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Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA)

Edgar Schreiber, Patrick Matthias, Michael M.Müller and Walter Schaffner

Institut für Molekularbiologie II der Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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The octamer sequence ATGCAAAT is found in the promoters of immunoglobulin (Ig) heavy and light chain genes and in the heavy chain enhancer and is a major determinant of the cell type specific expression of Ig genes in B cells. An apparent paradox is that the same sequence serves as an upstream promoter or enhancer element in a variety of housekeeping genes such as the histone H2B and U snRNA genes. The differential usage of this regulatory sequence motif is thought to be mediated by different species of octamer binding proteins. One species of 100 kd, designated OTF-1, is present in all cell types and may exert its activating function only when it can interact with additional adjacent transcription factors. The lymphoid cell specific octamer binding protein of 60 kd (OTF-2A) specifically stimulates Ig promoters which consist essentially of a TATA-box and an octamer sequence upstream of it. Here we present evidence for yet another B cell specific octamer binding protein of 75 kd (OTF-2B). From several findings, including the absence of OTF-2B (but not OTF-2A) from a lymphocyte line that cannot respond to the IgH enhancer, we propose a role of the novel octamer factor in the long range activation by the IgH enhancer. We have used the proteolytic clipping bandshift assay (PCBA) technique to distinguish the three different forms found in B cells. This anlaysis indicates that the 75 kd-species OTF-2B is closely related to the 60 kd species OTF-2A.

Key words: tissue specific gene expression/transcription factor/B lymphocytes/promoter/enhancer

Introduction

The B cell specific transcription of immunoglobulin (Ig) genes is controlled by a B cell specific enhancer (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983) and a B cell specific promoter (Mason *et al.*, 1985; Picard and Schaffner, 1985; Grosschedl and Baltimore, 1985). A considerable amount of work has demonstrated the central role of the octamer sequence motif ATGCAAAT (also referred to as the decanucleotide sequence ATGCAAATNA because of the conservation of another nucleotide) in B cell specific transcription (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Mason *et al.*, 1985; Gerster *et al.*, 1987; Lenardo *et al.*, 1987; Dreyfus *et al.*, 1987; Wirth *et al.*, 1987). The octamer sequence is present in promoters and enhancers of a variety of genes (reviewed in Müller *et al.*, 1988). Nuclear extracts from HeLa cells, and many other mammalian cell lines, contain a ubiquitous protein factor of 100 kd, designated OTF-1 (Fletcher *et al.*, 1987; Sive and Roeder, 1986; Singh *et al.*, 1986; Davidson *et al.*, 1986; Bohmann *et al.*, 1987; Sturm *et al.*, 1987), which specifically recognizes the octamer sequence. This factor has been implicated in the cell cycle regulation of histone genes (Fletcher *et al.*, 1987; LaBella *et al.*, 1988) as well as in the replication of adenovirus (Pruijn *et al.*, 1987; O'Neill and Kelly, 1988). Furthermore, a second, smaller protein species of 60 kd, designated OTF-2 by Scheidereit *et al.* (1987) is found only in B cells and also recognizes the same octamer sequence (Staudt *et al.*, 1986; Landolfi *et al.*, 1986; Gerster *et al.*, 1987). A simple model assumes that this protein, which we call here OTF-2A, mediates the B cell specific activity of promoters (Dreyfus *et al.*, 1987; Wirth *et al.*, 1987; Scheidereit *et al.*, 1987) and enhancers (Gerster *et al.*, 1987; Lenardo *et al.*, 1987) containing the octamer sequence.

In this paper we report that there is yet a second B cell specific octamer binding protein, which we call OTF-2B. It is larger in size (75 kd) and appears to be related, on the basis of limited proteolysis experiments, to OTF-2A. The possible role of this newly identified protein is discussed.

Results

Nuclear extracts from B lymphocytes contain three octamer binding complexes

In our initial studies we chose the *Dde*I−*Hin*fI fragment which contains the octamer sequence of the IgH enhancer (Figure 1). This DNA segment served as a target for binding of nuclear proteins, as detected by an electrophoretic mobility shift (bandshift) assay (Fried and Crothers, 1981; Garner and Revzin, 1981). The ^{32}P-end labelled DNA was incubated with nuclear extracts from HeLa cells (human cervix carcinoma epithelial cell line), BW 5147 cells (a human T cell line), Molt4 cells (a human T cell line), BJA-B cells (a human lymphoblastoid cell line of Burkitt lymphoma type), Namalva cells (a human Burkitt lymphoma line) and D1112 cells (a human spleen cell/mouse myeloma hybrid cell line) in the presence of poly [(dI)(dC)]as competitor to suppress non-specific binding. The mixture was electrophoresed through a native polyacrylamide gel and autoradiography identified specific protein−DNA complexes which migrated with a lower mobility than the unbound input DNA (Figure 2A). The slowest-migrating protein−DNA complex, which was seen in all extracts, is attributed to the binding of OTF-1, the ubiquitous octamer binding protein (Fletcher *et al.*, 1987). The mol. wt of purified OTF-1 has been reported to be 100 kd (Sturm *et al.*, 1987). The fastest-migrating protein−DNA complex, which was seen only in lanes 3−6 containing the extracts from B lymphocytes, represented binding of a second octamer binding protein (OTF-2A) which was first identified by Staudt *et al.* (1986).

