The EMBO Journal vol.7 no.3 pp.655 - 664, 1988

The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain

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Communicated by D.Richter

The matrix attachment regions of the chicken lysozyme domain were studied in an *in vitro* DNA binding assay by incubating oviduct nuclear matrices with labeled restriction fragments. A strong attachment region was localized between 11.1 and 8.85 kb upstream of the transcription start site and a weaker one between 1.3 and 5.0 kb downstream of the poly(A)$^+$ addition site. Both attachment regions co-map with the previously established boundaries of the chromatin domain. The upstream matrix attachment region is distinguishable from known enhancers and is composed of multiple binding sites. We find specific but weaker binding of the same restriction fragments to matrix preparations from transcriptionally inactive chicken erythrocytes indicating a cell-type and transcription-independent conservation of the sites for specific binding of matrix attachment sequences. We also demonstrate that the matrix attachment regions are located at the base of a chromosomal loop in histone-extracted nuclei. Thus, the lysozyme domain represents a topologically-sequestered functional unit containing the coding region and all known lysozyme-specific, *cis*-acting regulatory elements.

Key words: chicken lysozyme gene/chromatin domain/chromosomal loop/matrix attachment region

Introduction

Chromatin of interphase nuclei appears to be organized into topological domains or loops, which are constrained by a residual nuclear framework (Benyajati and Worcel, 1976; Cook and Brazell, 1976; Igo-Kemenes and Zachau, 1978; Lebkowski and Laemmli, 1982). This framework probably corresponds to the internal non-nucleolar component of the nuclear matrix, which can be isolated by extraction of nuclei with DNase I digestion and high salt (Berezney and Coffey, 1974). While earlier work established this model from studies of total chromatin, more recently the loop organization of specific genes was identified. In the tandemly repeated histone gene cluster of *Drosophila melanogaster*, one loop attachment region per cluster was found in a 657 bp segment of the H1 - H3 spacer (Mirkovitch *et al.*, 1984). Loop attachment regions were subsequently localized in the non-transcribed spacer between two divergingly transcribed heat-shock genes, in a 3 kb segment upstream of three tandemly repeated heat-shock genes, and in a 320 kb region surrounding the *rosy* and *ace* loci (Mirkovitch *et al.*, 1984, 1986). Studying three single copy genes (alcohol dehydrogenase, the glue protein Sgs-4 and fushi tarazu) Gasser and Laemmli (1986b) found attachment regions 5′ and 3′ of the genes. By employing an *in vitro* DNA binding assay, attachment regions were also localized on a DNA segment in the J-C intron of the mouse κ immunoglobulin gene and the mouse heavy chain immunoglobulin locus (Cockerill and Garrard, 1986; Cockerill *et al.*, 1987).

The special and functional relationship of the identified attachment regions to neighboring sequence elements with potential or known effects on transcriptional activity has attracted considerable interest. First, some of the attachment regions are enriched for sequences related to the consensus of the DNA topoisomerase II cleavage sequence (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986a,b; Sander and Hsieh, 1985; Udvardy *et al.*, 1985). As DNA topoisomerase II is a major structural element of interphase nuclei (Berrios *et al.*, 1985) and mitotic chromosomes (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986), a functional relationship between chromatin loop structure and the regulation of torsional stress was anticipated (Cockerill and Garrard, 1986). The second type of sequence elements suggested to be functionally related to matrix attachment regions are *cis*-acting sequences required for effective transcription. The mouse κ and heavy chain immunoglobulin matrix attachment sites were found to be located adjacent to tissue-specific transcriptional enhancers (Cockerill and Garrard, 1986; Cockerill *et al.*, 1987). Furthermore, the 5′ attachment regions of the *Drosophila* alcohol dehydrogenase, Sgs-4 and fushi tarazu genes apparently co-map with sequences required for the induction of high transcriptional level or for the transcriptional regulation during development.

A comprehensive view on the domain or loop organization of most higher eukaryotic genes or gene families is hard to obtain as their domains are usually fairly large, eventually reaching sizes of several hundreds of kbs (Cook and Brazell, 1976). The chicken lysozyme gene is a pleasing exception to this general situation since the size of its chromatin domain is comparatively small ($\sim$19 kb; Strätling *et al.*, 1986). In chicken oviduct chromatin, the domain is featured by a disrupted nucleosome structure, an elevated nuclease sensitivity, and a partitioning into two related chromatin fractions (S1 and P2) during chromatin fractionation (Strätling *et al.*, 1986). The domain encompasses nine nuclease hypersensitive sites apparently involved in the tissue-specific and developmentally regulated expression of the gene (Fritton *et al.*, 1983, 1984). Four of these hypersensitive sites were identified to contain transcriptional enhancers and a silencer element, respectively (Theisen *et al.*, 1986; Steiner *et al.*, 1987). In the present study, we have localized an upstream and a downstream matrix attachment region of the chicken lysozyme domain. Both attachment regions co-map with the previously determined boundaries of the chromatin domain

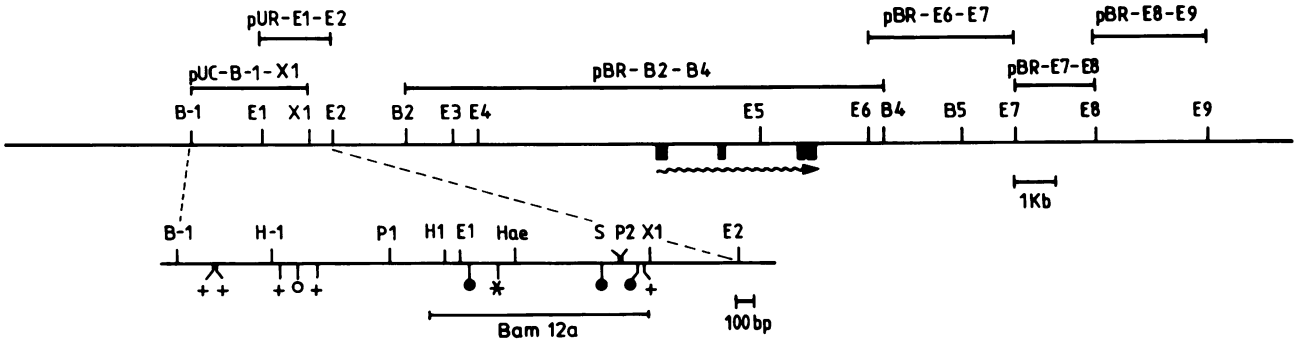

Fig. 1. Map of the chicken lysozyme domain with pertinent restriction sites and the relative positions of the plasmids used (Lindenmaier *et al.*, 1979; Nowock and Sippel, 1982; L.Phi-Van, unpublished results). B, *Bam*HI; E, *Eco*RI; Hae, *Hae*II; H, *Hin*dIII; P, *Pvu*II; S, *Sac*I; X, *Xba*I. The suffix numbers indicate the order of the restriction sites, defining the 5′ end (E0) of a previously studied clone as zero (Lindenmaier *et al.*, 1979). Filled boxes denote the four exons of the lysozyme gene; the wavy line indicates the lysozyme gene transcription product. The sequence between restriction sites B-1 and E2 is expanded 4-fold in the lower part of the figure. The horizontal bars above the map show the DNA fragments cloned into the indicated plasmids which, after appropriate restriction cleavage, are end-labeled and used in the *in vitro* binding assay. Closed circles along the expanded B-1−X1 sequence indicate three sequences in the coding strand conserving the central $\overset{A}{T}A\overset{T}{C}ATT$ of the topoisomerase II cleavage consensus and allowing 1−2 mismatches in the remainder of the consensus. A single one in the noncoding strand is indicated with an open circle. Crosses indicate T-rich stretches, and the star an A-rich sequence.

