Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3851–3855, June 1987
Immunology

Protein–nucleotide contacts in the immunoglobulin heavy-chain promoter region

(DNase I "footprinting"/octanucleotide/mobility shift assay)

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Communicated by Jean D. Wilson, February 17, 1987 (received for review November 18, 1986)

ABSTRACT **Immunoglobulin heavy-chain variable-region genes contain the octanucleotide ATGCAAAT upstream from the site of transcription initiation. The complement of this sequence, in the reverse orientation, is found at an identical location in light-chain variable-region genes. This sequence element is thought to be involved in the lymphoid-specific expression of immunoglobulin genes. Analysis of nuclear extracts from both lymphoid and nonlymphoid cells in a gel migration inhibition assay, using an immunoglobulin promoter region fragment containing the octamer, reveals multiple migration-retarded species that represent specific DNA–protein complexes. The number and relative level of these complexes vary with cell type; some complexes are detected with all extracts, whereas one complex is lymphoid-specific and may represent an interaction involved in the lymphoid-restricted expression of immunoglobulin genes. Mitogenic stimulation of a B-lymphoid line can increase the level of the protein responsible for this lymphoid-specific complex. Analysis of the complexes detected in the gel migration inhibition assay by DNase I protection ("footprinting") has revealed that all of these DNA–protein complexes involve contact of the protein with the nucleotides of the octamer. One complex, present in both lymphoid and nonlymphoid cells, displays an additional DNA–protein contact adjacent to the octamer. Our results also indicate that the interaction of proteins with the octameric sequence can cause a local alteration in the structure of the DNA helix.**

Selective gene expression is the basis of eukaryotic cell differentiation. Immunoglobulin gene regulation can serve as a model of this process because the members of this gene family are accurately transcribed and expressed only in cells of the B-lymphocyte lineage. The mechanism of this cell-type-specific expression is not understood. However, the identification of immunoglobulin gene promoter-associated regulatory sequences (cis-regulatory elements) that are required for this selective expression (1–7), in conjunction with the description of nuclear proteins that bind to these sequences (trans-acting factors) (8–11), implies that protein–DNA interaction(s) is the basis of this lymphoid-restricted expression.

One cis-regulatory element involved in the lymphoid-specific expression of immunoglobulin genes is the octameric sequence ATGCAAAT, which is found in the promoter region of heavy-chain variable-region (V_H) genes and is 70–90 nucleotides 5′ to the site of transcription initiation (1, 12). The complement of this sequence, in the reverse orientation, is found at an identical position in light-chain variable-region (V_L) gene segments (1, 12). The strict conservation of both location and sequence implies that the octamer has functional significance, and several studies have indicated that this sequence element is required for the accurate, lymphoid-specific transcription of immunoglobulin genes (1–7). This octanucleotide is not, however, limited to immunoglobulin promoter regions; it is also found in the immunoglobulin enhancer (1) and in the promoter regions of a variety of other genes (13–21).

Studies based on the alteration in electrophoretic mobility of a DNA fragment complexed with a protein as compared to the mobility of the free fragment (gel retention or migration inhibition assay) have detected nuclear proteins with binding specificity for the conserved octameric sequence (8–11, 22, 23). Further analysis of nuclear extracts prepared from cell lines of distinct lineages has revealed a cell-type-specific variation in the number and level of detectable octamer-specific interactions (8, 10), which leads to the hypothesis that a DNA–protein complex detected by the gel retention assay represents an interaction involved in the lymphoid-specific expression of immunoglobulin genes.

This report describes DNase I protection studies carried out to characterize more thoroughly the interaction of nuclear proteins from lymphoid and nonlymphoid cells with octamer-containing V_H promoter region fragments. Our analysis reveals that all of the DNA–protein complexes detected by the gel retention assay involve contacts with the nucleotides of the octamer. One complex, detected with both lymphoid and nonlymphoid extracts, also displays a DNA–protein contact area adjacent to the octamer, indicating that at least two distinct proteins are capable of interacting with octamer-containing fragments. Our results also indicate that the binding of specific proteins to this sequence element can cause a local alteration in the configuration of the DNA helix. In addition, we establish that mitogenic stimulation of a B-lymphoid line can alter the relative level of octamer-specific nuclear proteins and result in an increase in a DNA–protein complex unique to lymphoid cells.