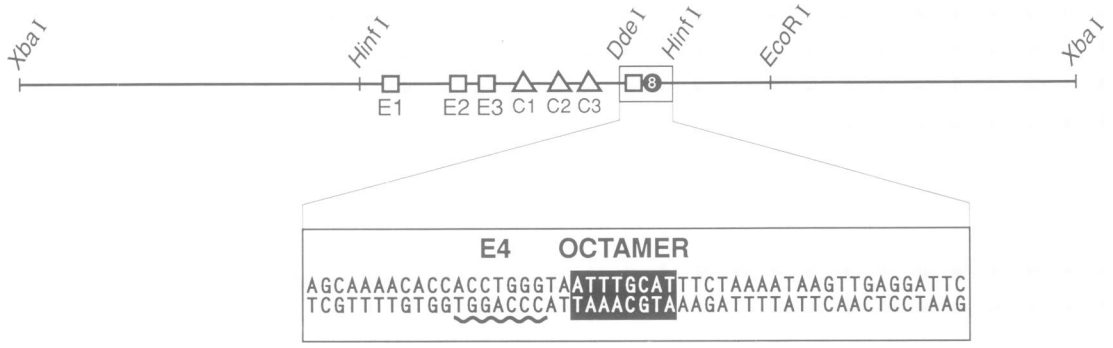

Fig. 1. Survey of the mouse Ig heavy chain gene enhancer (IgH). The original enhancer was isolated as a 997 bp *Xba*I−*Xba*I fragment (Banerji *et al.*, 1983). The internal 224 bp *Hin*fI−*Hin*fI fragment is sufficient for full activity in B cells (Gerster *et al.*, 1987). The boxes, designated E1−E4, represent the sequence elements that are protected from DMS-methylation due to proteins bound specifically in lymphoid cells (Ephrussi *et al.*, 1985). The triangles (C1, C2, C3) represent sequences which are related to the enhancer 'core' consensus sequence $GTGG^{TTT}_{AAA}$ defined by Weiher *et al.* (1983). The 51 bp *Dde*I−*Hin*fI fragment was shown to be the smallest fragment conferring features of a B cell specific enhancer (Gerster *et al.*, 1987). Within this fragment, which is shown enlarged with its sequence, both the E4 element (see above) and the octamer sequence (by some authors referred to as the decanucleotide sequence) can independently contribute to B cell specificity of transcription (Gerster *et al.*, 1987). The *Dde*I−*Hin*fI fragment was used as probe for the bandshift assay.

As will be outlined below, the bands observed in this experiment correspond to different octamer binding factors, as was verified by bandshifts with individual factors recovered from a SDS−polyacrylamide gel. OTF-2A seems to be a transcription factor crucial for lymphoid-specific gene expression (Scheidereit *et al.*, 1987). The protein occurs as a number of subtypes of 61, 60 and 58 kd. The smallest subtype of 58 kd could also be detected as a faint band which migrated slightly faster than the bulk of OTF-2A.

To our surprise, with extracts from B lymphocytes we consistently observed the appearance of a third octamer binding protein which migrated between the OTF-1 and OTF-2A protein−DNA complexes (Figure 2A, lanes 4−6). In the extracts from BJA-B lymphocytes the intensity of this intermediate band was even more prominent than the signal from OTF-1. However, in Namalva extract the extra OTF band was a minor one. This intermediate complex was typically seen in extracts from B lymphocytes. However, the same complex formation was also seen in extracts from Molt-4 cells (a human T-cell line which expresses immunoglobulin genes) as well as in B cells. By contrast, a different T cell line (BW 5147) which does not express Ig genes also lacked these two faster migrating complexes (Figure 2A, lane 2). A pattern of three complexes was also seen in bandshift experiments performed with extracts from human spleen or mouse myeloma X63Ag8 cells (data not shown). This indicated that the novel form was present also in non-human B cells. However, the human spleen cell/mouse myeloma hybrid cell line D1112 produces Igs but fails to support transcription of exogenously introduced test-genes under the control of the IgH enhancer (P.Matthias, unpublished results). Nuclear extracts from this cell line revealed complexes corresponding to OTF-1 and OTF-2A but the intermediate complex could not be detected (Figure 2A, lane 3). The appearance of the intermediate form was not dependent on a given octamer binding site, since it was also observed when different DNAs with octamer sequences were used for the bandshift assay.

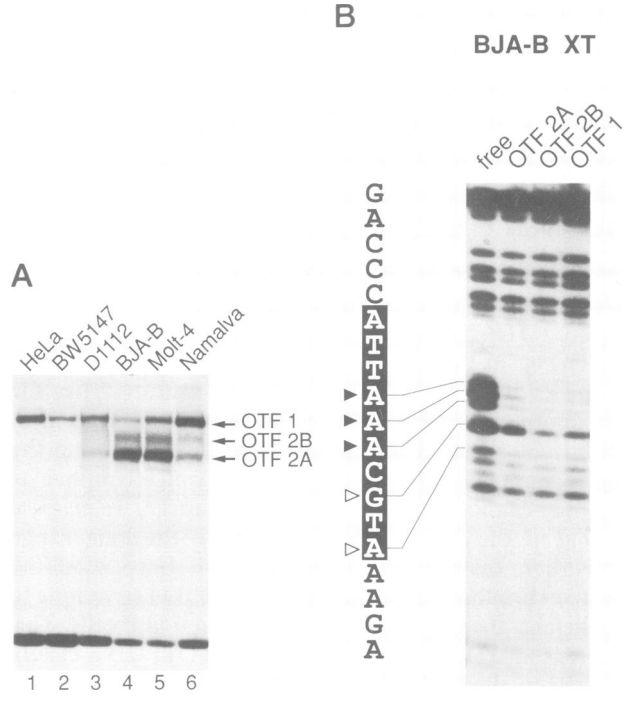

Fig. 2. (**A**) Bandshift assay with the octamer DNA probe and nuclear extracts prepared from different cell lines (for a description of the cell lines see text). (**B**) Methylation interference assay reveals the region of contact points of the octamer binding proteins. The partially *N*-methylated DNA (label at lower strand, Figure 1) which is still bound by these proteins, was isolated from the retarded complexes prepared from a bandshift assay done with nuclear extract from BJA-B cells, and subjected to cleavage with piperidine before separation on a sequencing gel. Black triangles indicate missing bands, i.e. strong interference with binding, open triangles indicate nucleotides with partial interference. No factor binding could be detected in the adjacent E4 sequence.

Methylation interference analysis reveals identical contact points for the three octamer protein−DNA complexes

We next asked where exactly the proteins present in the three complexes bind to the octamer sequence and determined the region of intimate contact by methylation interference analysis (Siebenlist and Gilbert, 1980).