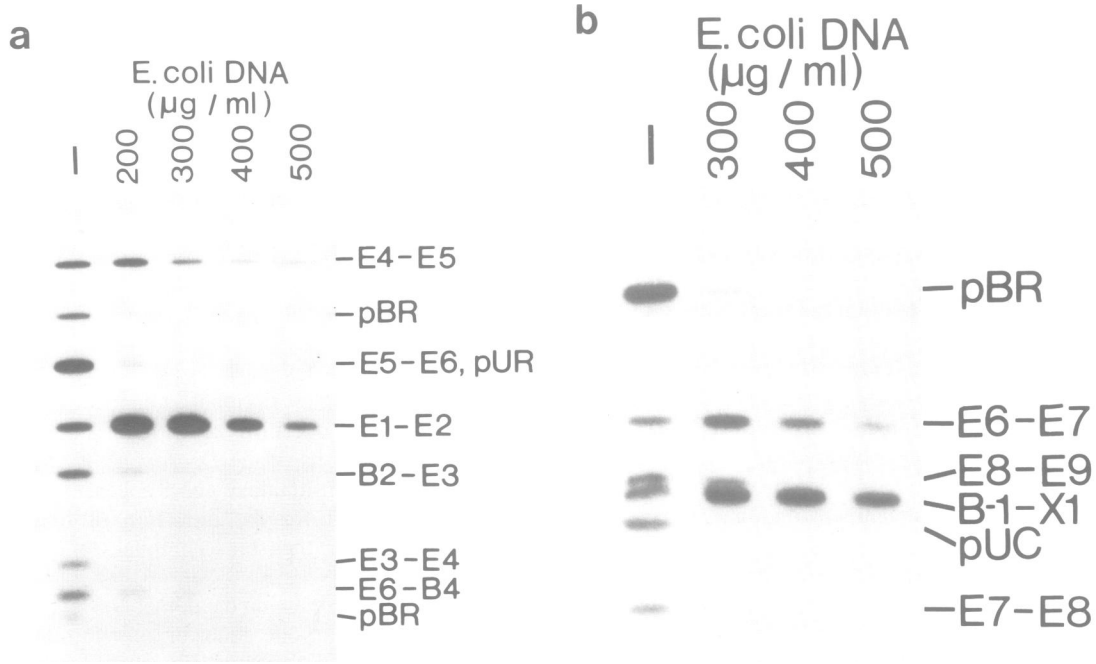

Fig. 2. Binding of specific restriction fragments of the lysozyme domain to oviduct nuclear matrices. (**a**) Nuclear matrices prepared from hen oviduct nuclei were incubated in a DNA-binding assay with end-labeled fragments derived from *Eco*RI−*Bam*HI-cleaved pBR-B2−B4 and *Eco*RI-cleaved pUR-E1−E2 in the presence of 200−500 µg/ml of *E.coli* competitor DNA. In (**b**) the matrices were incubated with end-labeled fragments derived from *Eco*RI-cleaved pBR-E6−E7, pBR-E7−E8 and pBR-E8−E9, and *Bam*HI−*Xba*I-cleaved pUC-B-1−X1 in the presence of 300−500 µg/ml of *E.coli* competitor DNA. The autoradiograms show the electrophoretically resolved DNA fragments purified from a 5% aliquot of the input sample (I) and from the matrix-associated samples. Fragments are identified on the right handed side by their map position.

(Strätling *et al.*, 1986). The upstream attachment region is distinguishable from known enhancers or nuclease hypersensitive sites. We also demonstrate that the matrix attachment regions are located at the base of a chromosomal loop in histone-extracted nuclei. Thus, the lysozyme domain contains in a topologically-sequestered functional unit the coding region and all known lysozyme-specific, *cis*-acting regulatory (nuclease hypersensitive) elements including three enhancers and one silencer element.

Results

Localization of two matrix attachment regions of the chicken lysozyme gene to the chromatin domain boundaries

The chromatin domain of the lysozyme gene in chicken oviducts was previously determined to encompass ~19 kb and to exhibit a disrupted nucleosome structure and an elevated micrococcal nuclease sensitivity (Strätling *et al.*, 1986). In order to determine the organization of the matrix

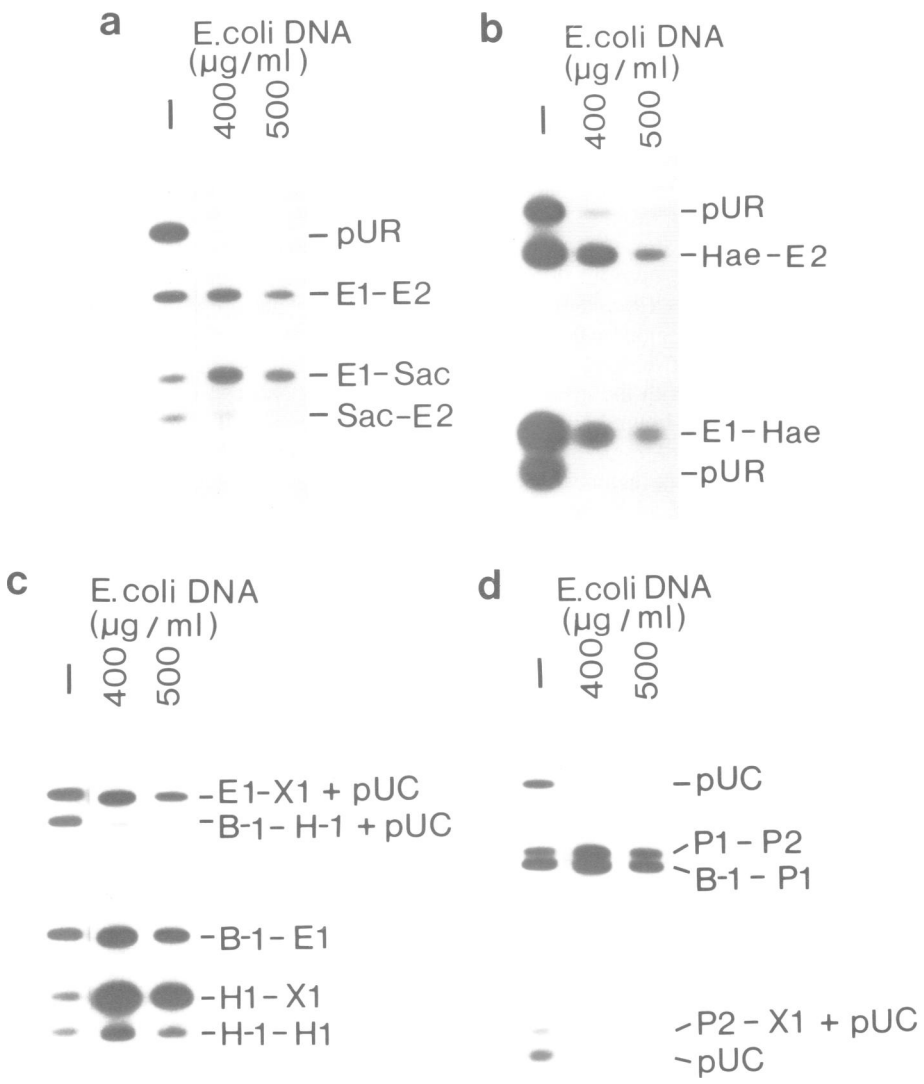

Fig. 3. Detailed mapping of the upstream matrix attachment region. (**a**) Oviduct nuclear matrices were incubated with end-labeled fragments derived from pUR-E1−E2 cleaved with *Eco*RI and *Sac*I, and with *Eco*RI alone in the presence of 400 and 500 µg/ml of *E.coli* competitor DNA. (**b**) Matrices were incubated with fragments derived from pUR-E1−E2 cleaved with *Eco*RI and *Hae*II. (**c**) Matrices were incubated with fragments derived from pUC-B-1−X1 cleaved with *Hin*dIII and separately with *Eco*RI. (**d**) Matrices were incubated with fragments derived from pUC-B-1−X1 cleaved with *Pvu*II and *Bam*HI. A 5% aliquot of the input DNA sample (I) and the purified matrix-associated DNA fragments are electrophoretically resolved and visualized by autoradiography. Fragments are identified on the right handed side by their map position.

