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Nuclear factors binding specific sequences within the immunoglobulin enhancer interact differentially with other enhancer elements

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The mouse immunoglobulin heavy chain (IgH) enhancer represents a *cis* essential control element that confers lymphoid-specific expression. Based on *in vivo* and *in vitro* competition experiments, as well as on *in vivo* dimethylsulfate (DMS) protection experiments, it has been inferred that cellular factors interact in *trans* with IgH enhancer sequences. In addition, transcription is stimulated *in vitro* by up to one order of magnitude in the presence of IgH enhancer sequences on an appropriate template. Thus, at least some of these factors have to be present in nuclear extracts. To examine the factors interacting with this lymphoid-specific enhancer in more detail we compared the binding pattern of nuclear factors present in B-cell, T-cell and HeLa cell extracts. We demonstrate here, using the DNase I and DMS protection methods, the specific interaction of three different nuclear factors with the central *PstI-EcoRI* fragment of the IgH enhancer. This fragment has previously been suggested to retain the major enhancing activity. Surprisingly, no or only minor differences were discovered when the footprints obtained with B-cell extracts were compared with those obtained with HeLa cell and T-cell extracts. Intriguingly, two factors binding specifically to different sequences of the IgH enhancer are shared by polyoma as well as Moloney sarcoma virus (MSV) and lymphotropic papova virus (LPV) enhancer, respectively. All three of these enhancer elements exhibit altered cell type specificities. This indicates the utilization of similar or identical factors for transcriptional enhancement in different cell types. A cassette model consisting of different factor binding sites will be discussed.

Key words: IgH enhancer/DNA binding factors/cell restricted enhancer activity/enhancer cassette shuffling model

Introduction

Beside promoter sequences located in the immediate vicinity of the mRNA start site, i.e. 'TATA-box' and 'upstream promoter sequences', another class of transcriptional control elements called 'enhancers' or 'activators', have been identified previously. These sequences, typically between 70 and 150 bp in length, are able to potentiate transcription dramatically (up to 1000-fold *in vivo*) even in inverted orientation, downstream of the respective gene or when experimentally located several kilobases from the responsive promoter. First described in a number of viruses and usually located ~100-300 bp upstream of the mRNA start site, it soon became evident that cellular genes also contain enhancers (for review, see Serfling *et al.*, 1985; Schlokat and Gruss, 1986).

Furthermore, many of the viral and cellular enhancers exhibit a striking cell- or tissue-specific pattern of expression. One of the best investigated examples to date of a cell-specific cellular enhancer is the immunoglobulin heavy chain gene enhancer IgH (Banerji *et al.*, 1983; Gillies *et al.*, 1983; for review, see Voss *et al.*, 1986). It is located in the large intron between the variable and constant Cμ region on a 1-kb *XbaI* restriction fragment (Figure 1). Since this fragment (termed 'Xba E') was shown to stimulate the transcriptional activity of the SV40 promoter 5- to 15-fold in a B-cell (BJA-B) nuclear extract *in vitro* (Schöler and Gruss, 1985) and had been demonstrated to compete for *trans*-acting factors, most likely proteins, *in vivo* (Mercola *et al.*, 1985) and *in vitro* (Wildeman *et al.*, 1984; Schöler and Gruss, 1985), we assumed that the cellular factors required for this activity were present in the extract. In order to identify sites of specific protein-DNA interactions on Xba E we employed two different DNA-binding assays using the central *PstI-EcoRI* fragment. This fragment has previously been suggested to retain the major enhancing activity (Banerji *et al.*, 1983). For the DNase I (Galas and Schmitz, 1978) and dimethylsulfate (DMS) (Siebenlist *et al.*, 1980) protection methods used it is assumed that specific binding of proteins to DNA will protect the sequences involved from digestion by DNase I and modification of G residues by DMS, respectively. Consequently, a protected area on an asymmetrically labelled DNA fragment appears as a spared region (footprint) after electrophoresis in denaturing polyacrylamide gels.

In this report we demonstrate the identification of three protected domains on the central *PstI-EcoRI* fragment of the IgH enhancer *in vitro* in BJA-B (i.e. B-cell line; Klein *et al.*, 1975) and MOLT-4 (i.e. T-cell line; Minowada *et al.*, 1972) nuclear extracts. In both of these cell lines the IgH enhancer has been shown to be able to potentiate transcription *in vitro* (Schöler and Gruss, 1985). Surprisingly, in HeLa (Gey *et al.*, 1952) nuclear extracts where the IgH enhancer is unable to mediate transcriptional activation (Schöler and Gruss, 1985) the same factors seem to be present. Furthermore, by DNase I protection competition experiments we demonstrate that one of the identified factors can also bind to Moloney sarcoma (MSV) and polyoma (Py) enhancer sequences, whereas another is shown to interact with specific sequences on the lymphotropic papova virus (LPV) enhancer. All of these enhancers exhibit cell type-restricted activity profiles. Finally, on the basis of these identified binding sequences, which are shared by different enhancers and interact with cellular factors, we discuss a model consisting of 'general' and 'cell type-specific' cassettes.