Both strands of the IgH enhancer *Dde*I−*Hin*fI fragment were partially methylated and used for a preparative bandshift assay with extracts from BJA-B cells. After separation of the complexes from the unbound DNA, the DNA in the various retarded complexes and the input DNA were recovered from the gel and subjected to cleavage by alkali-treatment. If a methyl group in a DNA molecule was situated

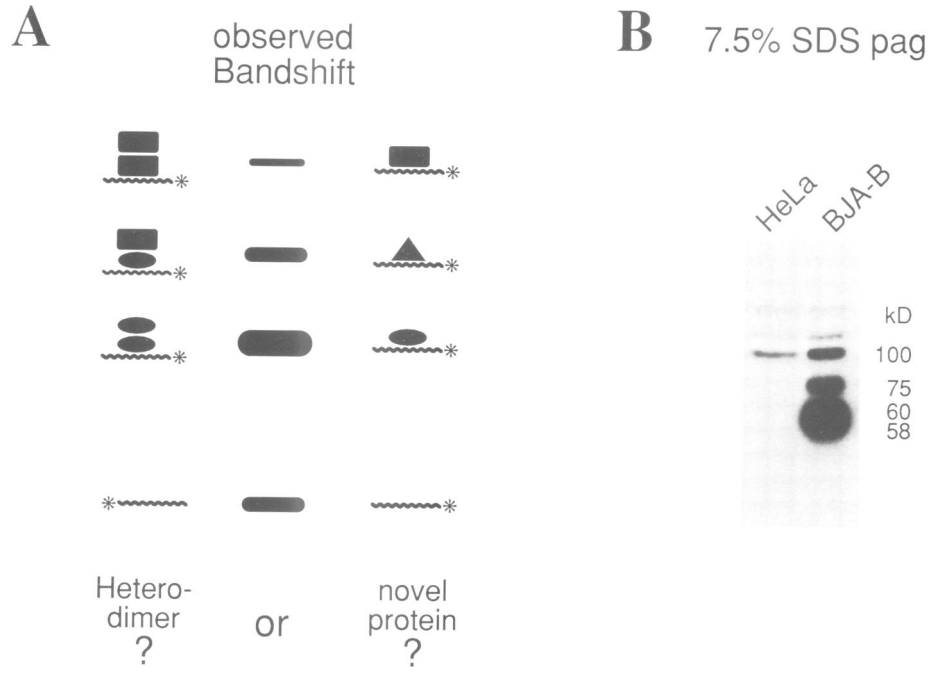

Fig. 3. 'Southwestern' blot analysis with nuclear proteins from BJA-B and HeLa cells. (**A**) The schematic outline on the left illustrates the original issue: are the complexes seen in the bandshift assay due to three monomeric proteins or due to heterodimerization? (**B**) Autoradiograph of the Southwestern blot. HeLa cell or BJA-B cell nuclear extracts were size-separated in a denaturing protein gel, blotted to a nitrocellulose filter, renatured and incubated with radiolabelled octamer DNA. Signals seen at 100, 75 and ~60 kd are specific for the octamer probe. The low intensity signals at positions of 120 kd and upwards are due to non-specific binding, as we have ascertained by incubating a control filter with a different probe which also binds to these high mol. wt proteins, but not to the regions in which the octamer proteins migrate (data not shown).

in the protein binding site in such a way that it interfered with binding of a specific protein, this DNA molecule was selectively underrepresented in the complexed form relative to the unbound DNA. This bias was detected as a missing band in the G/A ladder of the cleaved and gel-fractionated sample. As seen in Figure 2B, all three complexes displayed exactly the same interference pattern. This implies that the mode of DNA recognition is the same for all three octamer binding proteins.

'Southwestern' blot analysis identifies a novel 75 kd octamer binding protein

Since the DNA–protein contact points were identical for all three complexes, but only two octamer binding proteins (OTF-1 and OTF-2A) had been identified by others, we considered the following possibilities to explain the identity of OTF-2B: (i) it is a genuine B-cell specific protein, perhaps related to OTF-2A; (ii) it is a modified or precursor form of OTF-2A; (iii) it is a heterodimer between OTF-1 and OTF-2A (or another cellular protein); (iv) it is a specific degradation product of OTF-1. To test the heterodimer hypothesis (which is schematically outlined in Figure 3A) we decided to perform 'Southwestern' analysis of nuclear extracts (Hübscher, 1987). Denatured nuclear proteins from BJA-B cells and HeLa cells were size-separated in an SDS–polyacrylamide gel and then transferred to a nitrocellulose filter. After allowing the proteins to renature, the filter was incubated with ^{32}P-labelled octamer-DNA probe. Autoradiography of the filter revealed the position of the octamer binding proteins which were then compared to the position of appropriate size markers. As seen in Figure 3B, the octamer probe specifically bound to three regions. The most prominent binding occurred to three closely migrating bands of 58–61 kd (short exposure; not shown). This is in accordance with the published mol. wt of the B cell specific octamer binding protein OTF-2A (Scheidereit *et al.*, 1987). The ubiquitous octamer-binding protein, OTF-1, was seen in the 'Southwestern' blot at a position corresponding to 100 kd, which again agreed with published data (Sturm *et al.*, 1987). Specific binding of the octamer DNA probe to a region in the gel corresponding to a mol. wt of 75 kd identified a novel octamer binding protein which was seen only in nuclear extracts from B cells and not HeLa cells. To see whether the three proteins detected in the 'Southwestern' blot accounted for the three bands seen in the bandshift assay we also separated nuclear proteins of lymphoid cells in SDS–protein gel and renatured the eluted proteins for DNA binding (see Materials and methods). This analysis showed that octamer DNA binding activity was found at ~100, 75 and 60 kd (see below, Figure 4). We conclude that the lymphoid specific octamer binding protein of 75 kd, OTF-2B, is a genuine protein rather than a heterodimer between OTF-1 and OTF-2A.

