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Nuclear proteins TREF1 and TREF2 bind to the transcriptional control element of the transferrin receptor gene and appear to be associated as a heterodimer

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Two novel proteins that bind specifically to the transferrin receptor (TR) promoter, have been isolated from HeLa cell nuclear extract using a combination of ion exchange and oligonucleotide-affinity chromatography. TREF1 and TREF2, which have apparent molecular weights of 82 and 62 kDa, respectively, appear to be associated as a heterocomplex (TREF), and both proteins are able to contact target DNA directly. TREF interacts specifically with a region of the TR promoter which contains the TR transcriptional control element. This region is similar in sequence to the cAMP-responsive and phorbol ester-responsive elements found in several viral and cellular genes. Binding of TREF to the TR promoter results in modification of DNA topology over multiple helical turns, including a sequence revealed by a helical periodicity map as having an unusual structure.

Introduction

The transferrin receptor (TR), a transmembrane glycoprotein expressed on the cell surface, mediates iron uptake by binding and internalizing iron-loaded transferrin. Due to the essential role of iron in cell metabolism, a low level of TR is found on nearly all cell types. However, TR is abundant on tissues with high iron requirements, such as maturing erythroid cells and the placental trophoblast which provides iron to the fetal circulation. For example, exposure of the mouse Friend erythroleukemia cell line to agents that induce hemoglobin production results in increased expression of cell surface TR (Hu *et al.,* 1977).

The level of TR expression is determined not only by cellular iron requirements, but also is related to both the proliferative status of cells and the extent of cellular differentiation. Rapidly proliferating cells and many transformed cell lines have an elevated level of cell-surface TR (Larrick and Cresswell, 1979; Trowbridge and Omary, 1981; Hamilton, 1982). Indeed, increased TR expression in response to a mitogenic stimulus is observed in many systems, and in some cases this is known to be associated with increased steady-state levels of TR mRNA (Pauza *et al.,* 1984; Depper *et al.,* 1985; Miskimins *et al.,* 1986). Conversely, in many cell types, when cell division is halted upon differentiation, the level of TR expression is reduced (Tei *et al.,* 1982). Consistent with the role of TR in cellular differentiation and proliferation, experiments employing TR-specific monoclonal antibodies have established that expression of the receptor is a prerequisite for entry of cells into S-phase and subsequent completion of the cell cycle (Trowbridge and Lopez, 1982; Lesley and Schulte, 1985). It has been suggested that differentiation may involve co-ordinate synthesis of proteins subject to regulation by the transferrin cycle, an idea that is strengthened by the observation that antibodies which interfere with TR recycling in erythroid cells, but not with binding and internalization of transferrin, inhibit erythroid differentiation while proliferation remains unaffected (Schmidt *et al.,* 1986).

Determining the role of the TR in the control of cell proliferation and differentiation requires an understanding of the regulatory signals which control TR expression. Transcriptional modulation of the TR gene is mediated by interaction of transacting factors with discrete regulatory regions within the gene. A preliminary characterization of the TR promoter has revealed that <120 bp of GC-rich 5′-flanking sequence is necessary for promoter function in transient assays (Miskimins *et al.,* 1986) and contains sufficient information to allow a several-fold increase in expression of the transferrin receptor (Owen and Kuhn, 1987) or a bacterial chloramphenicol acetyltransferase (CAT) gene construct (L. Murphy and K. Miskimins, unpublished results) in response to stimu-

lation of cell growth. DNase 1 footprinting analysis has revealed at least two sequences which are protected from digestion when a 140 bp TR promoter fragment (TR140) is incubated with crude HeLa cell nuclear extract (Miskimins *et al.*, 1986), one of which includes an apparent Sp1 binding site. The adjacent upstream protected sequence exhibits a high level of sequence similarity to the transcriptional control elements present in a number of genes (Casey *et al.*, 1988). Furthermore, this element is essential for maximal activity of the TR promoter, as assayed either by transient transfection of HeLa cells (Casey *et al.*, 1988) or by in vitro transcription employing HeLa cell nuclear extract (M. R. Roberts, unpublished data). These observations prompted us to identify and isolate those factor(s) which interact specifically with this TR promoter element and therefore may be implicated in the transcriptional regulation of this gene.