Results

B-cell nuclear factors bind to IgH enhancer sequences in vitro

In our first experiment we prepared a nuclear extract from the human B-lymphoma line BJA-B. A terminally labelled fragment from the non-coding strand (i.e. the strand with the same polarity

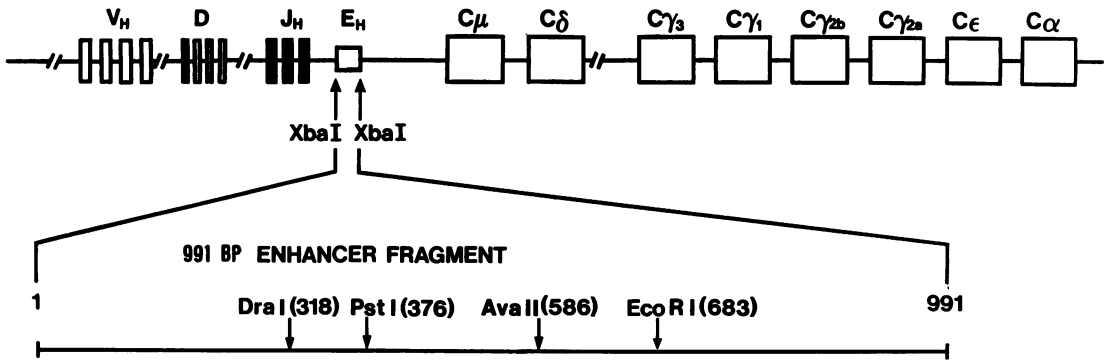
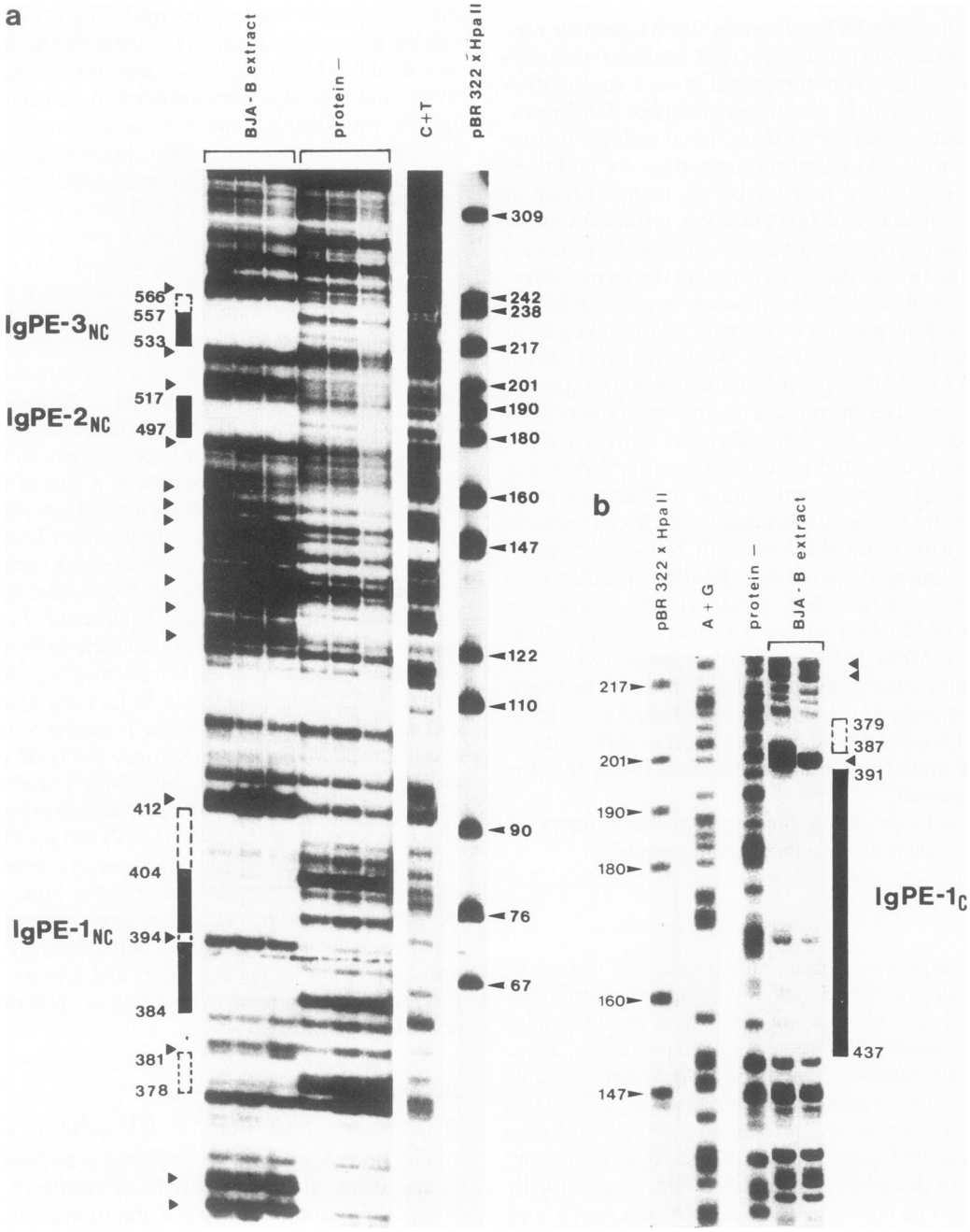


Fig. 1. Schematic representation of the immunoglobulin heavy chain gene enhancer and its location in immunoglobulin gene germline configuration. Top line, IgH gene germline configuration indicating the different elements V, variable; D, diversity; J, joining; and C, constant regions. Bottom line, enlarged display of the 991-bp IgH Xba E enhancer fragment E with important restriction sites and their location indicated (in nucleotides).