'Proteolytic clipping' of isolated octamer binding proteins reveals the different structure between the ubiquitous form and the lymphoid forms

To investigate the nature of the relationship between OTF-1, OTF-2A and OTF-2B we had to separate the three forms. Applying standard chromatographic procedures such as FPLC MonoQ and MonoS ion exchange, Heparin–Sepharose, Sephacryl S300 gel filtration and octamer-DNA specific affinity chromatography, we achieved a several hundred-fold purification of the octamer binding proteins,

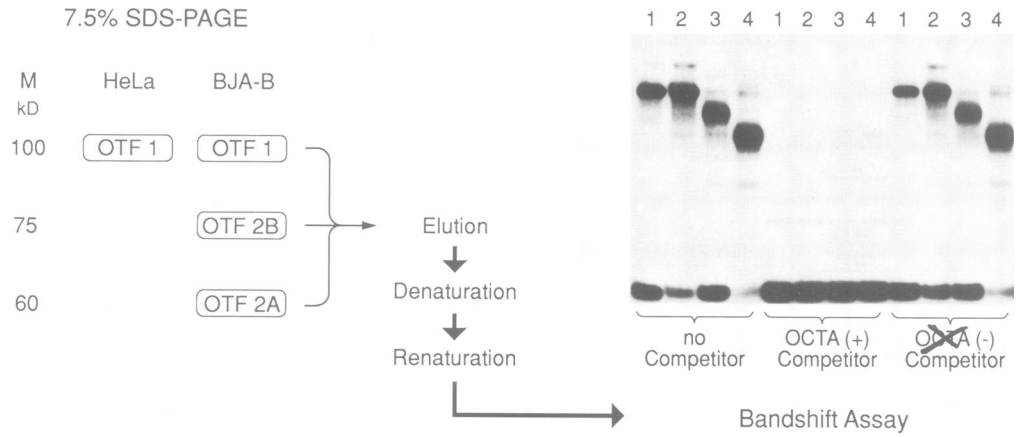

Fig. 4. Separation of the three octamer-binding proteins by preparative SDS−PAGE and renaturation. Nuclear proteins were separated on a denaturing protein gel. Gel slices were taken from the regions of 100, 75 and 60 kd, respectively. The eluted proteins were subjected to a denaturation/renaturation protocol as described in Materials and methods. A 5 μl from a total of 1500 μl renatured protein was used for each bandshift analysis. **Lane 1**: 100 kd proteins (OTF-1) from HeLa cell nuclear extract; **lane 2**: 100 kd proteins (OTF-1) from BJA-B nuclear extract; **lane 3**: 75 kd proteins (OTF-2B) from BJA-B cell nuclear extract; **lane 4**: 60 kd proteins (OTF-2A) from BJA-B nuclear extracts. The specificity of the complexes formed is demonstrated by addition of excess unlabelled competitor DNA; octa(+) is the *Dde*I−*Hin*fI fragment (see Figure 1); in the octa(−) DNA the octamer sequence is mutated in three out of eight nucleotides (see Materials and methods). The position of migration of the retarded complexes with the gel-isolated OTF-proteins was identical with the position of migration of the respective OTF-proteins in the unfractionated nuclear extract (data not shown).

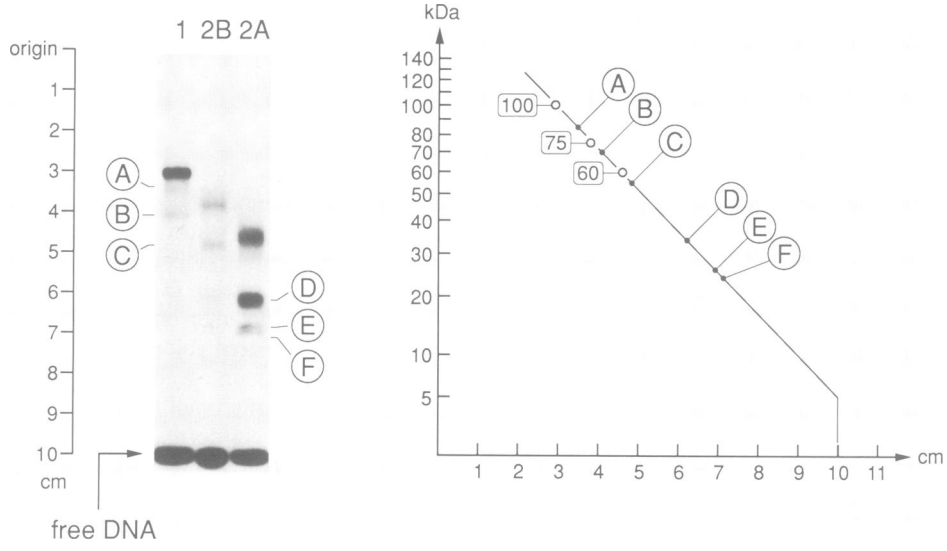

Fig. 5. Proteolytic clipping bandshift assay (PCBA); the isolated 100, 75 and 60 kd species of the octamer-binding proteins were subjected to limited proteolysis while they remain bound to the octamer DNA template in solution. Undigested and clipped proteins are separated from free DNA in a native bandshift gel. The distance of migration of the respective intact octamer proteins OTF-1 (100 kd), OTF-2B (75 kd) and OTF-2A (60 kd) are plotted versus their mol. wts on a logarithmic scale (open circles). The putative mol. wts of the truncated proteins (A−F) were deduced by extrapolation.

however, with all procedures the three forms co-purified almost completely so that the desired separation could not be achieved (data not shown).

We found that preparative SDS−PAGE of nuclear extracts was a simple, accurate and powerful method to separate the octamer binding proteins with respect to their mol. wts. OTF-1 from HeLa cells and OTF-1,2A and B from BJA-B cells were prepared from 7.5% SDS−protein gels (Laemmli, 1970) and renatured (Hager and Burgess, 1980) for octamer DNA-binding as described in Materials and methods. Figure 4 depicts the protocol and shows the bandshift analysis with the isolated and renatured octamer binding protein. Note that the proteins isolated from the 75 kd region of the SDS−gel gave rise to the intermediate band in the bandshift gel which confirms our previous conclusion that this is a distinct species. The specificity of these interactions is demonstrated by competition experiments. Figure 4 shows that addition of a 1000-fold molar excess of unlabelled octamer-DNA eliminated the binding of the octamer binding factors to a labelled fragment. An unlabelled competitor DNA with a mutated octamer binding site did not interfere with OTF binding to the labelled target site (Figure 4). These gel-purified and renatured protein samples were employed for the subsequent 'proteolytic clipping' analysis, the principle of which is outlined in Figure 5.

