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Purification of an octamer sequence (ATGCAAAT)-binding protein from human B cells

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ABSTRACT

The highly conserved octamer sequence ATGCAAAT or its inverse complement found in all human and murine immunoglobulin gene promoters has been demonstrated to be necessary in the lymphoid-specific transcription by deletion analysis[1-7]. Trans-acting factors that interact with the octamer motif are thought to be involved in this tissue-specific expression. Using a gel mobility shift assay, we have identified both lymphoid-specific and ubiquitous nuclear factors that interact with a human γ_1 heavy chain gene promoter region containing the octamer motif, consistent with the results obtained with murine heavy or light chain promoter regions. We have purified an octamer binding protein from human B cells by sequence-specific DNA affinity chromatography. Renaturation of gel-purified protein allowed the identification of a polypeptide with a molecular weight of 74 kilodaltons(kD) that is capable of recognizing and binding to the octamer motif. This 74kD protein seems to be also present in T-cells and non-lymphoid cells. The possible function of the factor is discussed.

INTRODUCTION

The immunoglobulin gene is specifically expressed in cells of B lymphocyte lineages. This tissue-specific transcription requires trans-acting factors that interact with each other and with cis-acting regulatory DNA elements of the immunoglobulin genes. The octamer sequence ATGCAAAT is found approximately 60-80 base pairs upstream from the site of transcriptional initiation in all human and murine immunoglobulin heavy chain variable region (V_H) genes[1,2]. In reverse orientation, this octamer sequence is also found in all light chain variable region (V) genes at an identical position[1,2], and in the immunoglobulin heavy chain enhancer[2]. The strict conservation of both location and sequence indicate that it is functionally significant. Transfection studies revealed that the immunoglobulin promoter regions containing this octamer motif directed lymphoid-specific transcription independently of the enhancer or even in the presence of viral enhancers[2-4,9-11]. A deletion or even a mutation of the octamer sequence destroyed this

tissue specific expression[2-7]. Using an electrophoretic mobility shift assay and DNase I protection (footprinting) assay, octamer binding factors have been identified in both lymphoid and non-lymphoid cells[12-18]. Staudt L.M. et al have reported that there were two species of nuclear protein binding specifically to the octamer, one was found in all cell types (named NF-A1) and the other (NF-A2) was restricted to lymphoid cells[14]. Landolfi N.F. et al reported up to four species of octamer binding factors in various cell types, and also found a lymphoid specific factor (species 3)[12]. Because both lymphoid-specific and ubiquitous factors recognize nearly identical sites in the DNA, as demonstrated by footprinting and methylation interference assay, it has been suggested that one factor may be a modified form of the other. So far, however, none of these factors has been purified and there is no direct evidence that the octamer binding protein(s) mediates the tissue-specific transcription of Ig genes. Purification of such factors will provide an important step toward understanding the mechanism of the regulation of the immunoglobulin gene expression and make it possible to determine directly whether the octamer binding factor(s) mediates tissue-specific expression by, for example, an in vitro transcription assay. In the present study, we show that nuclear extracts from various cell types interact differentially with the octamer containing human immunoglobulin γ_1 heavy chain promoter region. We describe the purification of one of the octamer binding factors from human B-Lymphoid cells by sequence-specific DNA affinity chromatography.

MATERIALS AND METHODS

Nuclear extracts and gel mobility shift assay

Nuclear extracts were prepared according to the method of Dignam et al[19]. In all the gel mobility shift assay, the AluI-HaeIII fragment (HP1) was used as a probe[20]. This fragment represents the sequence from -4 to -113 relative to the start site of transcription and contains the octamer sequence. The binding reaction was carried out in a total volume of 40µl containing the binding buffer (10 mM Tris pH 7.5, 50mM NaCl, 1mM EDTA, 1mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10%(v/v) glycerol), 2µg of poly(dI-dC)poly(dI-dC), 8µg nuclear protein and 0.1ng [γ-^{32}P]ATP-end labeled HP1 fragment. The reaction mixture was incubated for 30 minutes at 24 C. The resulting complexes were loaded on a 4.5% polyacrylamide gel (acrylamide: bisacrylamide weight ratio of 30:1) containing 6.7mM Tris-HCl pH7.5, 3.3mM Na-acetate and 1mM EDTA. The gel was pre-run for 30 minutes at 11V/cm, after which, the gel was run for about 2-3 hours at 4 C at the same voltage with

buffer recirculation. The gel was then dried and autoradiographed at -80 C with a screen. The synthetic octanucleotide (65 base pairs) used in the competition assay was derived by EcoRI and HindIII digestion of the PUC8 clone containing an octamer insert and purified by polyacrylamide gel electrophoresis.