MATERIALS AND METHODS

Immunoglobulin Promoter Region Fragments. The immunoglobulin promoter region fragments were derived from the plasmid pMK-3 (24), which contains the V_H gene expressed by the BCL_1 cell line. The restriction enzyme map of the 5′-most 593 base pairs (bp) of the insert is shown in Fig. 1*A*. The indicated fragments were labeled at either the 3′ or 5′ end (25) and were purified by polyacrylamide gel electrophoresis before use.

Nuclear Extracts. Nuclear extracts were prepared from the murine B-cell leukemia BCL_1, mitogen-activated murine B lymphocytes, and the human epithelial carcinoma HeLa as described (26). Mitogen-activated B lymphocytes were prepared as described (27). Mitogen stimulation of BCL_1 was carried out by culturing the cells in the presence of 50 μg of lipopolysaccharide (LPS) per ml for 2 days.

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Abbreviations: LPS, lipopolysaccharide; V_H, heavy-chain variable region; V_L, light-chain variable region.

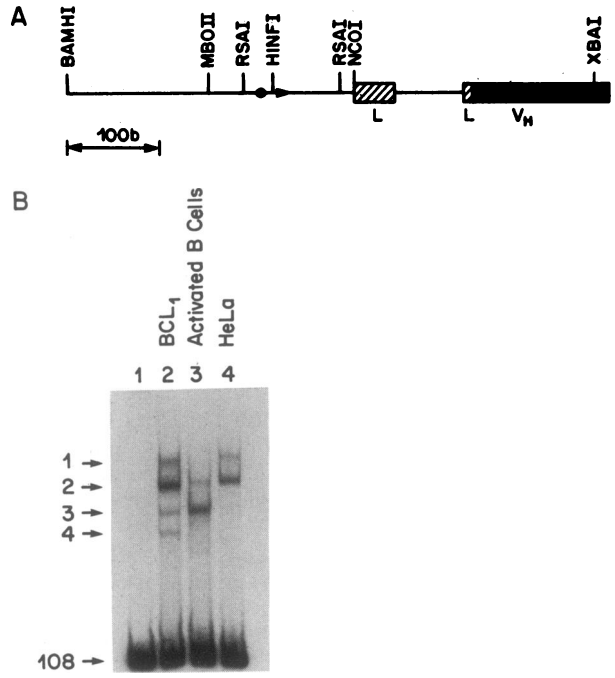

FIG. 1. (*A*) Restriction endonuclease map of the productivity rearranged BCL_1 V_H gene, which was used as a source of specific fragments. Only the relevant enzymes are indicated. The location of the conserved ATGCAAAT octamer (●), the "TATA" box (▸), as well as the protein-encoding leader (L) and variable-region (V_H) exons are indicated. (*B*) Autoradiogram of a gel retention (mobility shift) assay comparing the binding activity of nuclear extracts prepared from the BCL_1 line (lane 2), mitogen-activated B lymphocytes (lane 3), and HeLa cells (lane 4). The octamer-containing 108-bp *Rsa* I fragment shown in *A* was used in the assay. The migration of the free fragment (no extract; lane 1) and the position of the four arbitrarily numbered migration-retarded species are indicated on the left. None of the extracts exhibited binding activity to flanking fragments (data not shown and ref. 8).