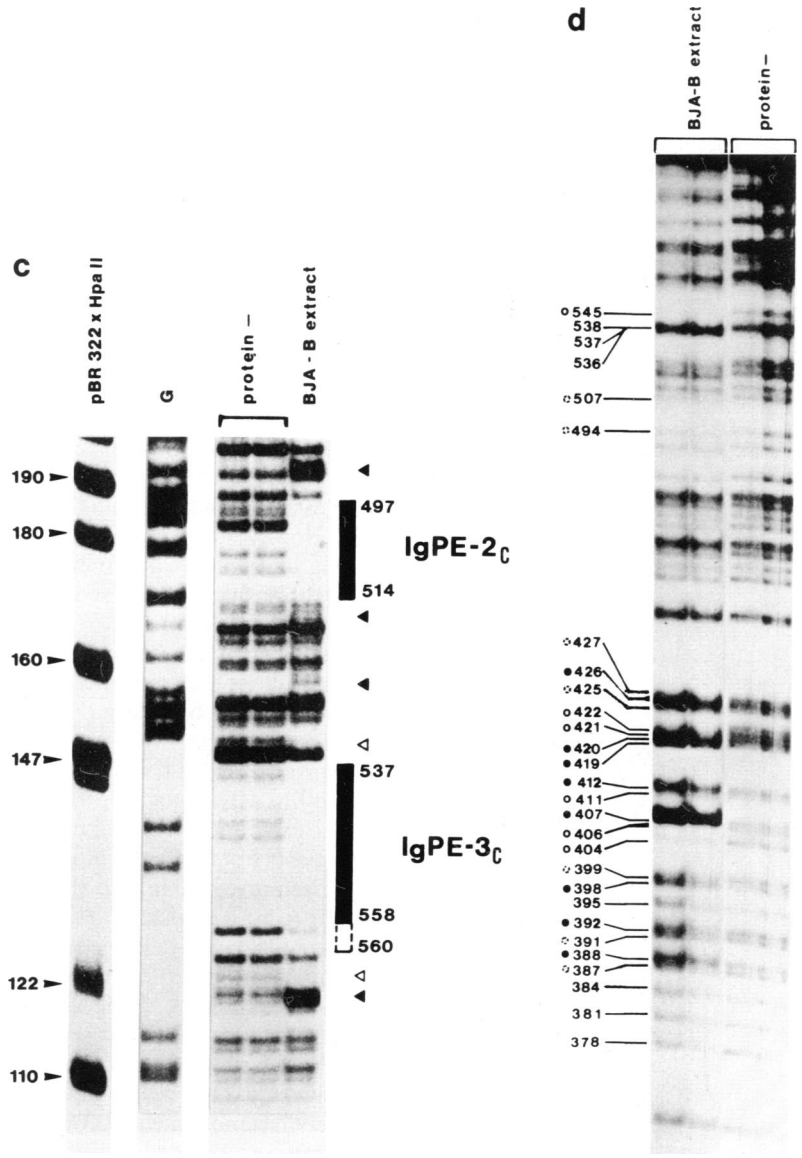


Fig. 2. DNase I footprinting and DMS protection pattern of Ig Xba E fragment (370–683) in B-cell (BJA-B) nuclear extract. **(a)** DNase I protection pattern on the non-coding strand reveals three areas of clear protection: IgPE-1_{NC}, IgPE-2_{NC} and IgPE-3_{NC}. From left to right: BJA-B binding reactions; control reactions without protein; C+T sequencing reaction and, finally, pBR322 cut by *Hpa*II as sizer markers. **(b)** Demonstration of the region protected from digestion by DNase I corresponding to IgPE-1_{NC} on the coding strand (IgPE-1_C). To the left, pBR322 size markers and A+G sequencing reaction are shown; the control reaction (protein minus); finally, the binding reactions with extract. **(c)** Sites of protein–DNA interaction on the coding strand corresponding to IgPE-2_{NC} and IgPE-3_{NC} mapped by DNase I protection. From left to right: pBR322 cut by *Hpa*II; G sequencing reaction; control reactions (protein minus); BJA-B binding reaction. **(d)** DMS protection pattern on the non-coding strand. Positions of G residues involved in binding are indicated by open (protected), dashed (partially protected) and closed (enhanced) circles. Areas of strong and weak DNA–protein interactions are specified as solid and dashed boxes. Regions of enhanced or decreased sensitivity to DNase I as compared with the protein-minus extracts are outlined by filled and open triangles, respectively. Numbers next to the pBR322 × *Hpa*II lanes represent the size of the resulting DNA fragments in nucleotides. Numbers along the opposite side of the figures indicate the boundaries of the protected areas relative to the numbering system shown in Figure 1.

as the mRNA; here nucleotides 318–683 according to the numbering system used by Ephrussi *et al.*, 1985) was incubated with the BJA-B nuclear extract prior to DNase I digestion and gel electrophoresis. In Figure 2a three protected areas (IgPE-1_{NC}, IgPE-2_{NC}, IgPE-3_{NC}) are clearly visible. In most cases these protected regions were flanked by sites of increased DNase I sensitivity. In order to confirm the specificity of these interactions and also to examine the nature of the binding in more detail we subsequently repeated this analysis with the coding strand. As demonstrated in Figure 2b and c, and as schematically outlined in Figure 5a, corresponding regions were found to be protected on the coding strand as well. Thus at least three different regions of the IgH enhancer ranging from ~20 bp to 40 bp in length interact specifically with factors present in a nuclear extract from BJA-B cells.

In vitro factors reflect the in vivo situation

To establish the relationship between the factors binding *in vitro* and those identified *in vivo* (Ephrussi *et al.*, 1985) we employed the DMS protection technique on the non-coding strand. Using this technique on living cells, Ephrussi *et al.* (1985) had demonstrated the interaction of nuclear factors with the IgH enhancer. Under the conditions used, sites of protein–DNA interactions are indicated by individual G residues protected from or increas-