Preparation of DNA affinity column

DNA affinity column was prepared according to the method described by Langer,P.R. et al[21] with slight modification. 3µg of the synthetic octanucleotide fragment was end filled with 0.1mM dATP, dCTP, dGTP and biotin-11-dUTP by klenow fragment of DNA pol I and then applied to a 0.5ml bed volume avidin sepharose column (prepared by covalently coupling the avidin to tresyl activated sepharose 4B). 1µci of [α-^{32}p]dCTP (3700µci/µmol) was also added to the reaction mixture as an indicator to estimate the amount of the synthetic octanucleotide retained on the avidin sepharose. Usually, more than 70% of the octamer containing DNA was retained on the avidin sepharose after washing with 5ml of the binding buffer containing 1.0M NaCl.

Column chromatography of the octamer binding proteins

Nuclear proteins extracted from 1x10^{10} cells (100mg) was dialyzed against buffer B[22] (20mM Tris pH7.9, 0.2mM EDTA, 100mM KCl, 1mM PMSF, 1mg/ml pepstatin A, 0.5mM DTT, 20% glycerol). The sample was applied to a 3x4.5cm (30ml bed volume) phosphocellulose column equilibrated with buffer B. 0.2M KCl fractions eluted from phosphocellulose column (containing 20mg proteins) were pooled and dialyzed against TM100[22] (50mM Tris pH7.9, 1mM EDTA, 12.5mM $MgCl_2$, 100mM KCl, 1mM PMSF, 1mg/l pepstatin A, 0.5mM DTT, 20% glycerol) and applied to a heparin column (1.5x6cm, bed volume 10ml) equilibrated with TM100. The proteins eluted at 0.2M KCl TMbuffer (containing 7mg proteins) were dialyzed against the binding buffer and applied to a 0.8ml bed volume avidin column. The fractions which flow through the column (5mg protein) were mixed with 80µg of poly(dI-dC)poly(dI-dC), incubated for 10 minutes at 4 C and then applied to a sequence specific DNA affinity column. All the chromatography operation was performed at 4 C.

DNase I protection (footprinting) assay

The assay was performed according to the method of Carthew et al[23]. The coding strand of the HP1 probe was 5' end-labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase. 5ng of DNA affinity column purified protein (second pass) was incubated with 0.5ng (50,000cpm) of 5' ^{32}P-end labeled HP1 fragment in a total volume of 100µl containing 10mM Tris ph7.5, 50mM NaCl, 0.1mM EDTA, 1mM DTT, 1mM PMSF, 1µg/l pepstatin A, 10% (v/v) glycerol, 5mM $MgCl_2$, 0.1µg

poly(dI-dC)poly(dI-dC) for 20 minutes at 24 C, and then digested with DNase I (0.25µg/ml) for 60 seconds at 24 C. The reaction was stopped by the addition of 3 µl of 0.5M EDTA, and the resulting complexes were resolved by a 4.5% low ionic polyacrylamide gel electrophoresis. Autoradiography of the wet gel was performed at 4 C overnight and the retarded band was excised, eluted at 37 C for 6 hours in a buffer containing 10mM Tris-HCl pH7.8, 1mM EDTA, 0.2% SDS, 0.3M NaCl, 1ug/µl tRNA. The eluted material was extracted sequentially with phenol/chloroform/isoamylalcohol (25:24:1,v/v), chloroform/ isoamylalcohol (24:1,v/v), and then ether. The DNA was precipitated with ethanol. The precipitates were electrophoresed on a 8% denaturing polyacrylamide /50% urea gel. A+G chemical cleavage ladders[24] of HP1 probe and the DNase I digestion of HP1 probe in the absence of the protein were co-eletrophoresed to map the protected region.

