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Upstream regulatory sequences of immunoglobulin genes are recognized by nuclear proteins which also bind to other gene regions

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ABSTRACT

The decanucleotide sequence (dc) TNATTTGCAT is an upstream regulatory sequence of immunoglobulin genes and occurs also upstream of certain other eukaryotic and prokaryotic genes (compiled in the accompanying paper). We now investigated the binding of proteins from nuclear extracts of a number of cell types and organisms to the dc sequence using a sensitive gel electrophoretic DNA binding assay. Binding studies with specifically designed oligonucleotides led to the following conclusions: (1) the central T of the dc sequence can be altered with only a slight decrease of protein binding activity: (2) the sequences in the neighborhood of dc have a positive or negative effect on the efficiency of protein binding; (3) C-rich sequences which occur in many K chain promoters have a protein binding activity independent of dc; (4) the dc binding protein(s) of human lymphoid cells elute from a Sephadex column in the 30.000-60.000 molecular weight range; (5) dc binding proteins were found in nuclear extracts of lymphoid as well as non-lymphoid human and murine cell lines, of Xenopus oocytes, and of yeast cells.

The finding of dc binding proteins in a wide variety of different organisms and the occurrence of dc-related sequences in the regulatory regions of several gene families point to a general role in the transcriptional regulation of the respective genes.

INTRODUCTION

The study of transcription factors and of proteins binding to promoter and enhancer elements has received much attention recently (reviews 3-5). Well characterized promoter binding proteins include the Sp1 protein and the heat shock transcription factor. Sp1, which was originally found as an activator of SV40 transcription, interacts with sequences containing the motif GGGCGG. The Drosophila heat shock factor similarly recognizes conserved upstream promoter sequences.

We have been interested in the upstream regulatory elements of immunoglobulin genes. We found the decanucleotide (dc)

TNATTTGCAT to be a highly conserved element in a region that is functionally important for the transcription of K light chain genes (6); independently the octanucleotide ATTTGCAT which is part of dc was defined by Parslow et al. (7). dc also occurs in the immunoglobulin heavy chain enhancer and, in inverted form, upstream of the heavy chain genes (6,7). The dc (or octanucleotide) sequence was recently studied in a number of transcription systems containing immunoglobulin gene regions (8-12). dc and dc related sequences were also found in several other eukaryotic and in prokaryotic gene regions (compiled in the accompanying paper, ref. 13).

It is generally assumed that sequences like dc which occur at strategic positions of gene regions are binding sites for regulatory proteins. It was indeed shown by in vivo methylation studies that the dc sequence within the enhancer of an immunoglobulin heavy chain gene participates in protein binding (14).

In a study which appeared while our experiments were in progress, Singh et al. (15) demonstrated that protein factor(s) from lymphoid cells and HeLa cells bind to restriction fragments of the heavy and light chain gene regions containing dc or dc related sequences; the factor was called IgNF-A. Recently Hromas and van Ness showed that the protein(s) which bind to the mouse V_K enhancer differ from the dc (or octanucleotide) binding protein(s) (16).

The method used in both studies (15,16) was essentially the same as the one of the present study, that is a gel electrophoretic retardation assay: in non-dissociating polyacrylamide gels protein-DNA complexes whose dissociation is prevented by using a low-ionic-strength buffer are retarded in comparison to free DNA. The method was first developed to investigate protein binding to prokaryotic DNA fragments (17-19) and was applied for instance in the identification of a protein factor which binds to α-satellite DNA (20).

Using synthetic oligonucleotides related to the K chain promoter region we have obtained similar results as those of Singh et al. (15) and Hromas and van Ness (16) and extend them mainly in two directions: we have analysed the sequence

specificity of the protein(s) and found analogous proteins in nuclei of as diverse cell types as human and mouse cell lines, Xenopus oocytes and yeast cells.