Gel Migration Inhibition Assay. The binding reaction was carried out in a total volume of 20 μl containing 20 mM Hepes (pH 7.9), 20% (vol/vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of sheared salmon sperm DNA, ≈10 μg of nuclear extract protein, and 0.5–5 ng of the ^{32}P-labeled octamer-containing fragment. The assay mixture was incubated for 15 min at 37°C and then was loaded onto a 4% polyacrylamide gel (acrylamide/bis, 25:1) containing 0.1 M Tris (pH 8.3), 0.1 M boric acid, 2 mM EDTA, and 2% glycerol. The samples were electrophoresed at 10 $V \cdot cm^{-1}$. The gels were then dried and autoradiographed.

DNase I Protection Assay. For DNase I protection (footprinting) analysis, the binding reaction was set up as above. Following the incubation, an equal volume (20 μl) of 5 mM $CaCl_2$/10 mM $MgCl_2$ was added to each sample. The samples were mixed and 250–500 ng of DNase I (1 μl) was added. The mixtures were incubated at room temperature for 1 min, and the digestion was stopped by the addition of 2.5 μl of 250 mM EDTA. The samples were then immediately loaded onto the gel and were electrophoresed as described above. After electrophoresis, autoradiography was performed on the wet gel to ascertain the position of the migration-retarded and unretarded DNA fragments. The appropriate areas of the gel were excised and eluted by soaking in 0.5 M ammonium acetate/0.2% sodium dodecyl sulfate/1 mM EDTA/10 μg of proteinase K per ml/100 μg of carrier tRNA per ml for 8–16 hr. The eluted material was extracted with phenol/chloroform (1:1), extracted with chloroform, and then ethanol-precipitated. The precipitate was resuspended in formamide, boiled, and electrophoresed on a 6% polyacrylamide/50% urea gel. The adenine- and guanine-specific chemical modification/cleavage reaction (28) was carried out on each fragment, and these samples were electrophoresed with the DNase I-digested samples to identify the protected nucleotides.

RESULTS

Cell-Type-Specific Variation in the Number and Relative Level of Octamer-Associated Interactions. Analysis of nuclear extracts prepared from the B-lymphocyte line BCL_1, mitogen-activated B lymphocytes, and the nonlymphoid HeLa cell line in a gel retention assay with an octamer-containing immunoglobulin promoter region fragment (see Fig. 1*A*) reveals similar but distinct migration inhibition patterns (Fig. 1*B* and ref. 8). BCL_1 extracts exhibit four distinct migration-retarded species (arbitrarily numbered 1–4 in Fig. 1*B*). The extract of mitogen-activated B lymphocytes results in two DNA–protein complexes that comigrate with species 2 and 3 of the BCL_1 pattern, whereas the extract of HeLa cells exhibits two complexes comigrating with species 1 and 2 of the BCL_1 pattern. Mitogen-activated B lymphocytes exhibit a high level of immunoglobulin transcription (29); therefore, if the gel retention assay detects interactions relevant to *in vivo* gene expression, the presence of one ubiquitous complex (species 2) and one lymphoid-specific complex (species 3) suggests that the latter may represent a protein–DNA interaction involved in the lymphoid-specific expression of immunoglobulin genes.

Our previous analysis had indicated that the DNA–protein interaction(s) that results in all of the migration-retarded species observed in Fig. 1*B* involves a 30-base *Rsa* I/*Hin*fI V_H promoter region fragment containing the conserved octamer flanked by 16 nucleotides on the 5′ side and 6 nucleotides on the 3′ side (8). To delineate more specifically the sequences involved, we have performed DNase I protection analysis (30) on each of these migration-retarded species.

Species 1 Displays Protein Contacts Within and Adjacent to the Octamer. Fig. 2 presents the area of protection (footprint) for each of the four migration-retarded species of the BCL_1 pattern. The slowest-migrating DNA–protein complex of the BCL_1 pattern (species 1) exhibits the most extensive area of protection. The protein(s) responsible for species 1 completely protects, at most, the 8 bases of the octamer and partially protects the 13 bases 3′ to the octamer on the noncoding strand (Fig. 2*A*, lane 3). The area of complete protection displayed by the DNA of species 1 on the coding strand includes, at most, the five 3′ nucleotides of the octamer; partial protection of the remaining 3 bases of the octamer and the 13 bases 5′ to the octamer is also apparent (Fig. 2*B*, lane 3). Since DNase I cleavage of the free fragment is not completely random (e.g., Fig. 2*A*, lane 2), it is impossible to assess the status of each nucleotide. The areas of protection we report, therefore, represent the maximum possible area protected.