^{32}P-labelled octamer DNA fragment was incubated with

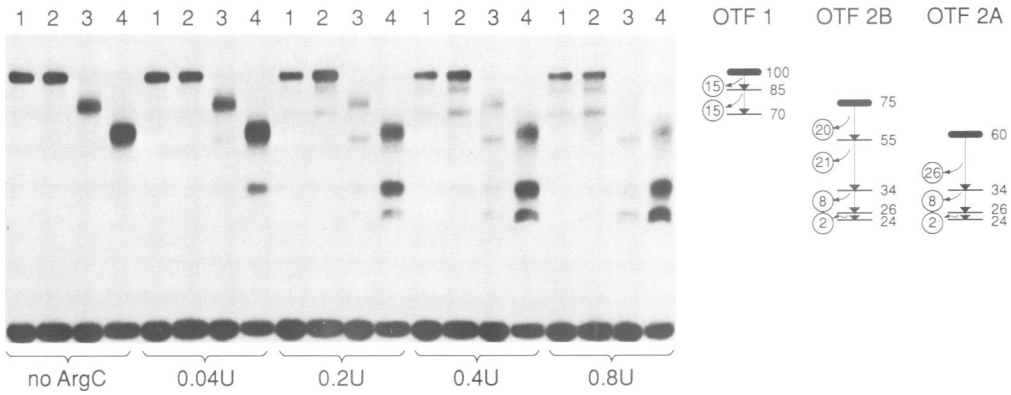

Fig. 6. Proteolytic clipping of octamer DNA–protein complexes with ArgC protease. A scheme is shown on the right part which depicts the putative sizes (in kd) of the original and the truncated proteins. The numbers in circles indicate the estimated sizes (in kd) of the peptides clipped off the core region. **Lane 1**: OTF-1 from HeLa cell nuclear extract; **lane 2**: OTF-1 from BJA-B nuclear extract; **lane 3**: OTF-2B from BJA-B extract; **lane 4**: OTF-2A from BJA-B nuclear extract.

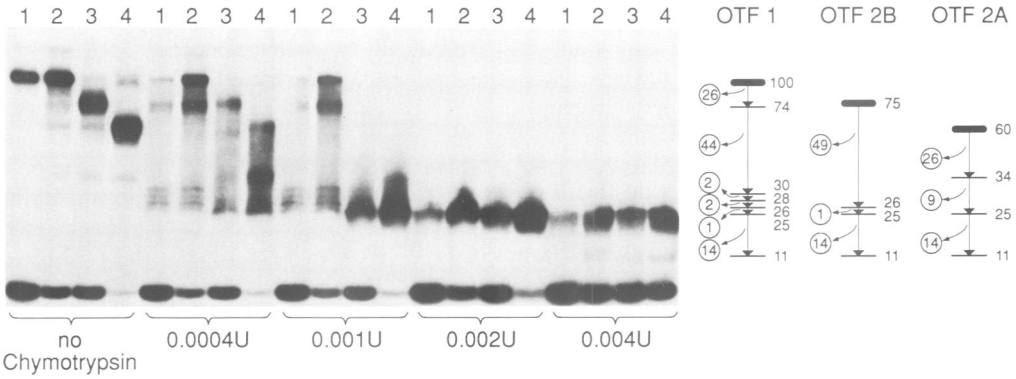

Fig. 7. Proteolytic clipping with chymotrypsin; **Lane 1**: OTF-1 from HeLa cell nuclear extract; **lane 2**: OTF-1 from BJA-B nuclear extract; **lane 3**: OTF-2B from BJA-B extract; **lane 4:** OTF-2A from BJA-B nuclear extract.

each of the OTF isolates under the usual bandshift conditions. Freshly diluted protease was added to the protein–DNA complex and allowed to react for 10 min at room temperature. The mixture was then loaded on a bandshift gel and electrophoresed as usual. Autoradiography revealed a collection of faster migrating bands which represent fragments of the particular OTF bound to the octamer DNA. Peptide chains which have been clipped off by the protease escape detection, since only the DNA-bound remainder is monitored. The position of migration of the original octamer binding proteins within the bandshift gel and their mol. wt followed a (semi-logarithmic) linear correlation (Bading, 1988). Plotting the distance of migration from the slot of the bandshift gel to the centre of the radioactive signal versus the natural logarithm of the mol. wts of OTF-1, OTF-2B and OTF-2A resulted in a straight line (see Figure 5). This line could be extrapolated further and enables us to determine the putative mol. wt of the DNA-bound protein fragments. The reason for the linearity may be that the net charge of the protein is overcome by the strong negative charge of the DNA which is the driving force to the anode. Any retardation in this mobility may therefore simply be a function of the molecular mass bound to the DNA. This approach worked out successfully for the different octamer binding proteins, but may not be valid for every DNA-binding protein. For reasons of simplicity we assume linearity over a wide range although we realize that the values given are only tentative and serve solely for a qualitative description of the following experiments.