MATERIALS AND METHODS

Preparation and purification of synthetic oligonucleotides

Synthetic oligonucleotides were prepared as described in ref. 21. The single stranded oligonucleotides were purified by a preparative version of the gel electrophoretic procedure of ref. 22 (R. Schneider, pers. communication). The lyophilized DNA was dissolved in 10 mM Tris, pH 7.5, 1 mM EDTA, 90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and electrophoresed at 50°C on a 1.5 mm thick 11% polyacrylamide gel containing 6 M urea for 7 h at 30 W. The full length oligonucleotide was identified under UV illumination and the band was excised from the gel. The minced gel slice was extracted overnight in H_2O. After addition of sodium acetate, pH 5.2, and $MgCl_2$ (final concentrations 0.3 M and 0.01 M, respectively) and 2.5 vol ethanol and standing overnight at -80°C, the oligonucleotides were centrifuged at 45.000 g for 30 min, redissolved in H_2O, extracted with phenol/Tris, pH 7.9, and chloroform and dialysed overnight against 5 mM Tris, 0.5 mM EDTA buffer, pH 8.0. The two complementary single stranded oligonucleotides were mixed in equimolar amounts and reassociated overnight at 4°C. The double stranded DNA was labeled with γ-^{32}P-ATP (specific activity 2.4x10^5 Bq/pmole) and T4-polynucleotide kinase (Boehringer Mannheim); ATP was removed by Millipore filter dialysis.

Cell culture

The origin of the cell lines has been described in previous papers from this laboratory (23,24). In addition, we used the acute lymphoblastic leukemia cell line GM3638 (25) and the human myeloma cell line GM2132 (26) which were obtained from the Human Mutant Cell Repository, Camden, New Jersey. HeLa and mouse L cells were grown as monolayers in minimum essential medium (Dulbecco) supplemented with 10% fetal calf serum. All lymphoid cells were propagated in suspensions containing RPMI 1640 medium, 2 mM glutamine, 18 µM 2-mercaptoethanol, and 10% or 20% fetal

calf serum depending on the cell line. The cells were grown to about 10^6 cells per ml prior to splitting or harvesting.

Mouse liver was from BALB/c mice. The mouse cell lines X63Ag8.653, NS1, and L cells were the same as used in previous work (6,9). Xenopus laevis oocytes (a gift of H. Feldmann) were obtained according to ref. 27. Nuclei of S. cerevisae were prepared by W. Hörz according to ref. 28.

Preparation of protein extracts

All protein extracts were made from fresh cells harvested by centrifugation at 1000 g. The S100 fraction and the nuclear extracts were prepared following the procedure of Dignam et al. (29) except that the extraction buffer C contained 0.35 M NaCl. Yeast nuclei and nuclei from Xenopus oocytes were minced with a glass homogenizer prior to extraction. The extracts were dialysed overnight against binding buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol). Protein concentrations were determined according to ref. 30. All preparations were shock frozen and stored in aliquots at -70°C.

Gel filtration

Gel filtration was performed on a Sephadex G200 column (80x1 cm). The running buffer was 25 mM Hepes, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, and 10% glycerole. 50 fractions were collected, concentrated with Sephadex G15, and tested for the presence of binding activity. The molecular weight range of the proteins eluted from the column was determined by subsequent SDS-polyacrylamide gel electrophoresis.

Gel electrophoretic DNA binding assay

The standard binding raction was performed according to ref. 21. The electrophoretic separation was similar to ref. 21. After the addition of bromophenol blue, the mixture was immediately loaded onto an 1 mm thick 11% polyacrylamide gel (acrylamide/bisacrylamide 40:1) containing 375 mM Tris-HCl, pH 8.8. The gel was electrophoresed at 65 V for 7-8 h in 50 mM Tris, 380 mM glycine (pH 8.3) as running buffer. For autoradiography the gel was frozen at -70°C and exposed for 5-48 h with an X-ray intensifyer screen.

RESULTS

Binding and competition experiments on the recognition of the decanucleotide dc by nuclear protein(s)

Initially three dc containing oligonucleotides and a control oligonucleotide with an Sp1 element (21) were synthesized for the protein binding studies (A,C,D, and H, respectively, in Fig. 1). The oligonucleotides correspond to sequences upstream of the mouse K light chain genes T1 (A; ref. 31) and K2 (D; ref. 32) and to the dc containing part of the ara BAD operon of E. coli (C; ref. 33). The oligonucleotide D contains in addition to dc a 15 bp sequence which is fairly well conserved upstream of the K light chain genes (pd in 6,9) and a C-rich sequence which turned out to influence protein binding (see below). As an additional feature oligonucleotide C contains the $\begin{matrix}5'\ TCACA\ 3'\\ 3'\ AGTGT\ 5'\end{matrix}$ motif of the binding site for the cAMP receptor protein (33; reviewed in the accompanying paper, ref. 13).