The Three Faster-Migrating Complexes of the BCL_1 Pattern Exhibit Identical Nucleotide-Protection Patterns of Octamer Residues. Analysis of species 2, 3, and 4 reveals that the maximum area of complete protection on the noncoding strand is the eight nucleotides of the octamer (Fig. 2*A*). On the coding strand, each of these three species exhibit complete protection of, at most, the five 3′ nucleotides of the octamer (Fig. 2*B*). In addition, the DNA from each of these complexes demonstrates a hypersensitivity of the guanine residue (third residue of the octamer) on the coding strand [i.e., this nucleotide was cut more efficiently by DNase I when the fragment was complexed with the protein(s)].

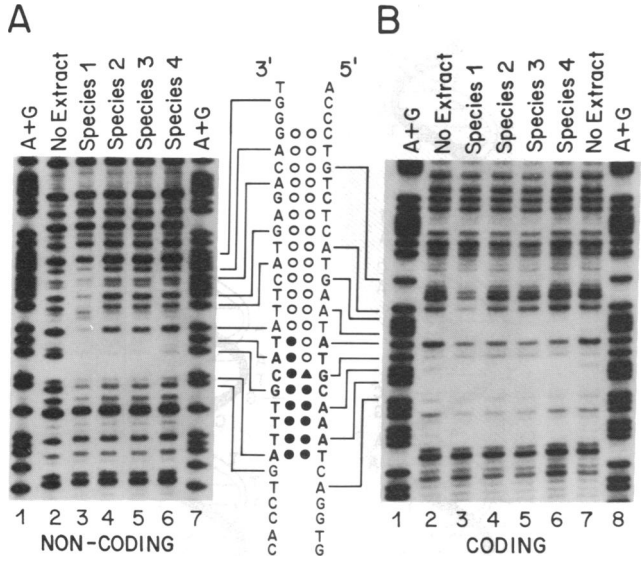

FIG. 2. Autoradiogram of DNase I protection analysis of the four migration-retarded species observed with the BCL_1 extract and the octamer-containing fragment in the gel retention assay (Fig. 1*B*, lane 2). The fragments utilized were the 322-bp *Bam*HI/*Nco* I fragment, which was 5′ end-labeled at the *Nco* I site to establish noncoding strand contacts (*A*), and the 165-bp *Mbo* II/*Nco* I fragment, which was 3′ end-labeled at the *Nco* I site to determine coding strand contacts (*B*). Lanes 1 and 7 in *A* and also lanes 1 and 8 in *B* represent the A+G-specific modification/cleavage reaction carried out on the respective fragments for orientation. Lane 2 in *A* and also lanes 2 and 7 in *B* represent the pattern of DNase I cleavage of the unbound DNA fragment (digested in the presence of nuclear proteins). The sequence of the 30-base *Rsa* I/*Hin*fI fragment is indicated between the panels. The ATGCAAAT octamer and its complement are in bold type. ●, Complete protection; ▲, the hypersensitivity observed for species 2, 3, and 4; ○, partial protection (only observed for species 1), which can be seen by comparing the intensity of the cleavage products of species 1 with those of neighboring lanes.