attachment and loop structure of the lysozyme domain, we used an *in vitro* binding assay described recently by Cockerill and Garrard (1986). In this assay, labeled restriction fragments are incubated in the presence of *Escherichia coli* competitor DNA with the operationally defined nuclear matrix prepared by the method of Berezney and Coffey (1974). Following centrifugation, the matrix-associated labeled fragments are purified, electrophoretically resolved and visualized by autoradiography. Specifically, the recombinant plasmid pBR-B2-B4, which contains the coding region (4.0 kb in length), 6.3 kb 5′, and 1.8 kb 3′ of the gene, was cleaved with *Bam*HI and *Eco*RI (see Figure 1 for restriction map), and the generated fragments were end-labeled with [^{32}P]dATP and the Klenow fragment of *E.coli* DNA polymerase I. Similarly, the fragments derived from an *Eco*RI digest of the plasmid pUR-E1-E2 containing a 1.75 kb *Eco*RI− *Eco*RI fragment (E1−E2) located between 10.0 and 8.25 kb upstream of the transcription start site of the gene were labeled. Following incubation of these fragments in the presence of 200−500 µg/ml *E.coli* competitor DNA with nuclear matrices from hen oviduct retaining between 0.5 and 1% of the nuclear DNA, the fragments bound to matrices and the input DNA sample (I) were displayed electrophoretically and visualized by autoradiography. We estimated the affinity of a particular restriction fragment to nuclear matrices by comparing the level of the fragment bound to matrices with increasing concentrations of competitor DNA to the level of that fragment in the input DNA sample. The autoradiogram in Figure 2a shows that the 1.75 kb E1−E2 fragment binds most prominently to nuclear matrices. The fragments E4−E5 and E6−B4 are weakly bound, while all other fragments bind very poorly. Since previous studies localized the 5′ boundary of the chromatin domain of the lysozyme gene close to the E1 site to which the E1−E2 frag-

ment abuts (Strätling *et al.*, 1986), the present results identify an upstream matrix attachment region very close to the 5′ boundary of the domain. A more precise mapping of this attachment region is described below, which shows in greater detail that it co-maps with the 5′ domain boundary.

The lysozyme gene domain extends over ~ 4 kb on the 3′ side of the gene (Strätling *et al.*, 1986), while the plasmid pBR-B2-B4 contains only 1.8 kb of 3′ flanking DNA. In order to search for a possible downstream attachment region, we thus applied the *in vitro* binding assay to three consecutive *Eco*RI fragments (E6−E7, E7−E8 and E8−E9), which expand the analysed 3′ flanking region by 8.1 kb (see Figure 1 for restriction map). The affinity of these fragments was determined by comparison with the affinity of fragments B-1−X1, which—as will be shown below—contains the complete upstream attachment region; this fragment includes that portion of the attachment region which we localized on the E1−E2 fragment as well as the portion expanding further upstream. Figure 2b shows that fragment E6−E7 efficiently binds to oviduct matrices, although weaker than fragment B-1−X1, while the further downstream fragments E7−E8 and E8−E9 do not exhibit a significant affinity to matrices. These results define a downstream attachment region within the E6−E7 sequence which covers from 1.3 to 5.0 kb downstream of the poly(A)$^+$ addition site. For unknown reasons, however, this downstream attachment region exhibits a weaker affinity to matrices than the upstream attachment region. In Figure 2a, the fragment E6−B4, which is contained in the fragment E6−E7, was found to bind very weakly to matrices, probably because it represents a portion of the downstream matrix attachment region. The 3′ boundary of the chromatin domain of the lysozyme gene was previously determined to be located within the 1.95 kb B4−B5 sequence and probably within adjacent downstream sequences (Strätling *et al.*, 1986). Since the fragment E6−E7 includes the sequence B4−B5 and the abutting downstream sequence, we conclude that the identified downstream matrix attachment region co-maps with the 3′ boundary of the lysozyme gene domain.

Detailed mapping of the upstream matrix attachment region

In order to map the upstream matrix attachment region with greater detail, we digested the plasmid pUR-E1-E2 with *Eco*RI and *Sac*I and, separately, with *Eco*RI alone (see Figure 1 for restriction map); cleavage with *Sac*I divides the 1.75 kb E1−E2 fragment into an upstream 1.00 kb E1−*Sac*I fragment and a downstream 0.75 kb *Sac*I−E2 fragment. A mixture of all end-labeled fragments was incubated with matrices from oviduct nuclei. The autoradiogram in Figure 3a shows that the 1.00 kb E1−*Sac*I fragment is retained by matrices as preferentially as the parental 1.75 kb E1−E2 fragment, while the 0.75 kb *Sac*I−E2 fragment binds poorly. In a second experiment, the plasmid pUR-E1-E2 was digested with *Eco*RI and *Hae*II. *Hae*II cleaves the E1−E2 insert into an upstream 0.35 kb E1−*Hae*II fragment and a downstream 1.40 kb *Hae*II−E2 fragment, while the vector DNA is cleaved into five fragments (see Figure 1 for restriction map; Rüther, 1980). Both subfragments derived from the E1−E2 fragment by cleavage with *Hae*II bind with approximately equal affinity to oviduct matrices (Figure 3b). Thus the results in Figure 3a and b localize the 3′ border of the upstream matrix attachment region to the *Hae*II−*Sac*I sequence.

Since we suspected that the attachment region extends further upstream from the E1 site, we constructed the plasmid pUC-B-1−X1. This plasmid contains a 2.95 kb *Bam*HI−*Xba*I fragment, which harbors the already analysed E1−*Sac*I

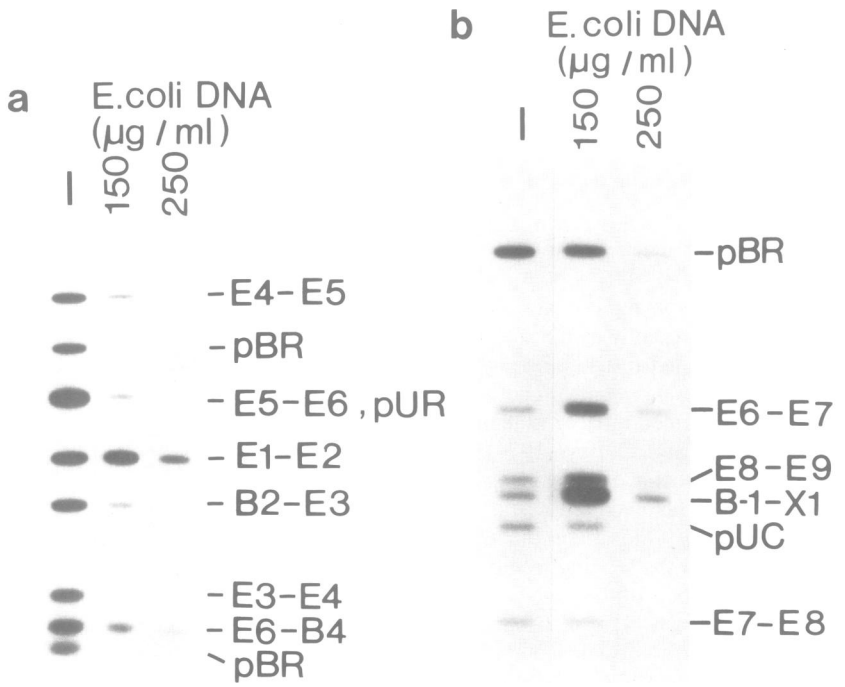

Fig. 4. Matrices from transcriptionally inactive erythrocytes bind specifically to the same fragments of the lysozyme domain as oviduct matrices. (**a**) and (**b**) Nuclear matrices prepared from hen erythrocytes were incubated with the same end-labeled fragments used in Figure 2a and b in the presence of 150−250 µg/ml of *E.coli* competitor DNA.