Gel slice and renaturation of purified octamer binding protein

Approximately 100ng of affinity purified protein was mixed with SDS sample buffer, allowed to stand at 4 C for two hours, and electrophoresed on a 10% SDS polyacrylamide gel at 11V/cm at 4 C. The gel was stained with ice cold 0.25M KCl and 1mM DTT for 5-10 minutes and destained with 1mM DTT for 1 hour at 4 C. The region containing the 74kD protein was excised, minced with sterilized scissors, then eluted for two hours at room temperature in 2 ml of elution buffer containing 50mM Tris-HCl pH 7.9, 0.1mM EDTA, 5mM DTT, 0.15M NaCl,0.1mg/ml BSA and 0.1%SDS (adapted from D.A.Hager et al[25]). The sample was centrifuged briefly, and the supernatant was precipitated with 4 volumes of cold aceton at -60 C overnight. The protein precipitated by centrifugation at 15,000rpm at 4 C for 30 minutes was resuspended in 100 µl of 6M guanidine-HCl containing 0.1%NP-40 and 0.1M KCl,and incubated for 1 hour at room temperature to denature the polypeptide chains. The guanidine-HCl was removed by passing the sample through a sephadex G-50 spun column(7x0.5cm) equibritated with the binding buffer. The sample passed through the spun column was incubated at room temperature for 1 hour to allow the refolding of the polypeptide chains. 10µl of the renatured protein was used in the gel shift assay.

RESULTS

Identification of octamer binding proteins in various cell types

As shown in lane 1 of each panel in fig.1, all nuclear extracts displayed a retarded band, species 1 (numbered 1 at the left of each panel), though quantitatively different. This band may be generated by a ubiquitous octamer

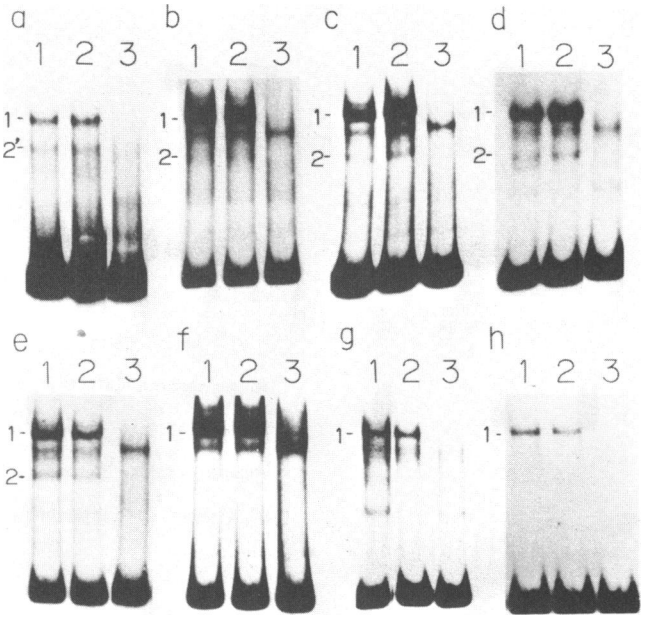

Fig.1 Binding activity of nuclear extracts from various cell types to HP1 probe. a. 70Z/3. b. AT10, c. WEHI-231. d. Manca. e. EL4. f. BW5147. g. L-cell. h. Hela. Lane 1 contained no inhibitor. Lane 2 and lane 3 in each panel contained 10ng of a nonspecific competitor (pBR322 digested with HinfI) and 10ng of the synthetic octanucleotide, respectively. The competitor was added to the nuclear extract mixture prior to the addition of the probe.

specific factor and possibly the same as NF-A1 defined by Staudt L.M et al[14]. In pre-B cell line AT10 (panel b), B cell lines WEHI-231 (panel c) and Manca (panel d) and in one T cell line EL4 (panel e), in addition to species 1, there was another retarded band, species 2 (numbered 2) that migrated faster. In pre-B cell line 70/Z (panel a), a different retarded band was detected (numbered 2') in addition to species 1. In T cell line BW5147 (panel f), L-cell (panel g) and Hela (panel h), only species 1 was observed. LPS stimulation of WEHI-231 and TPA stimulation of EL4 increased the relative levels of species 2 (data not shown). Species 2 or 2' may represent lymphoid-specific factor(s). Species 2 resembles NF-A2 characterized by Staudt L.M. et al in that it was found only in lymphoid cells and it could be induced by treatment with mitogen[14]. Competition assay showed that the formation of both species 1 and species 2 or 2' was completely inhibited by the synthetic octanucleotide (fig.1 lane 3), but not by a nonspecific fragment, such as pBR322 (fig.1 lane 2). Since HP1 and the synthetic octanucleotide had only