Clipping with ArgC protease. In the experiment shown in Figure 6, the octamer proteins were exposed to the protease ArgC which cleaves specifically after arginine residues. OTF-1, the ubiquitous 100 kd species, was clipped into remainder fragments of 85 and 70 kd. The 75 kd species OTF-2B yielded 55, 34, 26 and 24 kd fragments whereas the 60 kd species OFT-2A was clipped into 34, 26 and 24 kd fragments, i.e. to a set of fragments shared with OTF-2B. The lymphoid specific octamer binding protein OTF-2A and B were more sensitive towards this particular protease than OTF-1. With 0.8 units of ArgC protease, >80% of these proteins were cleaved to faster migrating species whereas the majority of OTF-1 remained unaffected. No difference in the clipping pattern of the ubiquitous octamer binding protein OTF-1 was seen whether it was isolated from HeLa cells or from BJA-B cells. We conclude from this result that they are most likely identical. As mentioned, OTF-2A and B shared DNA binding domains of 34, 26 and 24 kd. The regularity of this degradation pattern suggests a strong homology between both proteins. The band seen at 55 kd for OTF-2B, which was not seen in OTF-2A might indicate that OTF-2B (the 75 kd species) does not follow a 'precursor–product' relationship to OTF-2A (the 60 kd species) but is rather indicative of a novel octamer binding

protein. However, the possibility remained that the critical arginine residue is also present in OTF-2A but not accessible by the ArgC protease. We tried to test this issue by clipping with thrombin which is a particularly active arginine-specific protease. The same picture emerged as with ArgC except that the OTF-1 isolates were clipped one step further from 70 to 61 kd. Again, OTF-2B was clipped to 55 kd whereas OTF-2A did not give such an intermediate band (data not shown).

Clipping with chymotrypsin. Although chymotrypsin is a quite non-specific protease which cleaves peptide bonds after aromatic amino acids, as well as leucine, methionine and alanine, a defined clipping pattern was observed for the octamer binding proteins (Figure 7). OTF-1 was cut six times, leaving DNA-bound domains of 74, 30, 28, 26, 25 and 11 kd. OTF-2B was cleaved from 75 kd three times to 26, 25 and 11 kd and OTF-2A was clipped from 60 to 34, 25 and 11 kd. These results confirm our previous notion that OTF-1 is different from OTF-2A and provide further evidence (though circumstantial) that OTF-2B is probably not a precursor of OTF-2A, since if that were the case one might expect a 34 kd fragment for OTF-2, which however was not observed to any significant extent. So far, the largest fragment common to all octamer binding proteins was of 25 kd which probably indicates homology in the DNA-binding domain. The latter result was not unexpected since we have shown that all three forms bind identically to the octamer-binding site. This 'core' DNA binding domain of 25 kd could be clipped further to ~11 kd. We find it remarkable that removal of ~50% of the protein mass of OTF-2A did not lead to a decrease in the binding affinity to the octamer-DNA (compare unbound DNA in lane 4 in the panel without protease and with 0.001 U chymotrypsin).

Clipping with trypsin. Trypsin cleaves proteins after arginine or lysine residues. As seen in Figure 8, OTF-1 was clipped 5 times from 100 kd to fragments of 61, 28, 24, 17 and 10 kd. OTF-2B was clipped from 75 kd to 61, 45, 28, 24, 17 and 10 kd fragments. OTF-2A was clipped from 60 kd to 45, (28?), 24, 17, 10 and 8 kd. The clipping pattern of OTF-2B reveals intriguing clues to the 'identity' of this 75 kd octamer binding protein. The 61 kd band was common to OTF-1 and OTF-2B (we have surveyed autoradiographs of three independent experiments to verify that this fragment is slightly larger than intact OTF-2A). However, the band seen at 45 kd was common to OTF-2A and B and is therefore a lymphoid cell specific hallmark. These results also exclude the above-mentioned formal possibility that OTF-2B is a degradation product of OTF-1.

The most likely interpretation of this digestion pattern is that the 75 kd octamer binding protein (OTF-2B) displays features in common with both the ubiquitous type as well as the lymphoid type octamer binding protein. The largest DNA-binding domain common to all types is 24 kd which is similar to the value measured with chymotrypsin. This 'core' DNA-binding domain could be further clipped to fragments of 10 kd for all octamer binding proteins. With the OTF-2A sample the smallest fragment observed was 8 kd. If this value is taken as correct, this minimal DNA binding domain would consist of 75–85 amino acids.

Discussion

By applying 'proteolytic clipping bandshift assay' (PCBA) on size-separated nuclear proteins of human lymphoid cells we have identified a 75 kd protein as a novel factor that specifically binds to the octamer DNA motif. Like OTF-2A, its presence is restricted to lymphoid cells. Following the OTF-1/OTF-2(A) terminology of Fletcher *et al.* and Scheidereit *et al.* we have designated this protein OTF-2B. Regarding the binding specificity to the octamer sequence ATGCAAAT, the OTF-2B protein is indistinguishable from the other known octamer binding proteins from human cells, OTF-1 (Fletcher *et al.*, 1987) and OTF-2A (Scheidereit *et al.*, 1987) but it clearly differs in size as determined by band-shift and SDS–protein gels. We have shown that the clipping pattern of OTF-2B has features which are characteristic for both the ubiquitous type OTF-1 as well as for the B cell specific type OTF-2A. We consider it therefore likely, that the three proteins are encoded from different, but related, genes which probably share regions of homology. A good candidate for such an homologous sequence will be that coding for the DNA-binding domain since all three proteins bind identically to the octamer sequence and have similar DNA-binding domains as demonstrated when clipped by trypsin or chymotrypsin to a 'core' of ~25 kd. Alternatively, it is conceivable that OTF-2A and OTF-2B are encoded by one gene and transcribed into a common precursor transcript which is then differentially spliced.

The question arises why OTF-2B, which is a prominent band in the bandshift assay (Gerster *et al.*, 1987), has not been observed by other laboratories. Wang *et al.* (1987) reported the purification of a 70 kd octamer binding protein