In Fig. 2 the outcome of a typical protein binding experiment is shown. The four different oligonucleotides were radioactively labeled and incubated with a nuclear extract of the

		dc	pd	C-rich
A	5'-AATTCACTGGTTCCCAATGATTTGCATGCTCTCACTTCACTG	+	-	-
B	5'-AATTCACTGGTTCCCAATGATGTGCATGCTCTCACTTCACTG	altered	-	-
C	5'-AATTCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTGCATG	+	-	-
D	5'-AATTCTGCAGCTGTGCCTACCCTTTGCTGATTTGCATGTACCCAAAGCATAG	+	+	+
E	5'-AATTCTGCAGCTGTGCCTACCCTTTGCGTACCCAAAGCATAG	deleted	+	+
F	5'-AATTCGTTCGACTTGCCTACCCTTGGCGTACCCAAAGCATAG	deleted	altered	+
G	5'-AATTCGTTCGACTTGCCTCCTTGAGGCGTAATGAAAGCATAG	deleted	altered	altered
H	5'-AATTCGTGGCGCGGGGCGTGGGAACGGGGCGGGTGAACGT	-	-	-
I	5'-AATTCGTGGCGCGTGATTTGCATACGGGGCGGGTGAACGT	+	-	-

Figure 1. Sequences of the synthetic oligonucleotides. The complementary strands of oligonucleotides A, B, and D-G are constructed in a way to yield upon annealing the sticky ends of EcoRI sites (one EcoRI site and one SphI site in the case of oligonucleotide C). Oligonucleotides derived from each other (see text) are indicated by arrows. Asterisks designate the altered nucleotides. The dc sequence, a conserved pentekaideca nucleotide sequence (pd in refs. 6,9) and the C-rich region are underlined. The binding site for the Sp1 protein is indicated by a broken line and the one for the cAMP receptor protein by a dotted line.

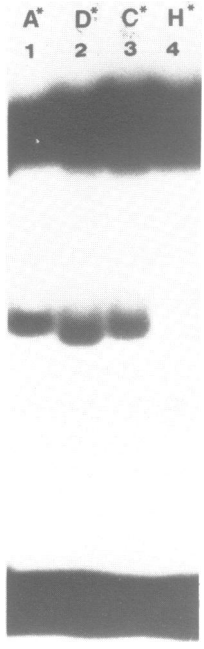

Figure 2. Binding of nuclear protein(s) from Raji cells to the radioactively labeled oligonucleotides A*, D*, C*, and H*. 20 fmoles oligonucleotide were incubated with nuclear extract containing 5 µg protein and subjected to gel retardation electrophoresis. The protein complexes with oligonucleotides B*, E*, F*, and I* had the same electrophoretic mobilities as the complexes shown in the figure.

human lymphoid cell line Raji; the mixtures were then separated by gel electrophoresis. Free oligonucleotides give rise to the most rapidly migrating band and oligonucleotides retarded by protein binding are in the middle of the gel. The band at the top of the gel probably represents oligonucleotides bound non-specifically to component(s) of the nuclear extract which do not enter the gel. As shown in Fig. 2, the protein-oligonucleotide complexes of the middle band appear only with the dc containing oligonucleotides A, C, and D but not with the control oligonucleotide H. Analogous results were obtained with nuclear extracts from other lymphoid cell lines (BL61, GM3638, GM2132) and from HeLa cells. No difference in the oligonucleotide binding was seen between the nuclear extract(s) and the cytosolic ("S100") fractions.

In a control experiment with purified cAMP receptor protein from E. coli (a gift of A.M. Gronenborn, Martinsried) only oligonucleotide C which contains the intact TGTGA motif was retarded. The appearance of the complex depended on the presence of cAMP. The mobility of the corresponding band was about the