sequence and 1.95 kb of abutting upstream DNA. The plasmid pUC-B-1−X1 was cleaved with *Hin*dIII and separately with *Eco*RI (see restriction map in Figure 1). Figure 3c shows that the 1.29 kb H1−X1 fragment binds most prominently to nuclear matrices confirming our results in Figure 3a, since this fragment contains the previously defined E1−*Sac*I sequence. Two fragments from the left side of the 2.95 kb *Bam*HI−*Xba*I fragment also bind efficiently to matrices: the 1.08 kb fragment H-1−H1 and the 1.76 kb fragment B-1−E1. On the contrary, the 3.28 kb fragment carrying the sequence B-1−H-1 in addition to vector DNA exhibits no affinity to matrices. The 3.89 kb fragment carrying the sequence E1−X1 binds less efficiently to matrices than the fragment H1−X1 but with an efficiency approximately equal to that of fragment B-1−E1. It is likely that the E1 site is located withing a binding site of the attachment region and that, therefore, cutting at E1 reduces the affinity of the abutting downstream fragment. In a last experiment, we cleaved the plasmid pUC-B-1−X1 with *Pvu*II and *Bam*HI (see restriction map in Figure 1). A comparison of the input lane in Figure 3d with the lane of fragments bound at 500 μg/ml *E.coli* DNA shows that the 1.32 kb B-1−P1 fragment binds with nearly the same efficiency to matrices as the 1.45 kb P1−P2 fragment. The 0.18 kb fragment P2−X1 does not bind to matrices, confirming our result in Figure 3a that the attachment region does not extend downstream of the *Sac*I site. In a comparison of Figure 3c with 3d, the fragment H-1−H1 appears to bind less efficiently than the fragment B-1−P1. A possible explanation for this might be that the H-1 site is located within a binding site of the attachment region and the cleavage at H-1 reduces the affinity of the abutting fragments. We thus conclude that the 5′ border of the upstream matrix attachment region is localized to the H-1−P1 sequence or, alternatively, resides slightly upstream of the H-1 site. Since all restriction fragments of the H-1−*Sac*I sequence bind to matrices, the attachment region is composed of multiple binding sites. In support of the multifocal structure of the attachment region, a modulation of the matrix affinity within the attachment region is indicated by the reduced binding of abutting fragments after cleavage at E1 and H-1.

Recognition of the same binding sites by matrices from transcriptionally inactive erythrocytes

In the chicken the lysozyme gene is expressed in the oviduct and in macrophages but not in other tissues such as liver, kidney and erythrocytes (Fritton *et al.*, 1984). Because of a less likely contamination by macrophages, we have chosen erythrocytes as the source to study the binding of lysozyme domain restriction fragments to matrices from a transcriptionally inactive cell type. It must be emphasized, however, that matrices prepared from hen erythrocytes lack most of the internal nuclear network and are composed of nearly empty shells of pore complex-lamina (Lafond and Woodcock, 1983). In Figure 4a and b we show that the 5′ located fragments B-1−X1 and E1−E2 bind specifically and strongly to matrices, while the 3′ located fragments E6−E7 and E6−B4 exibit a weaker affinity. A comparison of Figure 2 with Figure 4 thus shows that matrices from transcriptionally inactive erythrocytes recognize the same attachment sequences as oviduct matrices. However, the specific binding to erythrocyte matrices is competed out by lower concentrations of *E.coli* DNA than that to oviduct matrices. We estimate that oviduct matrices bind—on a nuclear basis—~5-times the amount of restriction fragments bound to erythrocyte matrices.

In order to assess whether the binding response of small restriction fragments from the upstream attachment region to erythrocyte matrices differs from that to oviduct matrices, we repeated the detailed mapping experiments shown in Figure 3a−c with erythrocyte matrices. Figure 5a and b shows that the fragments E1−*Sac*I and E1−*Hae*II bind

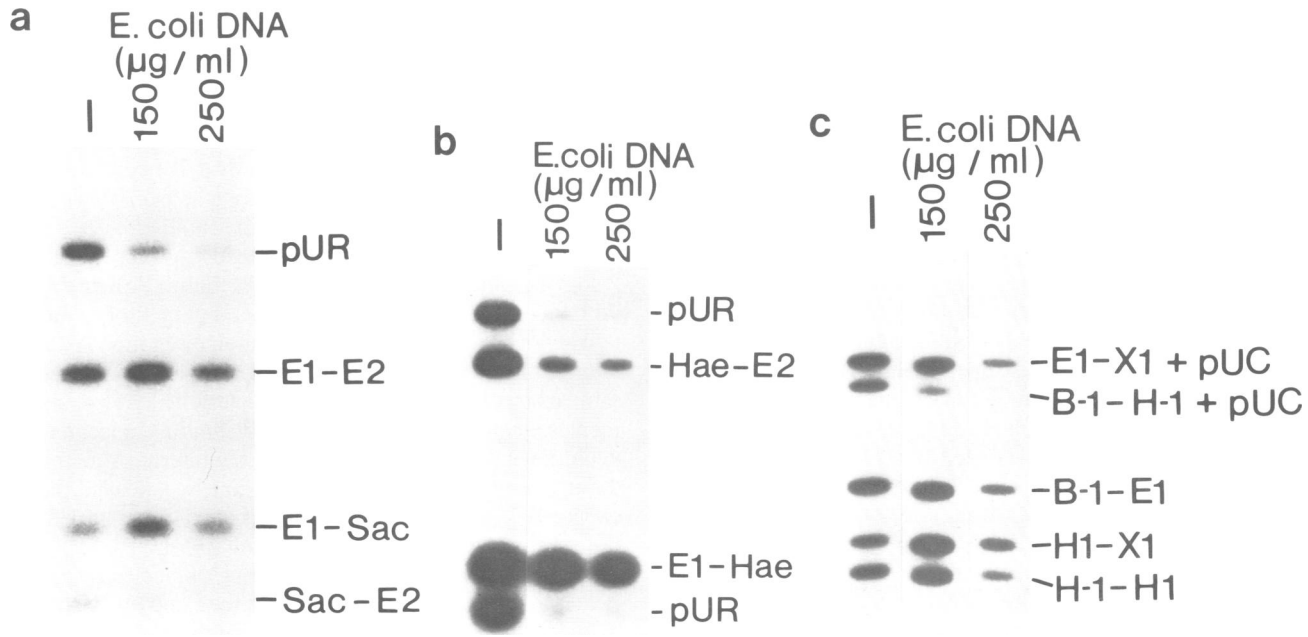

Fig. 5. Detailed mapping of the region attached to erythrocyte matrices. (**a**), (**b**) and (**c**) Nuclear matrices prepared from hen erythrocytes were incubated with the same end-labeled fragments used in Figure 3a, b and c in the presence of 150−250 μg/ml of *E.coli* competitor DNA.