The Two DNA–Protein Complexes Detected in Extracts of Mitogen-Activated B Lymphocytes Exhibit Identical Protection Patterns. Similar analysis was carried out on the two migration-retarded species observed with the extract of mitogen-activated B lymphocytes (which comigrate with species 2 and 3 of the BCL_1 pattern). These two DNA–protein complexes exhibit the same nucleotide-protection pattern—i.e., complete protection of at most the eight nucleotides of the octamer on the noncoding strand and the five 3′ bases of the octamer on the coding strand (Fig. 3 *A* and *B*)—that is observed with the three faster-migrating BCL_1 species. In addition, the DNA from these two species also displays a hypersensitivity of the third residue (guanine) of the octamer on the coding strand.

Octamer-Interacting Proteins of HeLa Display Areas of Protection Identical to That Observed in Lymphoid Cells. Delineation of the nucleotides protected in the two migration-retarded complexes detected with the HeLa nuclear extract (which comigrate with species 1 and 2 of the BCL_1 pattern) reveals that the slower migrating species exhibits a footprint indistinguishable from species 1 of the BCL_1 pattern on both the coding (Fig. 4*B*, lane 3) and noncoding strands (Fig. 4*A*, lane 3). The faster-migrating DNA–protein complex observed with the HeLa extract displays a footprint identical to that of both of the complexes detected with the mitogen-activated B lymphocyte extract and the three faster-migrating species of the BCL_1 pattern (Fig. 4 *A* and *B*, lane 4). This complex also clearly exhibits a hypersensitivity of the guanine residue of the octamer on the coding strand (Fig. 4*B*, lane 4).

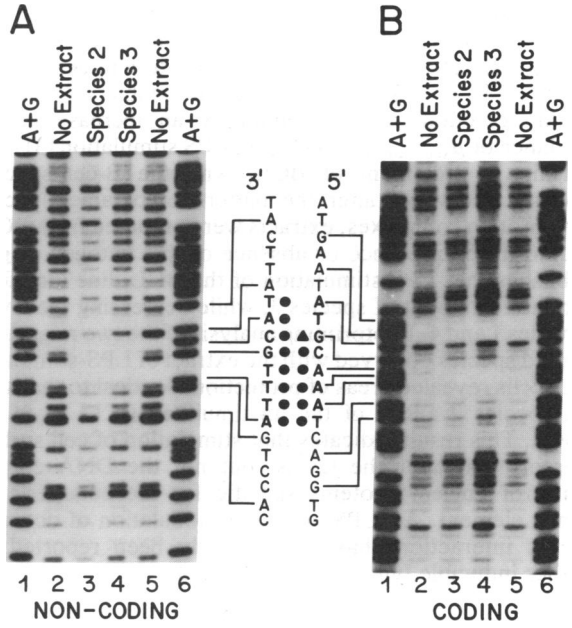

FIG. 3. Autoradiogram of DNase I protection analysis of the two migration-retarded species detected with an extract from mitogen-activated B lymphocytes and octamer-containing fragments in the gel retention assay (Fig. 1*B*, lane 3). (*A*) Noncoding strand. (*B*) Coding strand. The fragments utilized were the same as in Fig. 2. Lanes 1 and 6 represent the A+G-specific modification/cleavage reaction carried out on the respective fragments for orientation. Lanes 2 and 5 show the pattern of DNase I cleavage on the unbound DNA fragment (digested in the presence of nuclear protein). Symbols and details are as in Fig. 2.

Mitogenic Stimulation of BCL_1 Alters the Relative Amounts of Octamer-Interacting Proteins and Increases the Level of a Complex Specific to Lymphoid Cells. Mitogenic stimulation of