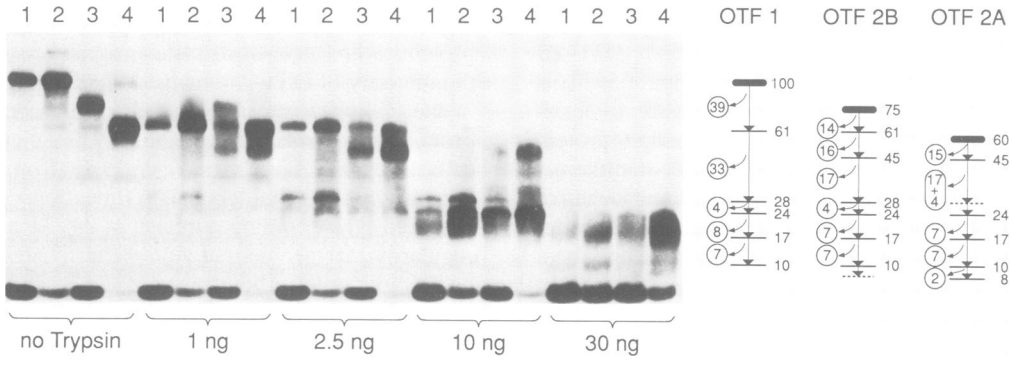

Fig. 8. Proteolytic clipping with trypsin; **lane 1**: OTF-1 from HeLa cell nuclear extract; **lane 2**: OTF-1 from BJA-B nuclear extract; **lane 3**: OTF-2B from BJA-B extract; **lane 4**: OTF-2A from BJA-B nuclear extract.

from human B cells. However, this protein is not OTF-2B because the authors have stated themselves that the protein which they isolated is a degradation product of the ubiquitous form OTF-1. The literature describing interactions of nuclear proteins from various cell lines with the octamer motifs is abundant (Hromas and Van Ness, 1986; Ballard and Bothwell, 1986; Singh *et al.*, 1986; Sen and Baltimore, 1986; Mocikat *et al.*, 1986; Staudt *et al.*, 1986; Rosales *et al.*, 1987; Lenardo *et al.*, 1987) but OTF-2B may have gone unnoticed due to early technical impediments. Using nuclear extract of mouse BCL-1B lymphocytes, Landolfi *et al.* (1986, 1987, 1988) and Hanke *et al.* (1988) have observed a similar lymphoid-specific bandshift pattern as ours, except that there was an additional complication due to protein binding to the heptamer sequence CTCATGA next to the octamer motif. They find a ubiquitous band of ~80–90 kd and two lymphoid cell-specific bands of 70 and 55 kd, the former of which increases in abundance upon induction of BCL-1 lymphocytes with bacterial lipopolysaccharides (LPS), a mitogenic stimulator specific for B cells. This LPS-mediated modulation of an octamer factor correlates with an up to 10-fold higher level of Ig transcription. After a close inspection of their data we propose that the 80–90 kd protein of Hanke *et al.* (1988) corresponds to the ubiquitous OTF-1 (100 kd), while their 70 and 55 kd bands correspond to OTF-2B (75 kd) and OTF-2A (~60 kd). Also, our side-by-side comparison of human and mouse extracts shows that there is no apparent size difference of the corresponding proteins between these species, i.e. the estimates of Hanke *et al.* (1988) and Landolfi *et al.* (1986, 1987, 1988) are probably too low. This may be the reason why their 70 kd factor was not recognized as a protein different from the lymphoid specific 60 kd factor (NF-A2 resp. OTF-2) described by others (Staudt *et al.*, 1986; Scheidereit *et al.*, 1987).

Is the novel octamer binding protein OTF-2B responsible for remote control by the IgH enhancer?

Several observations suggest a positive role of the 75 kd protein OTF-2B in Ig gene transcription. Although it may seem at first glance paradoxical, the mouse '70 kd protein' that corresponds most likely to OTF-2B is present at high level in nuclear extracts of EL-4 mouse thymoma cells (Landolfi *et al.*, 1986), but it is not detectable in extracts of BW 5147 T cell lymphoma cells (see Results). Interestingly, the EL-4 cell-line is permissive for the action of the IgH enhancer because it expresses abundant 'sterile' μ-transcripts (Kemp *et al.*, 1983) which typically initiate 43–86 bp downstream of the octamer sequence in the Ig heavy chain enhancer (Lennon and Perry, 1986). This correlation between the presence of the mouse '70 kd protein' (OTF-2B) and Ig gene transcription (which in this case initiates close to the octamer sequence in the IgH enhancer) is corroborated by our results that extracts of Molt-4 cells, which express heavy chain genes and respond to the IgH enhancer, contain both OTF-2A and OTF-2B. Along this line it is noteworthy to recall the observation that extracts of D1112 cells, which express Ig but are incapable of responding to a newly introduced IgH chain enchancer, did not display the OTF-2B complex in the bandshift analysis.

From all these observations we suggest a role of the 75 kd protein OTF-2B for the activation of the Ig gene enhancer, thereby contributing to the tissue-specific expression of the Ig genes.

The PCBA analysis reveals the subtle differences in otherwise indistinguishable DNA-binding proteins

In the work presented here we have introduced a technique for the refined analysis of protein–DNA complexes which we would like to refer to as 'proteolytic clipping bandshift assay' (PCBA). The principle of PCBA is based on indirect tagging of DNA-binding proteins by virtue of binding them to their ^{32}P-labelled DNA target site on which they are clipped by proteases until the DNA-binding domain is destroyed. The DNA-bound remainder is separated from unbound DNA by native gel electrophoresis and subsequent autoradiography reveals a pattern of retarded bands which is specific for a given protein clipped by a given protease. This feature may serve to distinguish a particular DNA-binding protein from another related but not identical species with the same DNA-recognition sequence. We can see potential applications of this procedure whenever the relationship between DNA binding proteins is under investigation. This technique could in principle be extended by employing certain carboxypeptidases and/or amino-peptidases which progressively remove amino acid residues from either end of the DNA-bound protein. It should thus be possible to approximately map the location of the DNA-binding domain within the protein studied. Recently, sophisticated techniques for screening cDNA expression libraries have been developed, which are especially designed for the isolation of genes coding for DNA binding proteins (Singh *et al.*, 1988; Vinson *et al.*, 1988). In consequence, rapid progress is to be expected in the analysis of a number of cDNAs coding for transcription factors. The PCBA technique can be performed with recombinant protein from bacterial lysates or from in vitro translations and then compared with the clipping pattern of the original protein from nuclear extracts. This should provide further evidence for the identification of the desired cDNA clone. The clipping pattern reflects indirectly (by accessibility of a protease) the intrinsic property of a native protein to display unique folding and arrangement of its domains.