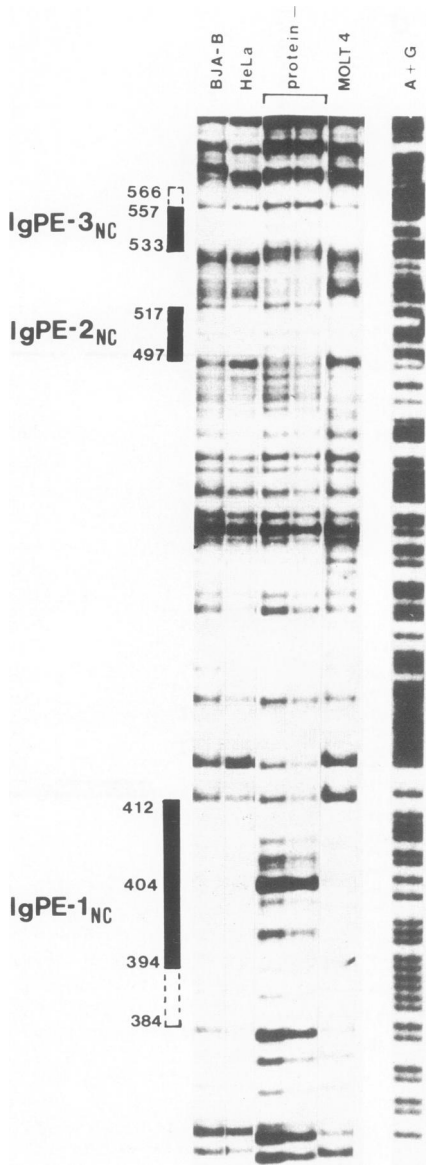


Fig. 3. Presence of similar IgH enhancer binding factors in cells as diverse in origin as HeLa, MOLT-4 and BJA-B. From left to right: DNase I footprinting reactions performed with BJA-B and HeLa extracts; control reactions without protein; protection reaction with MOLT-4 extract; finally, A+G sequencing reaction.

ingly sensitive to methylation by DMS. As demonstrated in Figure 2d, this strand essentially exhibits a similar pattern of enhancements and protections to that observed by Ephrussi and co-workers (Figure 5a). In particular, G545 and the region between G399 and G419 correspond perfectly to the ones seen most clearly *in vivo*. Though there seems to be some divergence in the DMS reactivity pattern of G residues G384–G398 and G420–G427 (as compared with the *in vivo* situation), it should be pointed out that these exhibit a rather marginal reactivity pattern *in vivo* and therefore are difficult to interpret. From this we conclude that the factors identified *in vitro* reflect the *in vivo* situation in the major protection pattern and, consequently, might have functional significance.

Corresponding factors are present in HeLa and T-cells

To determine whether any of these factors were specific for B-cells only, we next prepared nuclear extracts from MOLT-4 (a human acute lymphoblastic leukemia T-cell line) and HeLa cells. As seen in Figure 2, and in Figure 2a in particular, under the conditions used in crude nuclear extracts, multiple sites of increased sensitivity to DNase I as well as a few additional regions of partial protection could be observed. Using crude extracts at high protein concentrations (in order to saturate all putative binding sites on the labelled fragments) we account for these observations by the recognition of 'cryptic' binding sites, inadvertently occurring in each DNA sequence, by nuclear factors. We therefore sought to titrate out residual unspecific protein–DNA interactions by increasing the stringency of the DNase I protection method. This was done by further increasing the amount of unlabelled, unspecifically competing DNA [i.e. the synthetic heteropolymer poly d(I-C)]. Again, three specific footprints were obtained (Figure 3). As expected, the less pronounced footprints disappeared as did most regions of increased sensitivity to DNase I digestion (compared with the BJA-B binding pattern in Figure 2a). However, binding of IgPE-2 is also reduced, most likely owing to rather weak affinity for its binding site and/or lower abundance than IgPE-1 and IgPE-3.

Interestingly no major differences can be seen when BJA-B, MOLT-4 and HeLa cell nuclear extracts are compared. The only obvious difference concerns the IgPE-2 binding site in MOLT-4 extract. The 5′ boundary of IgPE-2 binding site is identical in all three extracts, whereas the 3′ boundary is located further downstream and is flanked by a region of increased DNase I sensitivity in MOLT-4 compared with HeLa and BJA-B extracts. These experiments have all been repeated with extracts and DNA fragments from different preparations and we have observed this hypersensitive area in both HeLa (not shown) and BJA-B extracts (see Figure 2a) under 'low stringency' conditions. Thus, we suspect that technical variations, such as quantitative differences of IgPE-2 in the different extracts (probably due to their preparation) and/or the stringency of the DNase I technique, are responsible for this increased sensitivity rather than binding of different factors. Since the factors are present in the three different extracts and seem to bind to identical sequences they might closely resemble each other or even be identical. Consequently, these factors alone might not be sufficient to confer the cell type-specific activity.

In this context, the previous findings of Ephrussi *et al.* (1985) and Church *et al.* (1985) using *in vivo* DMS protection should be noted. These authors observed protection in myeloma, B- and early B-cells, but not in cells of non-B lineage. This apparent discrepancy can be resolved if gene control requires nuclear matrix and/or higher order chromatin structure involvement. In such a case, an inactive gene that does not show DNase I-hypersensitive areas (Mills *et al.*, 1983) would not be accessible to the respective factors. When, on the other hand, purified DNA, as in the case of *in vitro* experiments, is employed the sequences involved are freely accessible to the *trans*-factors.

Exploiting a DNase I footprinting assay with increased sensitivity Singh *et al.* (1986) recently identified a factor, IgNF-A, that binds to a conserved sequence motif identified in all Ig promoters examined (Falkner and Zachau, 1984; Parslow *et al.*, 1984). Surprisingly, IgNF-A is present in B-cells as well as in HeLa cells, contrasting with the strict cell type specificity observed for Ig promoters (Grosschedl and Baltimore, 1985; Mason *et al.*, 1985; Picard and Schaffner, 1985). We strongly suspect IgNF-A to be responsible for binding to IgPE-3 binding site, since synthetic oligonucleotides of this region compete for binding of IgNF-A to the κ-promoter (Singh *et al.*, 1986).