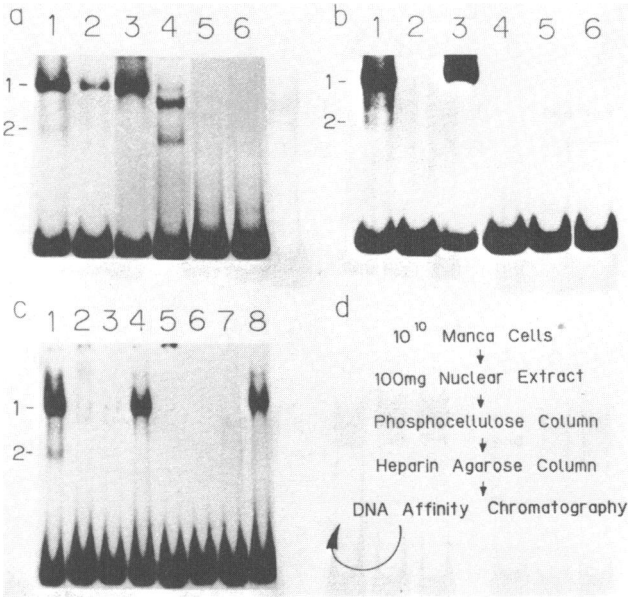

Fig.2 Column chromatography of the octamer binding proteins. The binding activity was examined by gel retardation assay. (a) phosphocellulose column. Lane 1, crude extract; lane 2, 0.1M KCl wash; lanes 3, 4, 5 and 6 show 0.2, 0.35, 0.5, 1M KCl fractions, respectively. 3µg of protein was used in each lane except for crude extract, where 8µg was used. (b) heparin agarose column. 0.2M KCl fractions eluted from phosphocellulose column (containing 20mg proteins) were pooled and applied to a heparin column. The proteins were eluted by stepwise with TM100 containing 0.1M, 0.2M, 0.35M, 0.5M and 1M KCl (shown in lanes 2-6, respectively). 8µg of crude extract (lane 1) or 1µg of protein from each fraction was used in the assay. (c) Purification by DNA affinity chromatography. The proteins eluted at 0.2M KCl from heparin agarose column (containing 7mg proteins) were dialyzed against the binding buffer, applied to an avidin column. The flow through were applied to a DNA affinity column, washed with the binding buffer and eluted successively by each 2ml of the binding buffer containing 0.1M, 0.2M, 0.3M, 0.5M and 1M NaCl. lane 1, crude extract; lane 2, 0.05M NaCl wash; lane 3, 0.1M; lane 4, 0.2M; lane 5, 0.3M; lane 6, 0.5M and lane 7, 1M NaCl fractions. lane 8, 0.2M eluate of the second DNA column chromatography. (d) Fractionation scheme for the purification of the octamer binding protein.

the octamer sequence in common, proteins responsible for species 1 and 2 or 2' did not recognize any additional sequences adjacent to the octamer or within the HP1. The bands found between species 1 and species 2 as well as other bands that migrated faster than species 2 were not inhibited specifically by the synthetic octamer.

Purification of an octamer binding protein

The purification scheme for sequence-specific DNA affinity chromatography

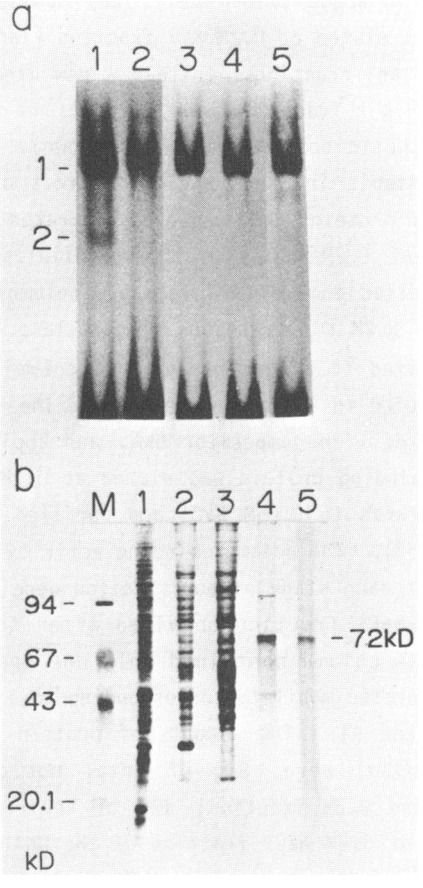

Fig.3 (a) Gel shift assay and (b)SDS polyacrylamide gel eletrophoresis[27] (PAGE) and silver staining[28] of fractions containing the octamer binding activity. In both panel a and b: lane 1, total nuclear protein. lane 2, phosphocellulose column 0.2M KCl fraction. lane 3, heparin column 0.2M KCl fraction. lane 4, DNA affinity column 0.2M fraction. lane 5, DNA affinity column 0.2M fraction (second pass). SDS PAGE was performed under reducing conditions. Numbers at the left refer to sizes (in kilodaltons) of molecular weight markers. The amounts of protein used in SDS PAGE were: 2µg of crude extract; 1.5µg of phosphocellulose column 0.2M KCl fraction; 1.5µg of heparin agarose column 0.2M KCl fraction; 10ng of DNA column 0.2M NaCl fraction (first pass); 2ng of DNA column 0.2M NaCl fraction (second pass).