The precise analysis of the relationships between the different octamer binding proteins will require comparative sequence analysis of the cloned genes. Recently, our laboratory has succeeded in isolating a cDNA clone (from a human B cell library) which codes for a lymphoid specific octamer binding protein with a size of 60 kd, presumably one of the subvariants of OTF-2A, and which has the ability to activate transcription of otherwise silent B cell specific promoters in a non-B cell (Müller *et al.*, in press). The analysis of cloned octamer factor genes should also be instrumental in sorting out the different functions of OTF-1, and OTF-2A and OTF-2B.

Materials and methods

Preparation of the probe for bandshift analysis

The *Dde*I–*Hin*fI fragment (nucleotides position 518–566) from the IgH enhancer was subcloned into the *Sal*I-site of pUC18. A 51 bp *Sal*I fragment was prepared from this clone, dephosphorylated with CIAP (Boehringer Mannheim) and 5′-end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A specific activity of 1×10^6/pmol 5′-end was usually achieved. For competition experiments an unlabelled fragment containing four copies of the above-mentioned sequence was used (octa+); the (octa−) fragment

contained four copies of a sequence in which the octanucleotide motif was eliminated by mutation from ATGCAAAT to CTGAACAT.

Cell lines and preparation of nuclear extracts

BJA-B cells and other lymphoid cells were cultured in 41RPMI-1640 medium (Sigma) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg streptomycin in suspension in 6 l round bottles. The cells were grown to a density of 1×10^6 cells/ml. HeLa cells were grown in suspension in DMEM-medium (GIBCO) supplemented with 2.5% horse serum. Frozen HeLa cell pellets were a kind gift of B.Lüscher. Nuclear extracts were prepared as described by Dignam *et al.* (1983) with minor modifications. After ammonium sulphate precipitation the extract was resuspended in, and dialyzed against, buffer X50 containing 20 mM Hepes, pH 7.9, 20% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF (phenylmethylsulphonylfluoride; Sigma).

Methylation interference assay

This assay (Siebenlist and Gilbert, 1980) was done as described by Petterson and Schaffner (1987).

Preparative SDS–PAGE and renaturation of DNA-binding activity

Nuclear extract (50 μl; 300–600 μg protein) was denatured by the addition of 10 μl 10% SDS and heating at 90°C for 10 min. The sample was loaded on a 1.5 mm 7.5% SDS–polyacrylamide gel (Laemmli, 1970) and size-fractionated. A lane with size-markers (BioRad high MW) was cut from the gel and stained in 0.3 M $CuCl_2$ for 5 min (Lee *et al.*, 1987). A self-made tool with two rods fixing 20 razor blades, separated from each other by a distance of 2.5 mm, was used for cutting the gel. The 19 slices were cut into small cubes and proteins were eluted by diffusion at 37°C for 24 h under agitation in 1 ml elution buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 150 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1% SDS, 200 μg/ml BSA). The eluate was precipitated by addition of 5 volumes cold acetone (−20°C) and left at −70°C for 4 h. The samples were centrifuged at 10 000 r.p.m. for 30 min in a Sorvall HB4-rotor. The pellet was washed with 10 ml 80% acetone (−20°C), centrifuged and air-dried. The proteins were resuspended in 20 μl 6 M guanidine–HCl in X50 buffer and left at 20°C for 20 min. For renaturation we also followed the protocol of Hager and Burgess (1980) except that we used X50 with 250 μg/ml BSA as a dilution buffer. Proteins were renatured for 20 h at 20°C. We estimated a value of 50% renaturation of octamer binding activity.

'Southwestern' blotting

This was performed according to the protocol of Hübscher (1987) with modifications. Nuclear extract (50 μg/ml) was denatured by adding 1/5 volumes 10% SDS, heated at 90°C for 10 min and size-fractionated by analytical 7.5% SDS–PAGE. Proteins were electrotransferred onto nitrocellulose filters (Schleicher & Schuell) in 25 mM Tris–190 mM glycine, 1 mM EDTA and 0.01% SDS. Proteins bound on the filter were renatured by incubation at 4°C for 24 h in 100 ml of 10 mM Hepes, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 5% milk powder (Carnation non-fat). For DNA-binding the filter was transferred into a bath containing 15 ml binding buffer (same composition as above except only 0.25% milkpowder is used) with 10 μg poly[(dI)(dC)] and 2 pmol labelled four tandem copies of the *DdeI–HinfI* fragment and was gently shaken at 4°C for 12–15 h. Filters were washed twice in 50 ml binding buffer each (without DNA and poly[(dI)(dC)]) for 15 min, air-dried and exposed to X-ray film.

Proteolytic clipping bandshift assay

Binding reactions (15 μl) were done by incubating a radiolabelled *SalI* fragment containing the *DdeI–HinfI* segment with the octamer site (0.2–0.5 ng; 3000–4000 c.p.m.) with 2 μg non-specific competitor poly[(dI)(dC)] in a buffer containing 4% Ficoll, 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT and 0.25 mg/ml bovine serum albumin (BSA, Boehringer Mannheim) and nuclear extract or renatured protein fractions which were added last (1–5 μl; 10 ng–5 μg). Poly[(dI)(dC)] (200 ng) was used for renatured protein fractions. After 10 min incubation at 20°C, 1 μl protease (freshly diluted in H_2O) at the indicated concentration was added to the reaction mixture and allowed to react for 10 min at 20°C. Due to the large excess of carrier protein (BSA) present in the renaturation and bandshift binding buffer, virtually the same overall protein concentration was present in each sample. ArgC protease and trypsin were from Boehringer Mannheim and chymotrypsin was from Worthington. The sample was loaded directly (without any dyes) on a 4% polyacrylamide gel (30:1 crosslink; SERVA chemicals) which was pre-run for 2 h at 20°C at 12 V/cm in 0.25 × TBE. Samples were electrophoresed at 12 V/cm for 100–120 min. Gels were dried and exposed to X-ray film (Fuji). Competition experiments were done by mixing the appropriate competitor DNA (in a 500- to 1000-fold molar excess over the labelled probe) to the binding reaction before adding the protein fraction.

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