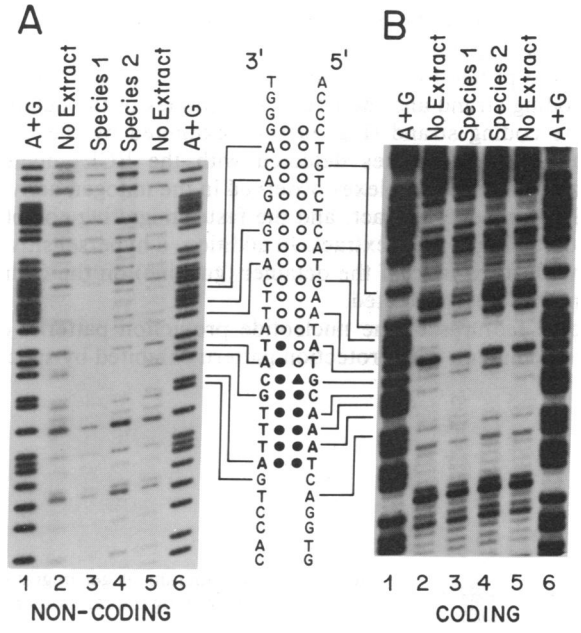

FIG. 4. Autoradiogram of DNase I protection analysis of the two migration-retarded species detected with a HeLa cell extract and octamer-containing fragments in the gel retention assay (Fig. 1*B*, lane 4). (*A*) Noncoding strand. (*B*) Coding strand. The fragments utilized were the same as in Fig. 2. Lanes 1 and 6 represent the A+G-specific modification/cleavage reaction performed on the respective fragments for orientation. Lanes 2 and 5 represent the pattern of DNase I cleavage of the unbound fragment (digested in the presence of nuclear protein). Symbols and details are as in Fig. 2.

lymphoid cells is an artificial correlate of antigen-specific cell surface receptor-mediated stimulation. A limited number of neoplastic B-cell lines exhibit a response to mitogenic stimulation. BCL_1 is an IgM^+ leukemia that exhibits an increase in IgM secretion and concomitantly an increase in the abundance of $\mu_{secretory}$ mRNA upon LPS stimulation (31). To determine if treatment of BCL_1 with the B-cell-specific mitogen LPS would alter the pattern of octamer-specific DNA–protein complexes, extracts were prepared from BCL_1 cultured in the presence or absence of this mitogen. Fig. 5 establishes that LPS stimulation of the BCL_1 line increases the relative amount of species 3, while decreasing the levels of species 1 and 4. Footprinting analysis of the two migration-retarded species observed with the extract of LPS-stimulated BCL_1 cells revealed areas of protection identical to that seen with species 2 and 3 of the unstimulated BCL_1 (data not shown). This result indicates that stimulation of cell surface receptors can alter the levels, but not the DNA contact points, of nuclear proteins specific for the octameric sequence element. An LPS-mediated modulation of octamer-specific interactions has also recently been reported for another inducible lymphoma, 70Z/3 (10).

DISCUSSION

All of the nuclear extracts we have examined in the gel retention assay using V_H octamer-containing fragments exhibit multiple migration-retarded species (Fig. 1*B* and ref. 8); therefore, we have employed a DNase I protection assay to determine precisely the nucleotide–protein contacts in each of these DNA–protein complexes. This analysis has revealed two basic patterns of nucleotide protection, which are schematically summarized in Fig. 6. Species 1, the slowest-migrating complex, which is detected with both BCL_1 and HeLa extracts, exhibits complete protection of, at most, the eight nucleotides of the octamer on the noncoding strand and the five 3′ bases of this element on the coding strand. Partial protection of the 13 bases 3′ to the octamer on the noncoding strand as well as the three 5′ nucleotides of the octamer and the 13 bases 5′ to the octamer on the coding strand was also observed (Fig. 6*A*). The second pattern of protection—i.e., complete protection of, at most, the entire octamer on the noncoding strand and the five 3′ nucleotides of the octamer on the coding strand (Fig. 6*B*)—is exhibited by the three faster-migrating species detected with the BCL_1 nuclear extract, the two complexes observed in the mitogen-activated B-lymphocyte extract, and the faster-migrating complex detected in the HeLa extract. In addition, a hypersensitivity of the third residue of the octamer (guanine) on the coding strand has been detected.