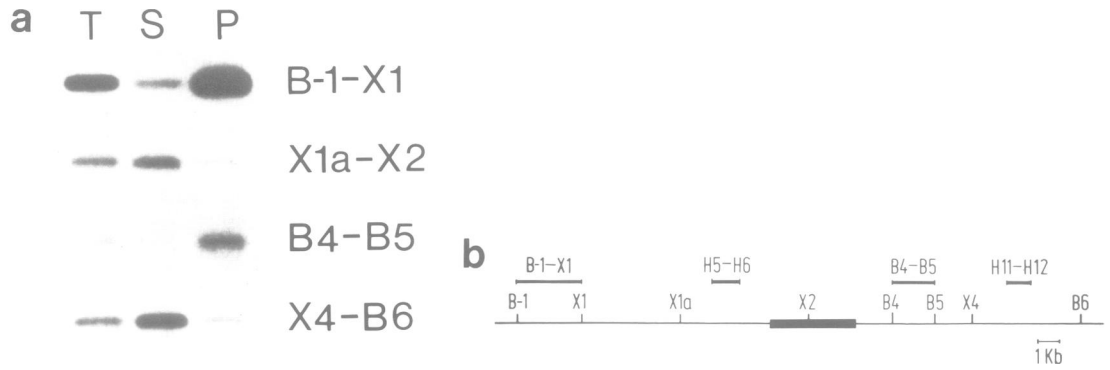

Fig. 6. Localization of the upstream and downstream matrix attachment regions at the base of a chromosomal loop in nuclear halos. (**a**) Nuclei isolated from HD11 cells were extracted with lithium diiodosalicylate and resulting halos were digested with *Bam*HI and *Xba*I. After centrifugation, DNA was purified from supernatant (S) and pellet (P) fractions. Equal amounts of DNA were electrophoretically resolved along with *Bam*HI−*Xba*I digested total DNA (T) and transferred to filters for hybridization and autoradiography. The fragments are identified by their map positions. (**b**) Map of the lysozyme domain indicating the relative position of the probes used (bars above the map), and of the restriction fragments detected by these probes. The filled box denotes the coding region. For identification of restriction sites see legend to Figure 1.

strongly to erythrocyte matrices, while the *Hae*II−E2 fragment exhibits only a weak affinity. This latter finding is in contrast to the strong binding of the *Hae*II−E2 fragment to oviduct matrices. A comparison of Figure 5c with Figure 3c shows that the binding response of all fragments to erythrocyte matrices is qualitatively identical to that of oviduct matrices; in particular cleavage at E1 appears to reduce the binding of the abutting downstream fragment. Thus erythrocyte matrices recognize the same binding sites within the upstream attachment region as oviduct matrices, with the exception that the *Hae*II−*Sac*I sequence binds weakly to erythrocyte matrices.

Nuclear envelopes, prepared from oviducts by use of the addition of RNase A and 2-mercaptoethanol according to Kaufmann *et al.* (1983), which lack the internal network, retain less than 3% of B-1−X1 fragments retained by nuclear matrices.

Chromosomal loop attachment regions in histone-extracted nuclei

To determine whether those fragments which bind to nuclear matrix preparations *in vitro* are anchored to the base of a chromosomal loop, we employed the procedure of Laemmli and coworkers to localize loop attachment sites (Mirkovitch *et al.*, 1984). Nuclei were prepared from HD11 cells, an established line of chicken macrophages transformed by the *myc*-containing retrovirus MC29 (Leutz *et al.*, 1984), and were extracted with lithium 3′,5′-diiodosalicylate. Halos were digested with *Bam*HI and *Xba*I and, after centrifugation, ~55−60% of the DNA was solubilized. Equal loads of DNA purified from the supernatant (S) and pellet (P) fractions were separated by gel electrophoresis adjacent to standards of restricted total DNA (T) and subjected to Southern analysis using the ^{32}P-labeled probes indicated in the map in Figure 6b. As shown in Figure 6a, ~89% of fragment B-1−X1 and 82% of fragment B4−B5, which encompass the upstream and downstream matrices attachment regions, respectively, are retained in the pellets, whereas ~81% of each of the fragments X1a−X2 and X4−B6 are released into the supernatants. Qualitatively similar results were obtained with nuclei from mature hen erythrocytes. These results established that, in histone-extracted nuclei, the upstream and downstream matrix attachment regions are located at the base of a chromosomal loop, while two control sequences covering 5.95 and 5.0 kb respectively do not contain anchoring points.

Competition between chicken lysozyme, Drosophila and mouse matrix attachment regions

We first established that the binding to oviduct matrices of fragment B-1−X1 in the absence of prokaryotic competitor DNA shows a typical saturation curve (results not shown). Scatchard analysis yielded a straight line and we calculated that oviduct matrices contain ~ 16 000 binding sites for the upstream matrix attachment region. We then determined whether the downstream lysozyme, a *Drosophila* heat-shock and a mouse immunoglobulin attachment region could compete for the same matrix binding sites recognized by the upstream lysozyme attachment region. Figure 7a shows that addition of increasing concentrations of fragment E6−E7 containing the downstream matrix attachment region reduced the binding of labeled B-1−X1 fragments to nearly the same extent as the addition of unlabeled B-1−X1 fragments. In contrast, the abutting fragment E7−E8 competed poorly (Figure 7b). Most significantly, a 1 kb *Xba*I−*Xba*I sequence (IgE) containing the matrix attachment region of the mouse heavy chain immunoglobulin locus (Cockerill *et al.*, 1987) and a 4.5 kb *Xho*I−*Xho*I fragment (hsp 70) containing the matrix attachment region of the *Drosophila* hsp 70 heat-shock genes at the 87A7 locus (Voellmy and Rungger, 1982; Mirkovitch *et al.*, 1984) compete as effectively as the upstream lysozyme matrix attachment region (Figure 7b). In Figure 7d the competition efficiency of each competitor is quantitatively evaluated by scanning the autoradiograms. As *Drosophila*, mouse and chicken attachment regions recognize the same matrix binding sites, those structural features of attachment sequences determining matrix binding appear to be evolutionarily conserved. To study the internal structure of the upstream matrix attachment region, we cleaved the sequence B-1−X1 with *Pvu*II into halves: an upstream 1.32 kb B-1−P1 fragment and a downstream 1.45 kb P1−P2 fragment. Figure 7c shows that the binding of labeled fragments B-1−P1 and P1−P2 was reduced to the same relative extent by unlabeled B-1−X1 and E6−E7 fragments. Interestingly, fragments B-1−P1 and P1−P2 were also competed at the same relative extents by unlabeled plasmid Bam12a, which contains a sequence from the downstream P1−P2 fragment (see map in Figure 1). These results show that the upstream

