal organization (22), has the same diagnostic value as the absence or presence of a nucleosomal ladder after micrococcal nuclease digestion.

The combination of Bsp and micrococcal nuclease digestion data provides a new basis for reconsidering structural models for active genes. As discussed before (8) active nucleosomes might have an increased nuclease sensitivity within the core DNA causing the disappearance of the typical regularly spaced bands after micrococcal nuclease digestion. If this were the case here the cleavage within core and spacer DNA must be similarly efficient since in our experiments a continuum of fragment sizes was observed (Fig.2A). Consequently, in comparison to inactive genes one should expect a considerably faster degradation of active chromatin by micrococcal nuclease and, analogously, a small residual protection of the Bsp sites. This is not in accord with our data. By a similar argument it appears unlikely that active nucleosomes should differ from inactive ones only by an extended spacer region. This would to some degree retain an alteration between accessible and protected segments on the DNA and, thus, micrococcal nuclease should still produce (ill resolved) bands.

At face value our data seem to indicate a complete absence of nucleosomes on the active immunoglobulin genes. One may think of a homogeneous protein cover providing an average 65% protection of the Bsp sites and also accounting for the observed variation in the accessibility of the sites by, e.g., a certain

sequence preference of some components. However, the transition between such a cover and an adjacent regular nucleosomal array must represent a major discontinuity of the chromatin structure which should be sensed by one of the endonucleases used here. This was not the case, though: According to our data (Fig.2) the transition should occur within a few nucleosomes around Bsp site 12 which is no different from other Bsp sites. Micrococcal nuclease shows no conspicuously frequent cleavage there either and the only DNAase I hypersensitive site in this region is located 1.5 kb further upstream (23). Apparently, the change from the typical active chromatin to a regular nucleosomal repeat is not a very dramatic one. This conclusion complements the one of Wood and Felsenfeld (11) which states that active and inactive chromatin are not too different in structure.

As an alternative a chromatin structure with nucleosomes but, at the moment of analysis, without the normal rather regular spacing between them may well explain the absence of a nucleosomal ladder and the analogous Bsp data. Since such a highly irregular structure need not have the same number of cores per unit length as inactive chromatin it may also provide for the somewhat faster rate of degradation and the reduced protection against Bsp RI. However, even such chromatin should yield discrete particles after digestion with micrococcal nuclease which should be observable as the bands corresponding to core particles and probably some kinds of small oligomers or compact oligomers (39). In our experiments (Fig.2A) no such bands have been observed, though, and this fact seems compatible with findings on the ovalbumin gene and the Drosophila heat-shock genes (36, 40). But a reliable decision in favor of, or against, this model must await a better characterization of the final cleavage products of the kappa chain genes by refined techniques because, at present, only the larger ones can be expected to be resolved by the blot-hybridization used here.

When we investigated the accessibility of the various Bsp sites in the inactive immunoglobulin gene in liver nuclei (22) we found some moderate differences from site to site which could be explained by, e.g., slight deviations from a completely random nucleosome positioning. Such modulations of an average access-

ibility have been found on the active gene as well; some of them are the same as in liver, e.g., the strong cutting at site 4 (Table 1). But overall, the active gene shows fewer chromatin induced modulations; on allele T1 there is only one major effect at site 4. Allele T2, which cannot be translated (21) shows several chromatin specific alterations in the accessibility of Bsp sites: Site -1 appears more protected than the other sites although it has normal accessibility on the DNA level and is preferentially cut in the inactive chromatin (22). Conversely, sites 1 and 9 appear particularly exposed within the chromatin. These findings confirm our earlier report that the chromatin structures of allele T1 and T2, as recognized by DNAase I, are different even in the regions of identical DNA sequences (i.e. downstream of the J gene segments) (23).

However, the allelic differences revealed by Bsp RI are not the same as those detected by DNAase I: There is a T2 specific DNAase I hypersensitive site close to site 6 which appears not particularly exposed to Bsp RI. Oppositely, the preferential Bsp cleavage at site 9 is found only on allele T2 although this site is located about 300 bp upstream of a DNAase I hypersensitive site found on both alleles. Therefore, considering all structural pecularities revealed by DNAase I and Bsp RI the chromatin structure of both alleles must be different at the following locations: at the 5' and 3' ends of the large intron, within the C gene segment (by a T2 specific DNAase I hypersensitive site (23)) and downstream of the coding region. These areas cover the entire region of sequence identity of both alleles. The hypothesis seems close at hand that these allelic differences are related to regulatory differences which, in turn, may result from the incapability of T2 of being translated. It appears intriguing that the one allele which is effectively transcribed and translated has a chromatin structure which provides no DNAase I hypersensitive site inside the gene and which affects the accessibility for Bsp RI at one or two sites, at best, (4 and possibly 1). Site 4 most likely coincides with a location in which the DNA sequence is particularly conserved (41) and which has been assigned a regulatory function in the expression of immunoglobulin genes (42, 43). The lack of other structural peculiarities on allele T1 may

indicate a highly dynamic chromatin structure. In contrast, the untranslatable allele T2 may carry more regulatory signals in form of specific proteins bound or/and non-randomly located histone complexes which are sensed by the various nucleases.

ACKNOWLEDGMENTS

We thank G.Ruhland for expert technical assistance. B.O.G. acknowledges the receipt of an Alexander von Humboldt fellowship. This work was supported by Deutsche Forschungsgemeinschaft, Forschergruppe Genomorganisation, München.