Some features of the nucleotide protection patterns deserve comment. The protection pattern exhibited by species 1 (21 nucleotides displaying some level of protection) encompasses approximately two turns of the helix on both strands. The lack of complete protection of the base pairs upstream of the octamer implies that the interaction with these residues is weaker than that with the nucleotides of the octamer. This may indicate an interaction that lacks true sequence specificity (e.g., electrostatic interactions with the phosphate backbone of the helix). The protection of nucleotides only within the octamer in species 2, 3, and 4 establishes this sequence element as the site of protein recognition in each of these complexes. The hypersensitivity of the third residue of the octamer on the coding strand, however, reveals that this residue is more accessible to DNase I when the DNA fragment is complexed with protein(s). This indicates a local alteration in the structure of the DNA helix upon protein binding, possibly analogous to the protein-induced DNA bending observed for the *Drosophila* heat-shock transcription factor (32).

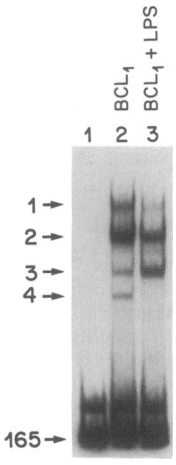

FIG. 5. Autoradiogram of gel migration inhibition assay examining the retention patterns of nuclear extracts prepared from BCL_1 and BCL_1 stimulated with LPS. The extracts were prepared from an identical number of cells and contained equivalent amounts of protein. The fragment used in the assay was the 165-bp *Mbo* II/*Nco* I fragment (see Fig. 1*A*). The migration of the free fragment (no extract; lane 1) and the position of the four arbitrarily numbered migration-retarded species are indicated on the left.

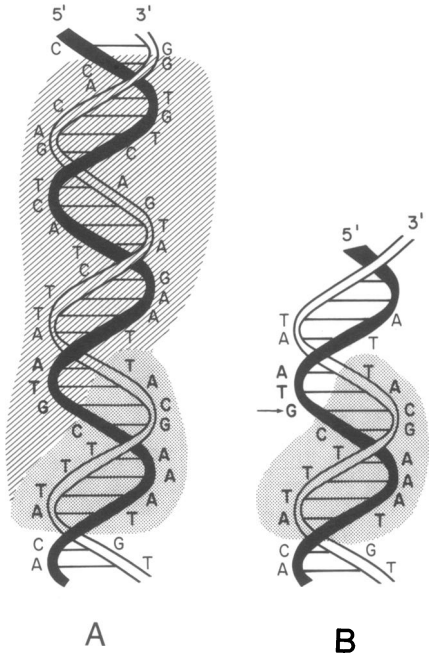

FIG. 6. A schematic representation of the two patterns of nucleotide protection observed on an octamer-containing immunoglobulin promoter region fragment. The coding strand is represented by the solid strand of the helix. The nucleotides of the octamer and its complement are in bold type. (*A*) The protection pattern observed for species 1. The stippled area represents the maximum area of complete protection. The hatched area represents the area of partial protection. (*B*) The area of protection observed for species 2, 3, and 4. The stippled area represents the maximum area of complete protection. The arrow indicates the guanine residue that displays DNase I hypersensitivity upon protein interaction.

The protection patterns we have observed differ slightly from those reported by other researchers for various nuclear proteins and octamer-containing fragments. Singh *et al.* observed a 19-base area of protection centered over the octamer on the coding strand of the κ light-chain promoter (9). Sive and Roeder have reported that the eight nucleotides of the octamer, as well as four nucleotides 5′ and two nucleotides 3′ to the octamer, are protected in the histone H2B promoter (22). Davidson *et al.* have detected 13–22 bases (including the octamer) protected on both strands of the immunoglobulin heavy-chain enhancer (33). We observe a low level of protection at the 5′ boundary of the octamer on the noncoding strand (Fig. 2*A*); thus, the differences in footprint size may be related to the different nucleotide sequences flanking the octamer in the respective DNA fragments. Variation in the amount of DNase I in the assay

does not alter the size of the area of protection that we observe (data not shown).