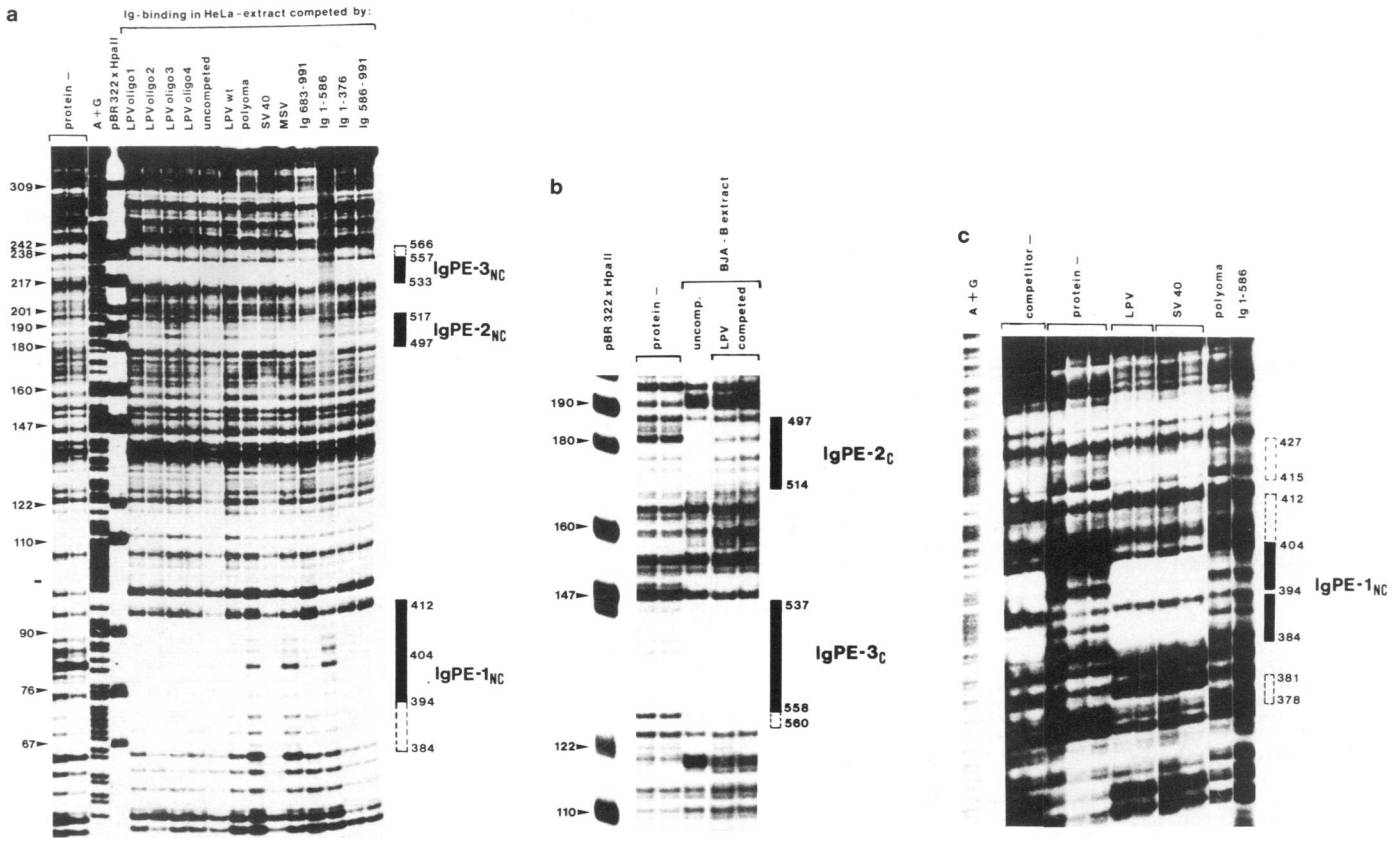


Fig. 4. DNase I footprint competition for IgH enhancer binding factors shared by other enhancer elements. **(a)** Differential competition pattern of the three binding sites on the IgH enhancer by different enhancer elements (in HeLa nuclear extract). From left to right: two control lanes without protein; A+G sequence; pBR322 × *Hpa*II size marker; competition reactions with four synthetic oligonucleotides of the LPV enhancer carrying different deletions or point mutations (as outlined in Figure 5a); control reactions without specifically competing DNA; reactions with different enhancer fragments used as competing DNA; a wild-type *Hae*III fragment carrying the LPV enhancer (Mosthaf *et al.*, 1985); the *Pvu*II-4 fragment of polyoma virus containing the B enhancer (Böhnlein *et al.*, 1985); the SV40 wild-type *Pvu*II−*Hin*dIII fragment including enhancer, early promoter, and TATA-box; an *Nco*I fragment of pMSV CAT (Schulze *et al.*, 1985) carrying MSV enhancer and SV40 21 bp repeats; finally, four different subfragments of the IgH enhancer as indicated at the top of the figure (see Figure 1). **(b)** Competition for IgH enhancer binding factor IgPE-2 by the LPV enhancer in BJA-B extract. From left to right: pBR322 × *Hpa*II size marker; two control reactions without protein; the positive control reaction without specific competitor DNA; two reactions demonstrating the competing effect exerted by the LPV enhancer (fragment as indicated above). **(c)** Competition pattern exhibited by Py, as compared with LPV and SV40 enhancers, for IgH enhancer binding factor IgPE-1 in BJA-B extract. From left to right: A+G sequencing reaction; two reactions without specifically competing DNA; three protein-minus control reactions; two competition reactions with LPV enhancer DNA; two reactions representing the SV40 enhancer competition pattern; finally, competition reactions with Py and IgH enhancer (No. 1−586) DNA. DNA fragments were used for competition as described in **(a)**. Areas of strong and weak protection are indicated by solid and dashed boxes, respectively. Numbers to the left of figures refer to the size of pBR322 fragments used as size markers, while numbers to the right indicate the positions of the respective protected areas on the Ig Xba E fragment.