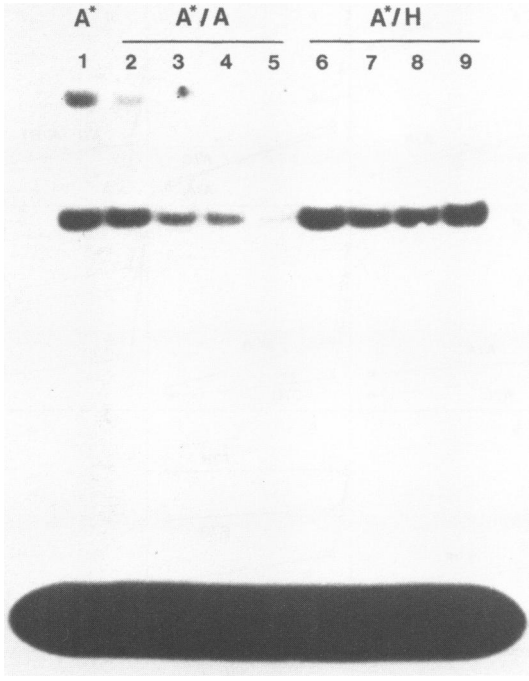

Figure 3. Sequence specificity of the dc binding protein(s) in the lymphoid cell system. Raji nuclear extract and 12 fmoles of the labeled oligonucleotide A* were incubated with increasing amounts of unlabeled oligonucleotide A (lanes 2-5) and H (lanes 6-9). Lane 1: no competitor; lanes 2,6: 12 fmoles; lanes 3,7: 60 fmoles; lanes 4,8: 120 fmoles; lanes 5,9: 1.2 pmoles of unlabeled competing oligonucleotides.

same as the one of the oligonucleotide-nuclear protein complex (experiment not shown). We could not detect any binding of the cAMP receptor protein to the dc element. This is interesting in the context of the observation reported in the accompanying paper (13) that dc or dc related sequences occur in or near several prokaryotic cAMP receptor binding sites (33).

The fact that the sequences of the three oligonucleotides A, C, and D are rather different except for the dc box makes it possible to study the specificity of the protein binding by a competition assay. A radioactively labeled oligonucleotide was mixed with increasing amounts of an unlabeled oligonucleotide, incubated with the nuclear extract and submitted to electrophoresis and autoradiography. Fig. 3 shows a typical result. The

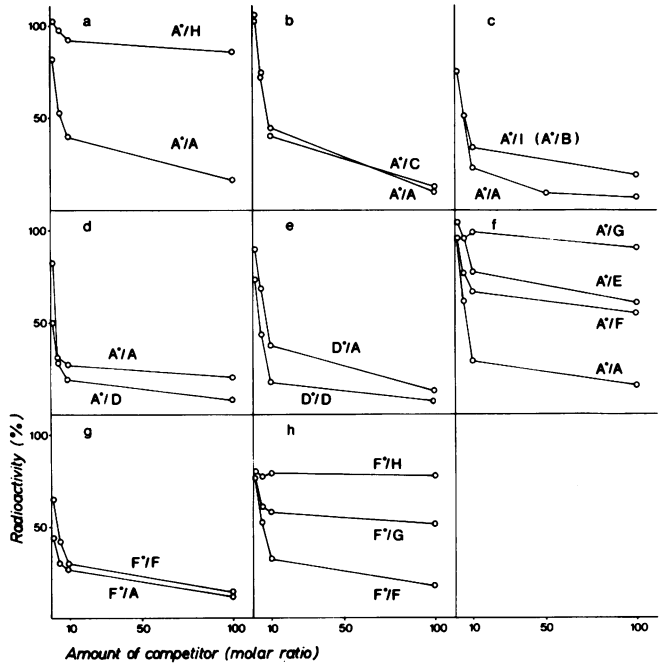

Figure 4. Quantitative evaluation of competition assays. In experiments of the type shown in Fig. 3 the bands with the oligonucleotide-protein complexes were excised and counted. The radioactivity (in percent of the one recovered from the band without competitor) is plotted against the molar excess of competitor. The letters refer to the radioactively labeled (marked by asterisks) and the competing oligonucleotides. The A*/B experiment in panel c gave practically identical curves as the A*/I experiment (see text). The F*/G experiment in the presence of α-satellite DNA (panel h and text) was carried out in the presence of 4 pmoles of a cloned 174 bp monomer of the African Green Monkey α-satellite DNA (a gift of W. Hörz).

experiments were quantitated by scintillation counting of the gel sections that contain the radioactive bands. In the panels of Fig. 4 selfcompetition, that is competition of the radioactive oligonucleotide by the same oligonucleotide in unlabeled form, is compared to competition by other (unlabeled) oligonucleotides.