of octamer binding proteins from Manca, a human B-lymphoblastoid cell line that produces large amounts of IgM protein, is summarized in fig.2d. The presence of the octamer binding activity was monitored by gel-mobility shift assay with HP1. Fig.2a shows a typical elution pattern when 100mg nuclear

proteins extracted from about $1x10^{10}$ cells was applied to phosphocellulose column. Species 1 was eluted at 0.2M KCl fraction (lane 3), however, species 2 was not detected in any fractions. Instead a new band that migrated faster was detected at 0.35M KCl fraction (lane 4), but its formation could not be inhibited by the synthetic octamer (data not shown). Species 2 generating protein(s) may be unstable in Manca nuclear extract or may be a complex of more than two kinds of proteins that could be separated by the chromatography. The proteins eluted at 0.2M KCl from phosphocellulose column were pooled, dialyzed and then applied to a heparin agarose column. The octamer binding activity was found in 0.2M KCl fractions (fig.2b,lane 3) and these fractions were pooled, and applied to a avidin-sepharose column to eliminate proteins that nonspecifically bind to the sepharose beads. The avidin sepharose column flow through were mixed with competitor DNA, and applied to a DNA affinity column. The octamer binding protein was eluted at 0.2M NaCl (fig.2c,lane 4). This fraction was diluted to 0.05M NaCl and purified again on the same DNA affinity column (lane 8). The octamer binding activity and SDS polyacrylamide gel electrophoresis at each stage of purification were shown in fig.3a and b, respectively. 0.2M NaCl fraction obtained after the second pass of the sequence-specific DNA column contained only one species of protein that exhibits an electrophoretic mobility corresponding to a molecular weight of 74 kilodaltons (fig.3b,lane 5). The amounts of protein used in the gel shift assay (shown in fig.3a) were: 8µg of total nuclear proteins; 3µg of phosphocellulose column 0.2M fraction; 1µg of heparin agarose column 0.2M fraction; about 10ng of 0.2M NaCl fraction of the DNA affinity column and 1-3ng of the second pass of the same column. Thus, the octamer binding protein has been purified to approximately 4000 fold in specific activity. The yield of the purified protein from 100 mg crude extracts was about 2µg.

DNase I protection assay

To define the area of protection of the purified 74kD octamer binding protein, DNase I protection (footprint) analysis was performed (fig.4a). The 74kD protein completely protected 8 bases of the octamer as well as 5 bases 3' and 4 bases 5' to the octamer (closed cycles) and partially protected additional 3 bases 3' to the octamer on the coding strand (open cycles), similar to the results obtained by singh,H et al[13].

Gel purification and renaturation of octamer binding protein

To provide more evidence that this 74kD protein represents an octamer binding protein, the proteins purified by second DNA affinity chromatography was loaded on a SDS polyacrylamide gel, electrophoresed at 4 C. The gel was

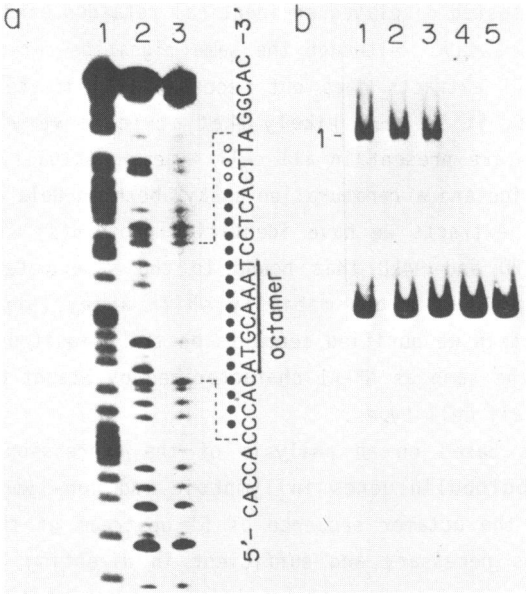

Fig.4 (a) DNase I protection assay. lane 1, A+G. lane 2, no extract. lane 3, 0.2M NaCl fraction obtained after the second pass of the DNA affinity column. (b) Renaturation of purified octamer binding protein. lane 1, native octamer binding protein obtained after the second pass of the DNA affinity column. lane 2 , 3 and 4, renatured protein. lane 5, proteins extracted and renatured from a region of the gel that did not contain the 74kD protein. lane 3 and lane 4 contained 10ng of pBR322 DNA and the synthetic octanucleotide as inhibitors, respectively.