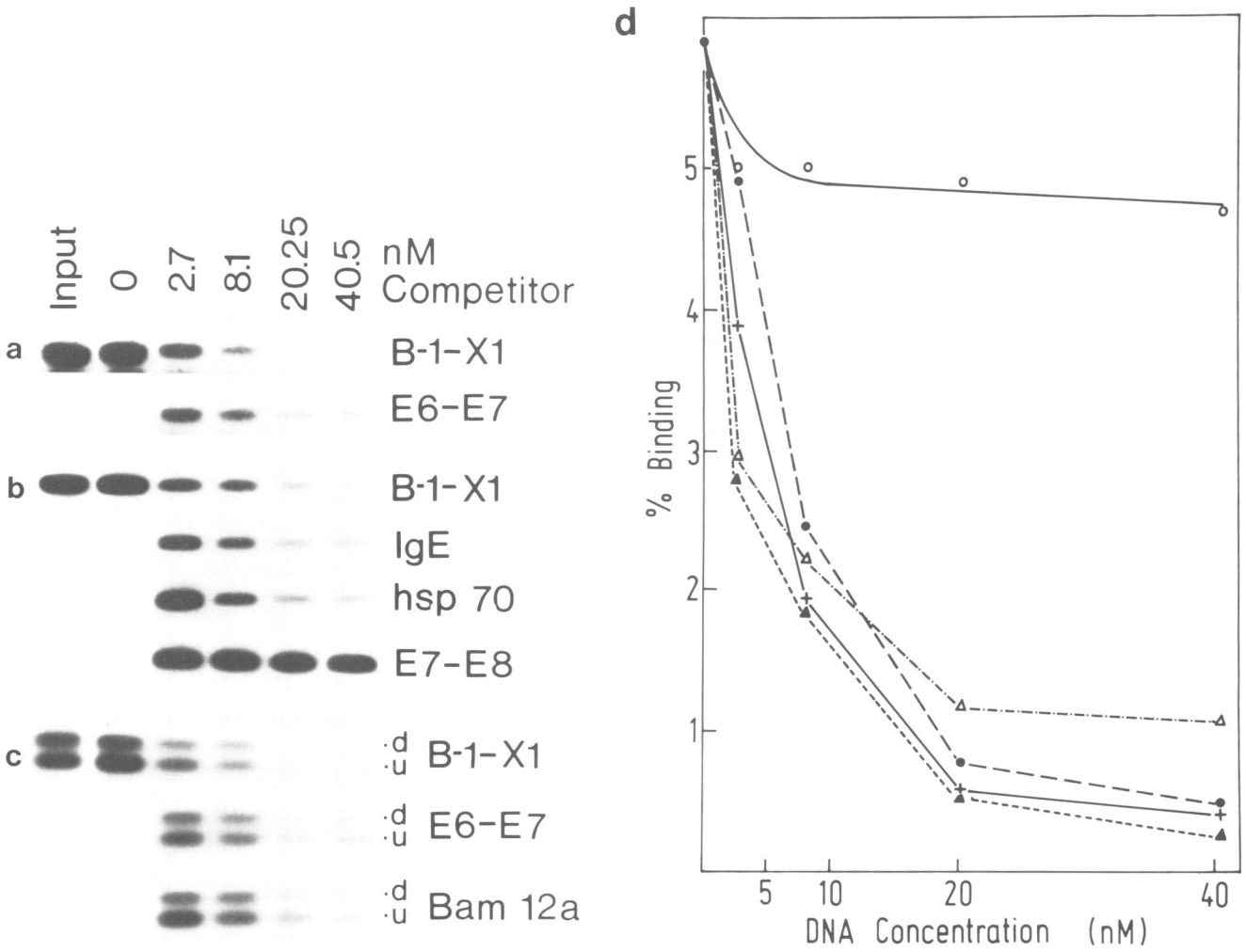

Fig. 7. *Drosophila*, mouse and the downstream lysozyme matrix attachment regions compete with the upstream lysozyme matrix attachment region for binding sites in oviduct matrices. Oviduct matrices (2.5×10^6 nuclei equivalents) were incubated for 2 h in a 50-μl DNA-binding assay with ^{32}P-labeled restriction fragments (0.27 nM) derived from *Bam*HI−*Xba*I-cleaved pUC-B-1−X1 (**a**) or with ^{32}P-labeled gel-purified fragment B-1−X1 (**b**) in the presence of unlabeled competitors at 2.7−40.5 nM: *Bam*HI−*Xba*I-cleaved pUC-B-1−X1, and *Eco*RI-cleaved pBR-E6−E7 (**a**); *Bam*HI-cleaved pUC-B-1−X1, *Bam*HI-cleaved pUC-IgE, *Eco*RI-cleaved plasmid 122X13 (hsp 70), and *Eco*RI-cleaved pBR-E7−E8 (**b**). (**c**) B-1−X1 was cleaved with *Pvu*II into an upstream (u) 1.32 kb B-1−P1 and a downstream (d) 1.45 kb P1−P2 fragment. Oviduct matrices were incubated with these ^{32}P-labeled fragments in the presence of unlabeled competitor *Xba*I-cleaved pUC-B-1−X1, *Eco*RI-cleaved pBR-E6−E7, and *Xba*I-cleaved plasmid Bam12a at 2.7−40.5 nM. The autoradiograms in (**a**)−(**c**) show the electrophoretically resolved matrix-bound fragments with a standard (5% of the input sample; first lane). (**d**) Specific binding of the B-1−X1 fragment was determined by scanning the autoradiograms in (**a**) and (**b**). Unlabeled competitors: ○, E7−E8; ●, hsp 70; +, IgE; △, E6−E7; ▲, B-1−X1. The low amount of binding of fragment E7−E8 is considered to be unspecific.

and downstream halves of the attachment region competed for the same matrix binding sites.

Sequences homologous to the topoisomerase II cleavage site and A- and T-rich stretches in the upstream attachment region

Previous studies on the matrix attachment regions of three *Drosophila* genes and the mouse κ immunoglobulin gene have revealed an enrichment of sequences related to the consensus of the topoisomerase II cleavage sequence (Gasser and Laemmli, 1986a,b; Cockerill and Garrard, 1986). We have analysed both strands of the 2.95 kb *Bam*HI−*Xba*I fragment containing the upstream matrix attachment region of the chicken lysozyme domain for sequences homologous to the central 6 bp (underlined) of the topoisomerase II cleavage sequence, whose consensus is

$$\mathrm{GTN}^{\mathrm{A}}_{\mathrm{T}}\underline{\mathrm{A}^{\mathrm{T}}_{\mathrm{C}}\mathrm{ATTNATNN}}^{\mathrm{G}}_{\mathrm{A}},$$

allowing various extents of mismatches in addition to the four undefined bases (labeled N). A perfect match of the consensus is not present but we find one 14/15 and two 13/15 matches in the coding strand (filled circles) in or near the 3′ portion of the attachment region and one 13/15 match in the noncoding strand (open circle) at the 5′ end of the attachment region (see Figure 1).

The attachment sequence from H-1 to S (see Figure 1) is AT-rich (61%), while the flanking sequences lacking matrix binding sites (B-1−H-1 and S−X1) are equally AT-rich (59% and 68% respectively). An analysis of repeated sequences within the *Bam*HI−*Xba*I fragment revealed two T-rich stretches within the H-1−S sequence and three outside of it (indicated by crosses in Figure 1). These stretches bear similarity to the T-box found in the attachment region of several *Drosophila* genes (Gasser and Laemmli, 1986b). An A-rich sequence (22 bases in length), which might be homologous to the A-box occurring in several *Drosophila* attach-