IgH enhancer binding factors can interact with other enhancer elements

Since these factors are also present in non-lymphoid cells (see Figure 3), it is conceivable that the factors identified so far represent common enhancer binding factors. In order to test this hypothesis, we performed footprint competition experiments using various enhancers as competitors. This strategy was based on the assumption that, firstly, only a limited amount of the respective factors would be present in the extracts and, secondly, these factors might also bind to other enhancer elements. Thus, prior to the addition of labelled IgH enhancer fragments either HeLa or BJA-B extracts were incubated with an excess amount of the respective enhancer-containing competitor DNA. As expected, and as demonstrated in Figure 4a and c, homologous DNA (No.1−586) competed for the binding of all three factors, whereas IgH enhancer fragments lacking the sequences involved in binding (No.1−376, 586−991 and 683−991) did not. Also, a DNA fragment carrying the SV40 enhancer and early promoter did not compete (at the molarity of fragment used) for binding of the factors involved. However, in band shifting experiments at lower protein concentrations, binding of IgPE-3 to the SV40 enhancer could be demonstrated (D.Bohmann, T.Dale, G.Tebb, H.Schöler, I.Mattaj and W.Keller, in preparation). Interestingly, LPV enhancer sequences competed for the binding of IgPE-2 (Figure 4a,b), whereas both Py and MSV enhancer sequences competed for binding of IgPE-1 (Figure 4a,c). Under 'low stringency' conditions, an additional partial protection of Xba E, No.415−427, could sometimes be observed (Figure 4c). This protection is not detected under high stringency conditions and upon competition with Py enhancer sequences. Therefore, to date we cannot clearly distinguish whether an additional specific factor is present or binding of IgPE-1 induces some rearranged (and therefore somehow protected) DNA structure adjacent to its binding site. This has to await purification and concentration of the respective factors. Homologous regions between IgPE-1 binding site, MSV enhancer and Py enhancer are outlined in Figure 5a using capital letters. Notably, this region in the polyoma B enhancer has recently been shown to be absolutely essential for