It is clear from Figs. 3 and 4a that oligonucleotide H which contains no dc sequence does not compete with the dc-containing oligonucleotide A. No competition was found also with another control oligonucleotide which contains a binding site for nuclear factor I (A1/B1 in ref. 21), with poly IC, poly AT or α-satellite

DNA (experiments not shown). All dc containing oligonucleotides, on the other hand, are efficient competitors. This is exemplified by oligonucleotide C which corresponds to the sequence of a prokaryotic promoter region (Fig. 4b). Also oligonucleotide I which is derived from the G,C-rich control oligonucleotide H by insertion of a dc sequence (Fig. 1) competes well with oligonucleotide A but the amount of residual radioactivity at high competitor concentrations is somewhat higher than in the case of selfcompetition of oligonucleotide A (Fig. 4c). Oligonucleotide D, on the other hand, competes with oligonucleotide A more efficiently than oligonucleotide A with itself (Fig. 4d). This observation which was surprising at first was confirmed by the inverse experiment: competition of radioactively labeled oligonucleotide D with unlabeled oligonucleotide A was significantly less efficient than selfcompetition with oligonucleotide D (Fig. 4e). We conclude from the competition experiments that the protein(s) of the nuclear extract bind to the dc sequence and that sequences in the neighborhood of dc exert an influence on the efficiency of binding (see below).

In order to find out whether the whole dc sequence is essential for protein binding we substituted the highly conserved central T of the dc sequence by a G (oligonucleotide B in Fig. 1). This oligonucleotide competed about as well as oligonucleotide I (Fig. 4c) indicating that some substitutions are possible in the dc sequence without significant loss of protein binding ability.

In all competition experiments the general shape of the competition curves and the relative intensities of competition are reproducible from one experiment to the next (most experiments have been carried out more than three times). The extent of competition given as percent residual activity at a certain molar excess of competing oligonucleotides, however, can be compared only within one experiment.

The protein-oligonucleotide complexes are rather stable: when the radioactive oligonucleotide A is incubated with the nuclear extract from Raji cells for 15 min before a 200-fold excess of unlabeled oligonucleotide A is added, a fairly strong band of the specific complex is still observed in the retardation

gel (experiment not shown). This radioactive band is practically absent when the labeled and unlabeled oligonucleotides were mixed prior the addition of the nuclear extracts (as in all competition experiments of Figs. 3, 4 and 6).

A rough estimate of the molecular weight of the dc binding protein(s) was obtained by separating a nuclear extract from Raji cells on a Sephadex G200 column. The fractions which bind to oligonucleotide A eluted in the molecular weight range 30.000-60.000.

The dc binding protein(s) also recognize a C-rich sequence

The effect of the sequences outside of dc which makes the protein binding to the dc sequence more efficient was investigated with the help of oligonucleotides E, F, and G (Fig. 1). Oligonucleotide E which is derived from D by deleting the dc sequence is a less efficient competitor than the parent oligonucleotide D but it seems to possess a definite residual binding activity for the nuclear protein(s) (Fig. 4f in comparison with Fig. 4d and e). This binding activity cannot be due to the pd sequence since its alteration in oligonucleotide F does not change its ability to compete (Fig 4f). Alteration of the two C-rich sequences of oligonucleotide F, on the other hand, led to an oligonucleotide (G; Fig. 1) which had no competing activity any more (Fig. 4f). From these experiments and from competition experiments with radioactively labeled oligonucleotide F and unlabeled oligonucleotide A (Fig. 4g) we conclude that the dc binding protein(s) also bind to the C-rich sequence of oligonucleotide F. The C-rich sequence does not seem to be the only region of oligonucleotide F which contributes to protein binding: oligonucleotide G which does not contain the C-rich sequence any more competed to a certain extent with oligonucleotide F (Fig. 4h). This competition appears not to be due to non-specific protein binding since the control oligonucleotide H showed no competition (Fig. 4h) and the F*/G competition is not influenced by the presence of 2 µg poly IC or poly AT or by the presence of α-satellite DNA.

The binding activities for the C-rich sequence and for dc were detected in the same fractions of the Sephadex G200 column (see above). On the basis of this experiment and of the competition data we assume for the time being that the same pro-

tein(s) recognize both sequences. It is not clear whether the C-rich sequence has the same efficiency of protein binding as the dc sequence. From the experiment of Fig. 4f it appears to be weaker; but the efficiencies of the A*/F and the A*/A competitions become about equal (experiment not shown) when the crude nuclear extract is replaced by protein fractions from small Sephadex G15 or G100 columns. A decision on the question of binding efficiencies has to await the purification of the binding protein(s).

dc binding proteins are present in several unrelated species

No significant differences were detected in protein binding or competition assays when the nuclear extracts of Raji cells were replaced by extracts from other lymphoid cell lines (see above) or from HeLa cells. At least in this type of experiment no cell type specificity of the binding protein(s) could be detected.