REFERENCES

1. Permanent address: Institute of Molecular Biology, USSR Academy of Sciences, Moscow, USSR
2. Abbreviations: V, variable, J, joining, C, constant region of the immunoglobulin kappa light chain and their corresponding gene segments; T1 and T2: functionally and aberrantly rearranged K (kappa) chain genes of myeloma T and DNA fragments containing these genes.
3. McGhee,J.D. and Felsenfeld,G.(1980) Annu.Rev.Biochem. 49: 1115-1156
4. Mathis,D., Oudet,P.,and Chambon,P.(1980) Prog.Nucleic Acids Res.Mol.Biol. 24:2-55
5. Igo-Kemenes,T., Hörz,W.,and Zachau,H.G.(1982) Ann.Rev.Biochem. 51:89-121
6. Weintraub,H. and Groudine,M.(1976) Science 193:848-856
7. Wu,C., Bingham,P.M., Livak, K.J., Holmgren,R.,and Elgin, S.C.R.(1979) Cell 16:796-806
8. Wu,C., Wong,Y-C.,and Elgin,S.C.R.(1979) Cell 16:807-814
9. Weisbrod,S.(1982) Nature 297:289-295
10. McGhee,J.D., Wood,W.I., Dolan,M., Engel,J.D.,and Felsenfeld,G.(1981) Cell 27:45-55
11. Wood, W.I.,and Felsenfeld,G.(1982) J.Biol.Chem. 257:7730-7736
12. Pfeiffer,W. and Zachau,H.G.(1980) Nucl.Acids Res. 8:4621-4638
13. Pfeiffer,W., Hörz,W., Igo-Kemenes,T.,and Zachau,H.G.(1975) Nature 258:450-452
14. Hörz,W., Igo-Kemenes,T., Pfeiffer,W.,and Zachau,H.G.(1976) Nucl.Acids Res. 3:3213-3226
15. Igo-Kemenes,T., Ōmori,A.,and Zachau,H.G.(1980) Nucl.Acids Res. 8:5377-5390
16. Max,E.E., Seidmann,J.G.,and Leder,P.(1979) Proc.Natl.Acad. Sci. USA 76:3450-3454
17. Sakano,H., Hüppi,K., Heinrich,G.,and Tonegawa,S.(1979) Nature 280:288-294
18. Altenburger,W., Neumaier,P.S., Steinmetz,M.,and Zachau,H.G. (1981) Nucl.Acids Res. 9:971-981
19. Steinmetz,M.,and Zachau,H.G.(1980) Nucl.Acids Res. 8:1693-1707
20. Early,P. and Hood,L.(1981) Cell 24:1-3

21. Altenburger,W., Steinmetz,M.,and Zachau,H.G.(1980) Nature 287:603-607
22. Weischet,W.O., Glotov,B.O.,and Zachau,H.G.(1983) Nucl.Acids Res.,accompanying paper
23. Weischet,W.O., Glotov,B.O., Schnell,H., and Zachau,H.G. (1982) Nucl.Acids Res. 10:3627-3645
24. Max,E.E., Maizel,J.V.,Jr.,and Leder,P.(1981) J.Biol.Chem. 256:5116-5120
25. Neumaier,P.S. and Zachau,H.G.(1983) Nucl.Acids Res.,accompanying paper
26. Hewish,D.R. and Burgoyne,L.A.(1973) Biochem.Biophys.Res. Commun. 52:504-510
27. Nedospasov,S.A. and Georgiev,G.P.(1980) Biochem.Biophys. Res.Commun. 92:532-539
28. Wu,C.(1980) Nature 286:854-860
29. Southern,E.M.(1975) J.Mol.Biol.98:503-517
30. Pech,M., Höchtl,J., Schnell,H.,and Zachau,H.G.(1981) Nature 291:668-670
31. Steinmetz,M., Altenburger,W.,and Zachau,H.G.(1980) Nucl. Acids Res. 8:1709-1720
32. Höchtl,J., Müller,C.R.,and Zachau,H.G.(1982) Proc.Natl. Acad.Sci.USA 79:1383-1387
33. Storb,U., Wilson,R., Selsing,E.,and Walfield,A.(1981) Biochemistry 20:990-996
34. Hörz,W. and Altenburger,W.(1981) Nucl.Acids Res. 9:2643-2658
35. Bryan,P.N., Hofstetter,H.,and Birnstiel,M.L.(1981) Cell 27: 459-466
36. Bellard,M., Dretzen,G., Bellard,F., Oudet,P.,and Chambon,P. (1982) EMBO J. 1:223-230
37. Jessee,B., Gargiulo,G., Razvi,F.,and Worcel,A.(1982) Nucl. Acids Res. 10:5823-5834
38. Cartwright,I.L. and Elgin,S.C.R.(1982) Nucl.Acids.Res.10: 5835-5852
39. Tatchell,K. and Van Holde,K.E.(1978) Proc.Natl.Acad.Sci.USA 75:3583-3587
40. Levy,A. and Noll,M.(1981) Nature 289:198-203
41. Hieter,P.A., Max,E.E., Seidmann,J.G., Maizel,J.V.,Jr.,and Leder,P.(1980) Cell 22:197-207
42. Parslow,T.G. and Granner,D.K.(1982) Nature 299:449-451
43. Chung,S.Y., Folsom,V.J.,and Wooley,J.C.(1982) J. Cell Biol. 95:76a