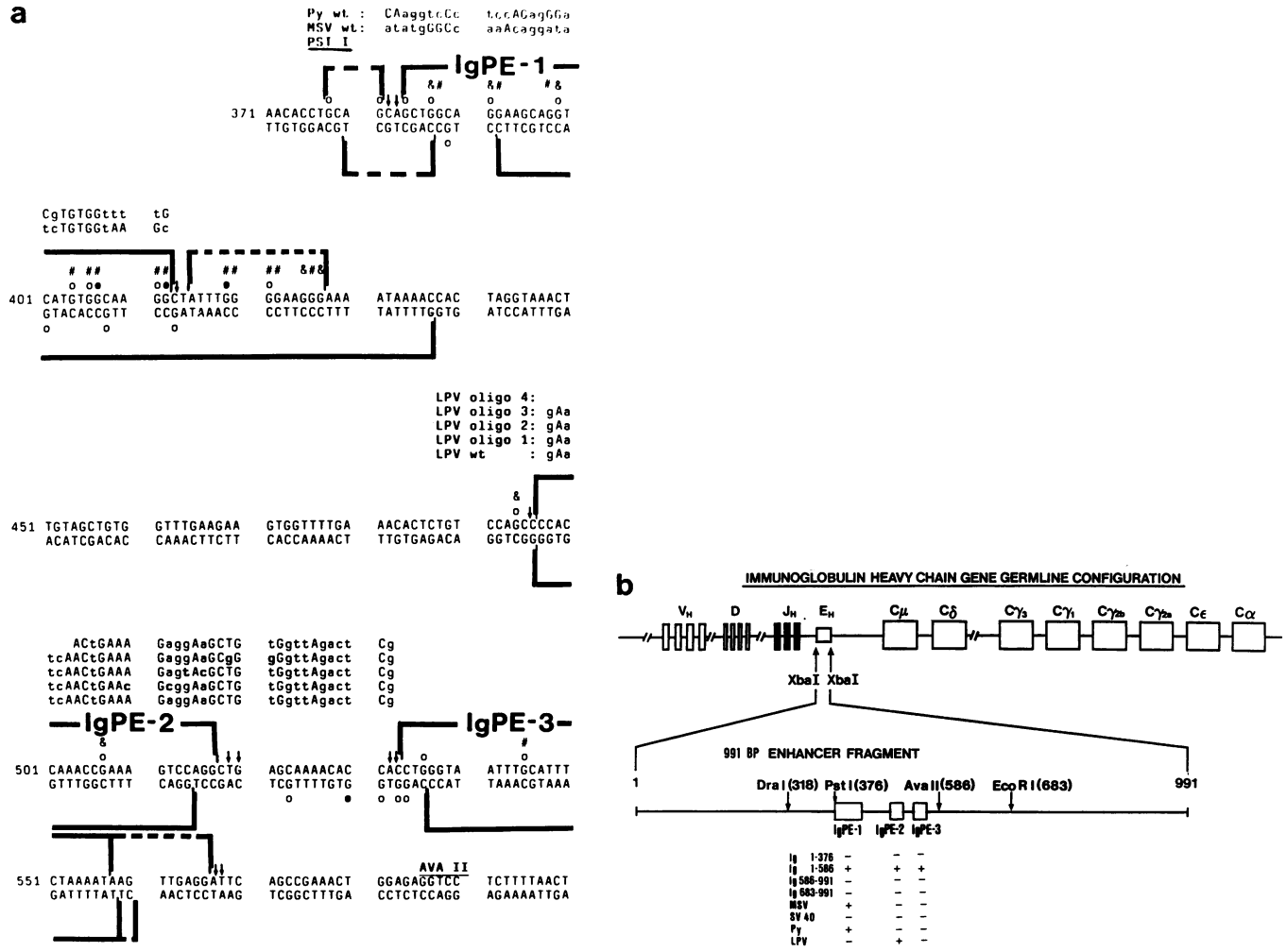


Fig. 5. Schematic representation of the protected areas demonstrated on the IgH enhancer and their diverging competition profiles with respect to other enhancer elements. **(a)** The nucleotide sequence of the IgH Xba E fragment, No. 371–600 (approximately *Pst*I–*Ava*II) is shown. Protections in BJA-B extract as identified by DNase I digestion and indicated as IgPE-1–IgPE-3 are outlined by brackets above (non-coding strand) and below (coding strand) the double-stranded sequence. Solid and dashed brackets represent strong and weak binding sites, respectively. At the same time, solid brackets on the non-coding strand also indicate clear protection in HeLa and MOLT-4 extracts. Arrows represent sites of increased sensitivity to DNase I digestion. Open and closed circles indicate protected and increasingly sensitive G residues identified *in vivo* in B-cells using DMS protection analysis (Ephrussi *et al.*, 1985). Similarly, reactive or protected Gs *in vitro* are represented by '#' (protected), '#' (enhanced) and '&' (partially protected). **(b)** Summary of the competition profile of the IgH enhancer for binding factors shared by other enhancer elements and homologous fragments. Ability and inability of the different DNA fragments to compete for binding factors IgPE-1 to IgPE-3 are indicated by '+' and '–', respectively.

functional activity as well as for binding of nuclear factors (Böhnlein *et al.*, 1985; Piette *et al.*, 1985; Böhnlein and Gruss, 1986). The central feature of the IgPE-1 binding site as compared with MSV and Py enhancers is the TGTGGCAA motif that exhibits strong homology with the 'core' consensus sequence TGTGGAAA, as previously identified by Weiher *et al.* (1983). However, the core by itself is not sufficient for factor binding, since SV40 enhancer sequences do not compete for IgPE-1. Therefore, additional features are necessary for binding of IgPE-1. The IgPE-1 binding site also includes two areas homologous to the consensus sequence CAGGTGGC as deduced by Ephrussi *et al.* (1985; depicted as boxes 2 and 3). Sequences homologous to this consensus sequence can be found in most of the factor binding sites as identified *in vivo* by Ephrussi and coworkers.

Surprisingly the LPV enhancer, which has a host-range restricted to human cells of the haematopoietic system (Mosthaf *et al.*, 1985), competes for the binding of IgPE-2 which, therefore, seems to be different from the factor (IgPE-1) interacting with Py and MSV enhancer sequences (Figure 4a,b). Sequence comparison between the LPV enhancer and the IgPE-2 binding site revealed a highly homologous domain (Figure 5a). Furthermore, recent footprinting studies (L.Mosthaf and P.Gruss, in preparation) have demonstrated binding of cellular factors to this sequence motif. Using four synthetic oligonucleotides carrying different point mutations within this LPV motif, we observed competition for binding to the homologous IgH motif only in the case of LPV oligo 3 (Figure 4a). Thus, the LPV sequences shown to bind specific cellular factors (L.Mosthaf and P.Gruss, in preparation) also compete with IgH enhancer sequences for factor binding.

Discussion

Using the DNase I footprinting and DMS protection techniques we have demonstrated specific binding of cellular factors to at least three different sites on the central *Pst*I–*Eco*RI fragment of the mouse IgH enhancer. Surprisingly, the three factors identified seem to be present in all cells (i.e. BJA-B, MOLT-4 and

HeLa) irrespective of the IgH enhancer's ability to mediate transcriptional activity in these cells. Employing a similar approach, an analysis of the SV40 enhancer has revealed different, separable domains that are required for its functional activity (Wildeman *et al.*, 1986; Zenke *et al.*, 1986). Although the IgH enhancer has not been examined in such detail, the availability of the specific footprints now allows a directed mutagenesis. Furthermore, we draw confidence from the observation that some of these factors similarly bind to homologous sequences in other enhancers such as those from Py, MSV and LPV. Interestingly, Py and MSV enhancers compete for IgPE-1, whereas the LPV enhancer interferes with IgPE-2 binding (as summarized in Figure 5b). Neither of these enhancer sequences was able to compete for IgPE-3. Though sequence comparison of the different *in vivo* binding sites revealed a consensus octamer and therefore implied binding of one factor to multiple sites (Ephrussi *et al.*, 1985), the differential competition profile of the respective factors *in vitro* argues against this possibility. Since different enhancers can bind some of the factors that have been shown to interact with the IgH enhancer, a combination of shared factors and factors unique to a given enhancer might be required for the activity of the enhancers mentioned above. Since the host range of Py, MSV and LPV enhancers is different from the activity profile of the IgH enhancer we like to speculate that possible combinations of certain binding sequences (generally active cassettes) with others (cell-specific cassettes) might lead to an active enhancer with altered cell type specificity.

At least for SV40 it seems to be clear that the combination of at least two cassettes (core and *Sph*I domain; Herr and Gluzman, 1985; Herr and Clarke, 1986) is sufficient for enhancer activity. Although the competition profiles of MSV, Py and LPV enhancers for immunoglobulin binding factors are different, we suspect that neither IgPE-1 nor IgPE-2 by itself confers cell type specificity. This statement is supported by the observation that these factors are present in all cell lines tested and by recent *in vivo* transfection experiments using deletion mutants of the IgH enhancer (Wasylyk and Wasylyk, 1986); in both HeLa and B-cells, IgH enhancer subfragments containing either IgPE-1 binding site or IgPE-1 and IgPE-2 binding sites are able to potentiate transcription ~10- or 50-fold, respectively. Since the presence of the respective binding sites increases IgH enhancer-mediated transcription at identical rates in both cell types, IgPE-1 as well as IgPE-2 seem to be functionally equivalent in fibroblasts and B-cells. The additional presence of an IgPE-3 binding site on the template, however, results in a further transcriptional increase in B-cells only. Since IgPE-3 also seems to be present in both cell types, any of the following explanations may account for the selective transcriptional activation by IgPE-3 in B-cells only. Firstly, in spite of the identical recognition sequence, the factor(s) interacting with the IgPE-3 binding site might actually be different in these cell lines. Secondly, if IgPE-3 were identical in the two cell lines, post-translational modification might render it either inactive in HeLa or active in BJA-B. Finally, protein–protein interaction between IgPE-3 bound to the enhancer and additional proteins might also be involved.