If one assumes that the pattern of nucleotide protection reflects, at least in part, the configuration of that portion of the protein that is involved in the interaction with the DNA, then complexes that exhibit indistinguishable footprints should contain proteins with similar, if not identical, sequence-specific DNA binding sites. Thus, identical areas of protection in complexes that exhibit similar migration among the extracts is most likely an indication of the presence of identical proteins in extracts of these cell types. The detection of identical protection patterns in DNA–protein complexes that display different migration rates (e.g., species 2, 3, and 4 of the BCL_1 pattern), however, is not so easily interpreted. Based on the above rationale, the indistinguishable footprints imply that these complexes contain a protein(s) possessing similar, if not identical, DNA-contacting regions. However, the clear difference in migration establishes that these complexes are distinct in some parameter. The basis of this difference in migration is unclear. It is possible that a single octamer-specific protein is responsible for the area of protection in each of these complexes and that the different migration rates between complexes are a result of additional protein interactions with this core complex. Alternatively, a related family of proteins, sharing a common octamer recognition site (domain or polypeptide chain) but differing sufficiently in the remainder of the molecule so as to result in diverse migration rates, could be responsible for the various complexes. The clear structural separation of sequence-specific DNA binding and transcription-modulating activity in the yeast GAL4 protein indicates that sequence specificity and effector activity can be distinct (34). Therefore, the multiple complexes may represent biochemically (and functionally) distinct nuclear proteins with the same (octamer-specific) binding activity. For example, octamer binding proteins with both positive and negative transcriptional activities may exist. Immunoglobulin expression in lymphoid cells may, thus, depend upon a transcriptional activator displacing a protein with negative regulatory activity.

The existence of two patterns of nucleotide protection among the complexes indicates that at least two distinct proteins are involved in the interaction with octamer-containing fragments. The additional protection observed in species 1 can most easily be explained as a result of another protein combining, possibly through a protein–protein interaction, with an octamer-bound protein. Alternatively, a single protein, distinct from that which just protects nucleotides of the octamer in that it additionally exhibits upstream contacts, may be responsible for species 1. In any case, our data are consistent with the existence of at least two different V_H promoter region-specific proteins.

The observed alteration in the relative levels of protein–octamer complexes in BCL_1 upon mitogenic stimulation (Fig. 5) implies that a cell surface stimulatory event can modulate the level of octamer-specific nuclear proteins. This stimulation results in a selective increase of a protein(s) responsible for a migration-retarded species that displays a lymphoid-specific pattern of expression (species 3) and a concomitant decrease in the level of a complex detected in nonlymphoid cells (species 1). Although it is not clear if a true reciprocal relationship exists between species 1 and 3, it should be pointed out that little or no species 1 is detected with extracts of mitogen-activated B lymphocytes (Fig. 1*B* and ref. 8). Thus, it is unlikely that the protein(s) responsible for species 1 is involved in immunoglobulin gene expression. Our result is, however, consistent with the hypothesis that species 3 represents a DNA–protein interaction involved in the lymphoid-restricted expression of immunoglobulin genes. Yet, using nuclear run-on analysis we have been unable to detect a significant increase in the rate of V_H gene transcription in BCL_1 upon LPS stimulation (unpublished results). This may be due to an already maximal level of transcription of the immunoglobulin locus in BCL_1, as a consequence of its neoplastic state. Alternatively, factors in addition to those represented by the presence of species 3 may be required to observe an increase in immunoglobulin-specific transcription. Nevertheless, the lymphoid-restricted expression of the protein(s) responsible for species 3 is suggestive of some role in the tissue specificity of immunoglobulin expression.

The authors thank Maureen Willard for technical assistance and Chhaya Das for preparation of plasmid DNA. N.F.L. is a Fellow of the Leukemia Society of America. This work was supported by National Institutes of Health Research Grants AI-18016, GM-1751, and GM-31689.

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