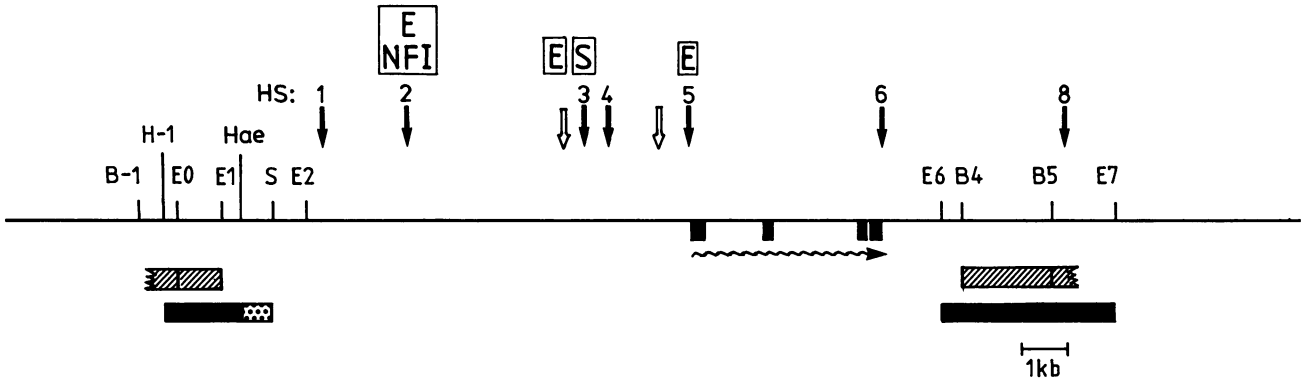

Fig. 8. Diagrammatic representation of the loop organization of the chicken lysozyme gene. The matrix attachment regions localized in the present paper are shown schematically as filled bars. The dotted region adjacent to the upstream attachment region binds only to oviduct matrices but not to erythrocyte matrix preparations. The boundaries of the lysozyme domain mapped by Strätling *et al.* (1986) are shown by hatched bars. The upstream boundary is located in the sequence E0−E1 and the adjacent upstream sequence, and the downstream boundary in the sequence B4−B5 and the adjacent downstream sequence. The toothed ends of the hatched bars indicate that the length of the adjacent boundary sequences is not known. The solid vertical arrows designate the position of seven nuclease hypersensitive sites (HS) in oviduct chromatin (Fritton *et al.*, 1983). [Site HS7 is no longer considered to be hypersensitive by Jantzen *et al.* (1986)]. In macrophages, hypersensitive sites HS3 and HS4 are lacking while two others (indicated by open arrows) appear (Fritton *et al.*, 1984). Three of the hypersensitive sites contain enhancers (E) and one a silencer (S) element (Theisen *et al.*, 1986; Steiner *et al.*, 1987). A binding site for nuclear factor I (NFI) (TGGCA-binding protein) resides in hypersensitive site HS2; three other binding sites, which do not coincide with hypersensitive sites, are not indicated (Nowock and Sippel, 1982). The filled boxes, the wavy line and the restriction sites are the same as in Figure 1.

ment regions, was found only once in the upstream attachment region (indicated by a star in Figure 1).

Discussion

In the present study, we localized, using an *in vitro* DNA binding assay, two matrix attachment regions to the chicken lysozyme gene: an upstream strong one to a *Hin*dIII−*Sac*I sequence located between 11.1 and 8.85 kb upstream of the transcription start site, and a downstream weaker one to an *Eco*RI−*Eco*RI sequence located between 1.3 and 5.0 kb downstream of the poly(A)$^+$ addition site. We further determined that these sequences are located at the base of a chromosomal loop in histone-extracted nuclei. The 5′ boundary of the chromatin domain of the lysozyme gene was previously localized to a region containing the sequence E0−E1 and the adjacent upstream sequence, and the 3′ one to a region containing the sequence B4−B5 and the adjacent downstream sequence (Strätling *et al.*, 1986). Figure 8 summarizes these data and clearly shows that the upstream matrix attachment region co-maps with the 5′ boundary of the domain, while the downstream attachment region co-maps with the 3′ boundary. It has been found for many genes and gene families that the open chromatin structure of these genes when they are expressed extend into the flanking regions (e.g. Weintraub *et al.*, 1981; Burch and Weintraub, 1983; Lawson *et al.*, 1982). However, this paper shows for the first time—in the case of the lysozyme gene—that the boundaries of the 'active' chromatin sequence co-map with matrix attachment regions and loop anchorage points. The alternating distribution of disrupted nucleosomes and 'phased', nuclease-protected particles along the *Drosophila* histone gene repeat might be a precedent to our work, although the use of micrococcal nuclease and the unresolved nature of the nuclease-protected particles limits the interpretation (Samal *et al.*, 1982). The lysozyme domain harbors nine nuclease hypersensitive sites (Fritton *et al.*, 1983, 1984; Jantzen *et al.*, 1986). Interestingly, the most upstream one of these sites (HS1) is positioned 1.05 kb away from the *Sac*I site that abuts the *Hae*II−*Sac*I fragment, to which we localized the 3′ border of the upstream matrix attachment region. Further, hypersensitive site HS2, which contains an enhancer (Theisen *et al.*, 1986) and a binding site for nuclear factor I (TGGCA-binding protein) (Nowock and Sippel, 1982) is positioned 2.8 kb away from the *Sac*I site. Thus, the upstream matrix attachment region is distinguishable from known enhancers and putative *cis*-acting regulatory elements. For three developmentally regulated genes of *D.melanogaster* a cohabitation of upstream matrix (and scaffold) attachment regions with enhancer and upstream regulatory elements was reported (Gasser and Laemmli, 1986b). Also the matrix attachment regions of two immunoglobulin genes reside next to the enhancer elements of these genes (Cockerill and Garrard, 1986; Cockerill *et al.*, 1987). Nevertheless, the present study shows that matrix attachment regions are not always located adjacent to transcriptional control sequences.

The upstream matrix attachment region is featured by a multifocal structure composed of multiple binding sites. This is indicated by the binding of subfragments of the attachment region to matrices and, after cleavage at a central *Eco*RI site (E1) or a peripheral *Hin*dIII site (H-1), by a reduced binding affinity of abutting fragments relative to fragments abutting neighboring sites. Thus, these two restriction sites are likely to be located within separate binding sites of the attachment region. The smallest fragment binding to matrices (E1−*Hae*II) is 346 bp in size; it is further likely that the restriction fragments of plasmids pUC-B-1−X1 and pUR-E1−E2 bind independently to matrices (but not cooperatively). Thus, it may be postulated that the upstream attachment region contains multiple discrete binding sites. The packaging of the upstream attachment region into a nucleosomal structure (Strätling *et al.*, 1986) also argues against one large continuous binding site. However, the minimal size and the localization of the individual binding sites remains to be determined. Multiple binding sites have previously been found in the distal and proximal attachment regions of the alcohol dehydrogenase and the Sgs-4 gene of *D.melanogaster*

(Gasser and Laemmli, 1986b).

The sequence requirements for attachment are not at all clear. The upstream matrix attachment region exhibits a 61% A-T content. However, A-T richness *per se* is not sufficient to define matrix attachment regions, since those flanking sequences of the B-1−X1 fragment which do not bind to matrices are equally A-T-rich as the central portion (from H-1 to S) containing the attachment region. Rather, A-T richness may be a general property of matrix attachment sites (Gasser and Laemmli, 1986b; Cockerill *et al.*, 1987). Second, the significance of A and T boxes as attachment sequences should be considered with caution, since three out of a total of six of these boxes reside in those parts of the B-1−X1 sequence which are not involved in binding (see Figure 1; Gasser and Laemmli, 1986b). Third, the lack of topoisomerase II in hen erythrocytes and the observation that topoisomerase II levels within a particular cell are a reflection of the proliferative state of that cell (Heck and Earnshaw, 1986), suggest that topoisomerase II is not mediating the attachment in erythrocytes.

We find that the same restriction fragments which bind to oviduct matrices also bind specifically to matrix preparations from transcriptionally inactive erythrocytes, although the latter binding is competed out by lower concentrations of *E.coli* DNA than that to oviduct matrices. This is surprising as the matrices prepared from chicken erythrocytes are composed of nearly empty shells of nuclear pore complex-lamina (Lafond and Woodcock, 1983). Assuming that matrices from chicken leukocytes contain as many binding sites as oviduct matrices, the (5-fold reduced) level of specific binding to erythrocyte matrix preparations cannot be accounted for by the contribution of leukocytes (0.85%) to the blood cell population utilized. Instead, it has to be concluded that the binding occurs to the remnants of the internal nuclear network, which are hardly visible in electron micrographs. The binding to pore complex-lamina structures appears to be unlikely, since envelopes from oviduct nuclei lacking the internal nuclear network (Kaufmann *et al.*, 1983) exhibit <3% of the binding of oviduct matrices. In any case, our results with three different sources (oviduct, erythrocytes and macrophages) indicate that the sites for specific binding of matrix attachment sequences are conserved in a manner independent of the cell type and transcription.