The identification of IgH enhancer binding factors now allows their purification and the subsequent investigation of the underlying mode of action by which these factors function. Thus, in conclusion, the domains identified so far by footprint analysis could represent constitutive and cell-specific elements of the IgH enhancer. Furthermore, though clear evidence to date is lacking, suggestions have been put forth based on *in vivo* mutational analysis (Wasylyk and Wasylyk, 1986) and on *in vitro* competition experiments (Schöler and Gruss, 1985) that sequences adjacent to the central *Pst*I–*Eco*RI fragment of Ig Xba E might also be involved in the tissue-specific expression of the IgH enhancer. Our present experiments are directed towards examination of these regions and purification of the factors described above.

Materials and methods

Cell growth and extract preparation

Ten liters of cells were grown to a density of 5×10^5 cells/ml. HeLa cells, adapted to growth in suspension, were grown in MEM Spinner medium (Flow), 1 × non-essential amino acids (Gibco). Medium for BJA-B and MOLT-4 cells was RPMI 1640 (Gibco), 3 g of $NaHCO_3$/l. All media were supplemented with 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% fetal calf serum.

The extracts were made as described previously (Wildeman *et al.*, 1984; Schöler and Gruss, 1985) and typically contained between 10 and 20 mg protein/ml.

DNase I and DMS protection reactions

Sequencing reactions (Maxam and Gilbert, 1980) and radioactive labelling were performed according to standard procedures (Maniatis *et al.*, 1982) with [γ-^{32}P]-ATP (≤5000 Ci/mmol; Amersham).

For Figure 2 the DNase I footprinting reactions were performed as follows: 8 μl of extract were pre-incubated with 2 μl of pBR322 × *Hpa*II (28 ng in 1 × ED; see below) on ice for 20 min, followed by addition of 2 μl of end-labelled fragment (10 000 c.p.m. in 10 mM Tris pH 8.0, 1 mM EDTA). The reaction mixture was kept on ice for 10 more minutes and transferred to 20°C for 1 min. Finally, 2 μl of DNase I (Serva; 1 mg/ml DNase I stock solution in 150 mM NaCl, 50% glycerol) either concentrated or appropriately diluted in 1 × DD [1 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol (DTT), 20 mM KCl] were added. The reaction was stopped after 90 s by addition of 100 μl of 0.2% SDS, 0.6 M NaAc pH 5.2, 30 μg/ml tRNA and 100 μl of phenol/chloroform/isoamylalcohol (PCI; 6:24:1). After chloroform/isoamylalcohol (CI; 24:1) extraction and ethanol precipitation, the pellet was resuspended in formamide loading buffer (Maniatis *et al.*, 1982) prior to electrophoresis on denaturing 5% polyacrylamide gels in 1 × TBE (Maniatis *et al.*, 1982). For the control reactions without protein the extract was simply substituted with 8 μl 1 × ED (1 mM spermidine, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT). The DNase I concentrations for the extract containing reactions were in the range between 0.25 and 2 μg DNase I/reaction. In the case of the protein-minus controls 2.5–10 ng were used.

For the DMS protection technique, binding was performed as for DNase I protection. After transfer to 20°C, 2 μl of DMS at appropriate concentrations in H_2O were added for 2 min (final concentration between 0.2 and 1%). Reactions were terminated by 100 μl of 0.3% SDS, 0.3 M NaAc pH 5.2, 15 μg/ml tRNA, 1.4 M mercaptoethanol and immediate freezing in liquid nitrogen. After subsequent PCI extraction, the same procedure as described for the DNase I protection was followed to prepare the probes for electrophoresis. In contrast to the DNase I footprints where the DNase I concentration had to be titrated carefully and independently for the protein-minus and the extract-containing reactions, DMS concentrations could be kept constant for these reactions.

Labelled Ig Xba E subfragments used were: No. 318–683 labelled at the *Dra*I site for a and d; 1–586 labelled at the *Ava*II site for b; finally, 1–683 labelled at the *Eco*RI site for c.

For Figure 3 the DNase I protection reaction was performed as described for Figure 2 with the following exceptions. For pre-incubation, 1 μl of 50 mM EDTA was added prior to addition of unlabelled competitive DNA. pBR322 was replaced by 900 ng of poly d(I-C) (Boehringer). Prior to transfer of tubes to 20°C, 1.2 μl of 75 mM $MgCl_2$ was added. The 318–683 fragment was labelled at the *Dra*I site.

Footprinting reactions for Figure 4a were performed as described for Figure 3. Competition experiments were accomplished by addition of 200 ng of the respective DNA fragments for pre-incubation. For the uncompeted control 200 ng of poly d(I-C) was used instead. Competition experiments shown in b and c were done as specified for Figure 2 with the unspecifically competing pBR322 DNA substituted by equivalent amounts of the respective enhancer - carrying DNA fragments. In a and c an Ig Xba E subfragment from *Dra*I–*Eco*RI (318–683) was labelled at the *Dra*I site. In b an *Xba*I–*Eco*RI (1–683) fragment labelled at the *Eco*RI site was used.

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While this manuscript was submitted/in press, D.Baltimore (*Nature*, **322**, 846–848; *Cell*, **46**, 705–716) and P.Chambon (*EMBO J.*, **5**, 1791–1797) and their co-workers described the identification of corresponding factors. Furthermore, preliminary band shifting experiments (U.S., data not shown) indicate that in B- and T-cells IgPE-3 represents multiple proteins rather than a unique binding factor (as seems to be the case for HeLa).