The amounts of the dc binding protein(s) in nuclear extracts of Raji and HeLa cells, as calculated according to ref. 21, were found to be about the same. Assuming that the protein(s) bind to the oligonucleotide at a molar ratio of 1:1 we estimate that approximately 10^{-20} moles of the binding protein(s) were solubilized from one nucleus (3.2 fmoles of complex at saturation per binding assay which contains the protein of approximately 3.6×10^5 cells).

After it was clear that the dc binding protein(s) are not lymphoid cell specific it seemed interesting to look at their distribution among different species. Nuclear extracts from mouse liver and the three mouse cell lines mentioned in Materials and Methods gave similar results as the extracts from human cells (see also ref. 16) although our experiments with the mouse nuclear extracts were less easily interpretable because of a high background in the autoradiograms and because of the occasional appearance of additional band(s) (not shown).

Nuclear extracts from oocytes of Xenopus laevis and from Saccharomyces cerivisiae were found to contain dc binding proteins with similar properties as those from human or mouse cells (Fig. 5). The complexes with the yeast protein(s) migrate slightly faster than the ones with mammalian or frog protein(s). A weak binding of proteins to the control oligonucleotide H which

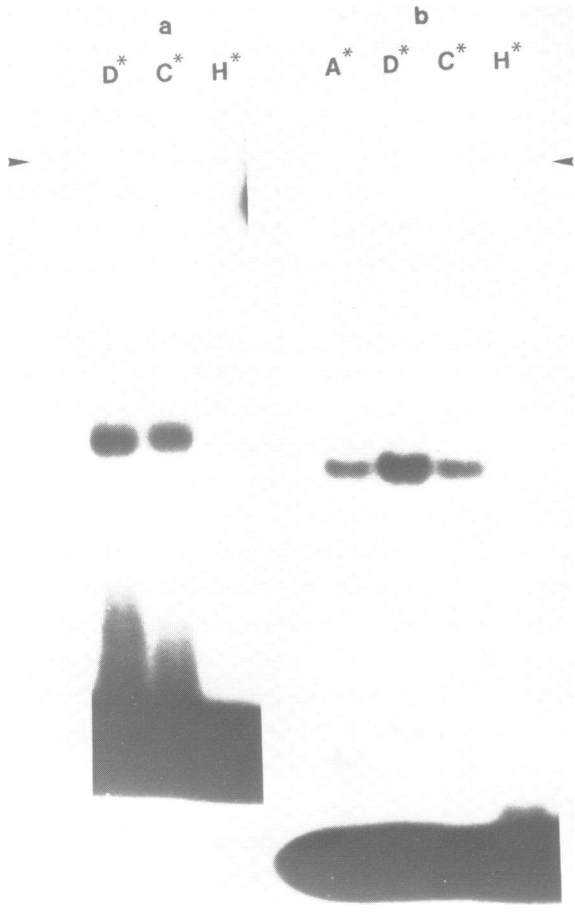

Figure 5. Binding of nuclear proteins from Xenopus oocytes (panel a) and yeast cells (panel b) under the same conditions and to the same oligonucleotides as in Fig. 2. The start points of migration are marked by arrows. The protein complexes with oligonucleotides A*, B*, E*, F*, and I* had the same electrophoretic mobilities as the complexes shown in the picture.

does not contain the dc sequence can be detected (Fig. 5) but in competition experiments (Fig. 6a,c) this oligonucleotide is similarly ineffective as in the corresponding experiments with nuclear extracts from human cells (Fig.4a). The competition of radioactive oligonucleotide A with increasing amounts of oligonucleotide C (Fig. 6b,d) is similar to the corresponding experi-