It is important to emphasize that artefacts induced by the matrix isolation procedure and inherent in the *in vitro* DNA binding assay cannot be fully ruled out. For example, the harsh method to prepare matrices by DNase I digestion and extraction with 2 M NaCl may rearrange matrix organization and block binding sites operating in intact nuclei and, conversely, generate binding sites not operating *in vivo*. Notably, the DNA binding assay, as well as the extraction method using lithium diiodosalicylate, identify the attachment of *naked* DNA fragments. This bears potential pitfalls, since deproteination may uncover non-functioning attachment sequences. Further, since nuclei extracted with lithium diiodosalicylate can specifically bind fragments containing scaffold attachment regions during digestion with restriction enzymes (Gasser and Laemmli, 1986b), the possibility exists that the method also identifies fragments whose matrix attachment is blocked in intact nuclei. Therefore, details of the matrix attachment observed here should be viewed with caution; however, the fact that various lysozyme domain sequences respond differently to the binding assays used strengthens the minimum conclusion that the chicken lysozyme domain contains at its boundaries sequences specifically binding to nuclear matrices.

In oviduct chromatin the lysozyme gene is flanked by seven nuclease hypersensitive sites (filled arrows in Figure 8; Fritton *et al.*, 1983; Jantzen *et al.*, 1986). In macrophages, two of these sites are lacking while two others appear at different positions (open arrows; Fritton *et al.*, 1984). The specific expression of these sites in different tissues and at different developmental and functional states suggests that they are implicated in the cell-type-specific and developmentally regulated expression of the gene (Fritton *et al.*, 1984). Three transcriptional enhancers (E) and a silencer (S) element were recently identified at four of these sites (Figure 8; Theisen *et al.*, 1986; Steiner *et al.*, 1987). Figure 8 clearly shows that the established matrix attachment regions bracket the coding sequence as well as all known hypersensitive sites. Thus, the lysozyme domain represents a topologically sequestered functional unit including the gene-specific, *cis*-acting control sequences. The generation of a topologically closed loop can deliver the structural basis to produce a modulated torsional stress on the lysozyme gene. Such a stress had been anticipated to occur on cellular and viral genes during transcriptional activation (Luchnik *et al.*, 1982; Villeponteau *et al.*, 1984; Ryoji and Worcel, 1984). It may be generally speculated that the limitation to specific genes of transcriptional stimulation by enhancers is provided by the sequestration into topological loops. Finally, the topological sequestration of functional genomic units by matrix attachment may confer onto these an independence of their relative positions, a notion which can be tested in gene transfer experiments.

Materials and methods

Preparation of nuclei

Oviduct nuclei were prepared from the magnum portion of the oviduct of laying hens (Lohmann Selected Leghorn). 3−5 g of tissue stored in liquid nitrogen were homogenized in 50 ml RSB−0.25 M sucrose by 10−15 strokes in a glass-Teflon potter. RSB contains 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris−HCl, 0.5 mM PMSF, pH 7.5. After passage through four layers of cheesecloth, nuclei were pelleted at 2200 r.p.m. in an HB4 rotor of the Sorvall centrifuge. Nuclei were then washed twice with RSB−0.25 M sucrose and resuspended in 13 ml of this buffer. Following the addition of 53 ml RSB−2 M sucrose, 24 ml aliquots of the suspension were layered in a tube of the Beckman SW 28 rotor onto 15 ml RSB−2 M sucrose. After centrifugation at 20 000 r.p.m., the nuclear pellet was washed twice and resuspended in RSB−0.25 M sucrose. To prepare erythrocyte nuclei, blood from adult hens was collected after decapitation in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate). Erythrocytes were washed three times in 1 × SSC, resuspended in RSB and stored in aliquots at −80°C. Lysis was achieved by incubating thawed samples for 10 min at 4°C. Nuclei were pelleted by centrifugation at 750 *g* for 10 min and washed three times in RSB.

In vitro DNA binding assay

Nuclear matrices containing <1% of the nuclear DNA were prepared following the method described by Cockerill and Garrard (1986) and stored at 20°C after combining with an equal volume of glycerol. The DNA fragment binding assay was also performed as described by Cockerill and Garrard (1986). Briefly, washed DNA matrices (from ~1.5 × 10^7 nuclei) were incubated in binding buffer (50 mM NaCl, 10 mM Tris−HCl, 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml BSA, pH 7.5) with ^{32}P-labeled DNA fragments (at 20 ng/ml) and unlabeled, sonicated *E.coli* competitor DNA (at 50−200 μg/ml). Following incubation for 90 min at 23°C, 500 μl of binding buffer were added and the matrices collected by centrifugation at 10 000 *g* for 60 s. After washing with binding buffer, matrix-bound fragments were purified, electrophoretically resolved on agarose gels, transferred to nitrocellulose filters by the method of Southern (1975) and autoradio-

graphed. Nuclear envelopes lacking the internal nuclear network were prepared following the method described by Kaufmann *et al.* (1983).

Chromatin fractionation of nuclei extracted with lithium diiodosalicylate

HD11 cells were cultured as described previously (Leutz *et al.*, 1984). 1×10^7 cells were washed once in phosphate-buffered saline, detached by treatment with trypsin, and washed four times in isolation buffer [5 mM Tris−HCl, 20 mM KCl, 0.125 mM spermidine, 0.05 mM spermine, 0.1% digitonin (Fluka), 0.5 mM Na−EDTA, 0.5% Trasylol, 0.1 mM PMSF, pH 7.5]. Following suspension in isolation buffer without EDTA, extraction of nuclei was performed as described by Mirkovitch *et al.* (1984) except that the concentration of lithium diiodosalicylate was reduced to 12.5 mM and the pH of the extraction buffer was carefully adjusted at pH 7.4. Nuclear halos were digested with 1000 U *Bam*HI and 1300 U *Xba*I for 2 h at 37°C and DNA was purified from solubilized (S) and insoluble (P) fractions. Naked DNA from intact nuclei (T) was digested similarly. After electrophoresis, DNA was transferred according to Southern (1975) to nitrocellulose filters and hybridized with ^{32}P-labeled, nick-translated (Rigby *et al.*, 1977) DNA probes as described previously (Strätling *et al.*, 1986).

DNA sequencing

The DNA sequence of fragment B-1−X1 was determined by producing progressively deleted clones from both ends using the exonuclease III/S1 method and sequencing these clones by use of the plasmid sequencing method.

Molecular probes

The chicken lysozyme probes used are subclones from pFF_2-lys-16 (Baldacci *et al.*, 1981), λlys30 and λlys31 (Lindenmaier *et al.*, 1979), and λlys 4/2.

Acknowledgements

We would like to thank A.E.Sippel, T.Igo-Kemenes, D.Rungger and J. Nowock for generous gifts of plasmid DNA, M.Hallupp for skilful technical assistance and J.Nowock for helpful comments on the manuscript. The work was funded by a fellowship from the Hermann & Lilly Schilling-Stiftung (to L.P.) and by a grant from the Deutsche Forschungsgemeinschaft (Str 145/11-1) and the Hamburgische Wissenschaftliche Stiftung.

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Received on December 1, 1987