stained and the 74kD region was excised. The protein was eluted and renatured. As shown in fig.4b, the renatured protein (shown in lane 2) gave rise to the same pattern as the native protein (lane 1) in a gel mobility shift assay, and the retarded band was competed for by the synthetic octanucleotide (lane 4) but not by non-specific DNAs (lane 3). A control sample containing excised and renatured proteins from a region of the gel other than 74kD polypeptide did not display any retarded band (lane 5). We conclude that the protein with a molecular weight of 74kD is responsible for the formation of octamer binding band species 1.

DISCUSSION

In the present study, we succeeded in purifying an octamer binding protein responsible for species 1 from a human B-lymphoblastoid cell line to an estimated homogeneity of more than 95%. All the cell types including non-

lymphoid cells we tested displayed an identical retarded band (species 1) in a gel mobility shift assay. Although the same migration-retarded band derived from different cell extracts does not necessarily indicate the presence of identical factors, it is very likely that at least very similar, if not identical, factors are present in all cell types. Actually, using SDS-PAGE followed by gel slice and a renaturation assay, both in Hela and Peer (a human T cell line) cell extracts we have identified protein(s) with the molecular weight between 67kD and 94kD that bound to the same octamer sequence and formed the species 1 in a gel mobility shift assay (unpublished data). Therefore, the protein we purified seems to be the ubiquitous octamer binding factor (possibly the same as NF-A1 characterized by Staudt L.M. et al)[14] and may be present in all cell types.

Recent studies based on an analysis of the expression of exogenously introduced immunoglobulin genes in lymphoid and non-lymphoid cells have demonstrated that the octamer sequence of 5' upstream of the immunoglobulin promoter region is necessary and sufficient in directing lymphoid specific transcription[2-8], and that the octamer binding protein(s) may mediate this tissue specific expression. On the other hand, this octamer sequence is not limited to immunoglobulin genes, it is also present in the transcription control elements of several genes such as human U2, U4c and mouse U6 small nuclear RNA (snRNA) gene promoters, and histone H2B gene promoter which do not show tissue specificity. The octamer motif is required for accurate transcription of these genes, therefore the ubiquitous octamer binding factor may not be a repressor molecule. Species 1 was detected in all cell types, whereas species 2 was restricted to lymphoid cells. Recently, Wirth et al[8] claimed that NF-A2 (corresponding to species 2) may be only a protein which expresses B-Lymphoid-Specific promoter activity and that NF-A1 (corresponding to species 1) may be needed to interact with other molecules to achieve the activation of the immunoglobulin gene. Thus, the 74kD protein, which is responsible for species 1, may be involved in the transcriptional regulation of not only immunoglobulin genes but also of other genes that contain a functional cis-acting octamer motif but do not show tissue-specificity, such as histon H2B genes. Species 1 and species 2 may derive from two functionally distinct nuclear proteins that possess the same octamer binding domain. Alternatively it is possible that the ubiquitous factor corresponding to NF-A1 or species 1 may be activated after modification such as phosphorylation (such a regulation has been identified in the yeast heat shock genes[26]). This modification may require the interaction with other molecules which could be

tissue-specific or induced by stimulation such as by treatment with mitogen. With the purification of the octamer binding protein, it is now possible to examine its transcriptional activity by an in vitro transcription assay[27] or by an in vivo assay such as by using microinjection of the protein into the cells[28] and to probe the possible interactions between the octamer binding protein and other binding factors, especially those interacting with immunoglobulin enhancer regions. And it is also possible to determine a partial amino acid sequences and prepare appropriate DNA probes to clone the gene encoding this factor. We are now undertaking these experiments. Purification of the B-cell-specific version of the octamer binding protein has been also in progress in our laboratory. All of these will facilitate the understanding of the mechanism of tissue specific transcription of the immunoglobulin genes.

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