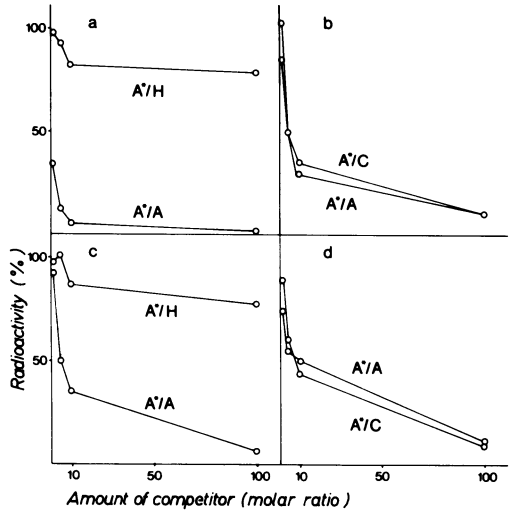

Figure 6. Quantitative evaluation of the competition assays in the Xenopus (panels a,b) and yeast systems (c,d). The experiments were carried out and are plotted as the ones of Fig. 4.

ment with nuclear extracts from Raji cells (Fig. 4b). The dc binding proteins from frog and yeast cells are similar to the human protein(s) also with respect to their binding to the C-rich sequence of oligonucleotide F (not shown). Addition of poly AT, poly IC or α-satellite DNA had no influence on the outcome of the experiments with yeast nuclear extracts; the corresponding experiments with extracts from frog oocytes were not done.

DISCUSSION

In the present paper we describe the binding of protein(s) from nuclear extracts of several different cell types and organisms to a series of specifically designed oligonucleotides from the K chain promoter region. The method applied is a very sensitive gel electrophoretic DNA binding assay. Since we used synthetic oligonucleotides of the same size range (40-50 bp) in all experiments competition experiments could be carried out without the danger of a non-specific protein binding to large outside sequences. It was found that the conserved decanucleotide (dc) sequence TNATTTGCAT of the K gene promoter is the main binding site for

the nuclear protein(s). In this sequence the central T can be altered with only a slight decrease of binding activity. This points into the same direction as the observation that restriction fragments from two K gene promoters which differ at the third position of dc (A vs C) were similarly active in an electrophoretic protein binding assay (16). We show by systematic variation of our oligonucleotide sequences that the surroundings of the dc element significantly influence the efficieny of protein binding. The G-rich sequences in the neighborhood of dc (oligonucleotide I; Fig. 1) seem to lower the efficiency of protein binding while the presence of the sequence ACCC (perhaps generally a C-rich sequence) leads to an increase in binding efficiency. The C-rich sequence has a protein binding activity independent of dc but, judging from the Sephadex G200 and the competition experiments, it seems to be the same protein(s) which bind to the two sequences. It may be noteworthy that C-rich sequences occur in many K gene promoter regions. In the MOPC41 promoter region, ACCC is located directly adjacent to dc and is protected together with dc in DNAase I footprinting experiments (15). Footprinting experiments will have to show for an isolated C-rich sequence how far the protection extends beyond the conserved sequence ACCC.

Little is known about the properties of the dc binding protein(s). The dc binding activity of human lymphoid cells elutes from a Sephadex column in the 30.000-60.000 molecular weight range but nothing is known yet about the molecular weight and the protein composition in the actual binding reaction. We have no indication from our experiments that more than one protein participates in the dc binding but any firm statement to this effect must await the purification of the protein(s). On the basis of competition experiments with α-satellite DNA and oligonucleotides containing the NFI or Sp1 binding sites we can be sure that the dc binding proteins are different from the proteins binding to these sequences.

Most of our experiments were carried out with nuclear extracts from human lymphoid cell lines. In agreement with Singh et al. (15) we could not detect any difference between the dc binding protein(s) of such extracts and of HeLa cell extracts.

Also the observation of Hromas and van Ness (16) that nuclear extracts of HeLa cells and of various mouse cell lines and tissues gave similar results could be confirmed. The finding of dc binding proteins in nuclear extracts of Xenopus oocytes and yeast cells reported in this paper indicates that these proteins are of widespread occurrence. Considering the occurrence of dc related sequences in several eukaryotic and prokaryotic promoter regions and in eukaryotic and viral enhancers (compiled in the accompanying paper, ref. 13) one may assume that the dc binding proteins serve a general function in the transcriptional regulation of a number of gene systems. Furthermore, in lymphoid cells dc and, consequently, the dc binding protein(s) contribute, together with other regulatory elements, to the cell type specificity of expression of immunoglobulin genes (9-12). It is an open question whether also in other cell types or in other gene families the dc related sequences and binding proteins have, in addition to the general function, also a specific function.

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