In this report we describe the isolation and characterization of two novel proteins which interact as a heterocomplex and bind specifically to the transcriptional control element of the transferrin receptor gene.

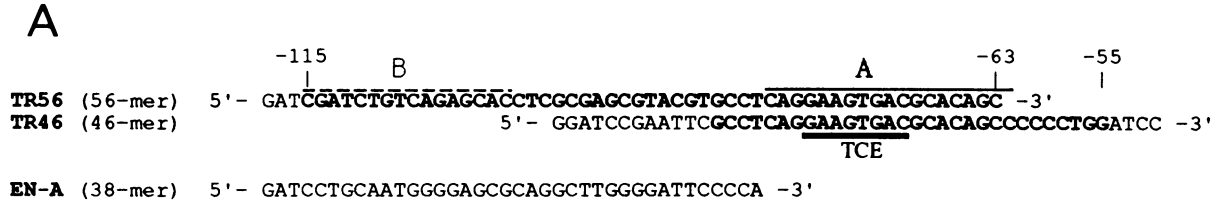
Results

Purification of TREF: ion-exchange and DNA-affinity chromatography

Nuclear extract was prepared from HeLa cells by the method of Dignam *et al.*, (1983) and fractionated on a TSK DEAE-5PW FPLC column as described in Methods. Bound protein was eluted with a 0.05–0.6 M KCl gradient, and the DEAE fractions were assayed for binding activity by gel retardation analysis, employing a 140 bp fragment (TR140) spanning −114 to +26 of the TR promoter (Miskimins *et al.*, 1986). The major promoter-binding activity eluted in fractions 29 to 32 of the gradient, between ~0.22 and 0.27 M KCl (data not shown). These fractions were subsequently pooled and fractionated further by DNA-affinity chromatography as described below.

The doublestranded (ds) oligonucleotide TR56 (Figure 1A) contains 53 bp of the TR promoter sequence (−115 to −63). DNase 1 protection studies employing the TR140 promoter fragment and crude HeLa cell nuclear extract have previously demonstrated that region A (located at the 3′ end of TR56, Figure 1A) binds nuclear protein(s) in vitro (Miskimins *et al.*, 1986). Furthermore, region A contains a sequence (−77 to −70) defined by Casey *et al.* (1988) as being essential for transcription in vivo. Subsequently, we have demonstrated transcriptional dependence upon this region in vitro (M. R. Roberts, unpublished results). Region A shares some sequence characteristics with the transcriptional control elements of a number of genes and also with an upstream region, B (Figure 1, A and B). In light of these observations, TR56 was employed in subsequent DNA-affinity chromatography.

The pooled DEAE fractions from the gradient were applied directly to an oligonucleotide-affinity column in which the ds oligonucleotide TR56 was



B

	TCE
TR region A (-80/-62)	CAGGAAGTGACGCACAGCC
TR " (rev)	GGCTGTGCGTCACTTCCTG
Ad E1a enhancer	CAGGAAGTGAC
U1 element B	GGGCAAGTGACCGTGTG
Ad MLP	GGCCACGTGACCGG
Somatostatin CRE	CTGACGTCAGAG
" (rev)	CTCTGACGTCAG
Enkephalin CRE	CTGCGTCAGC
" (rev)	GCTGACGCAG
Stromolysin TRE	TGAGTCAG
" (rev)	CTGACTCA
MT IIA TRE	GTGACTCA
" (rev)	TGAGTCAC
TR region B (-116/-100)	GCGATCTGTCAGAGCAC
TR " (rev)	GTGCTCTGACAGATCGC

Figure 1. **(A) Nucleotide sequences of the oligonucleotides employed in DNA-binding analysis.** Bold type corresponds to the sequence of the TR promoter present in TR56 and TR46. Region A (solid line) is protected from DNase 1 digestion upon incubation of HeLa cell nuclear extract with the TR promoter and contains the TR transcriptional control element (TCE, shown by a bar) defined by Casey *et al.* (1988). Region B (dotted line) exhibits limited sequence similarity to region A (see Figure 1B). EN-A corresponds to a regulatory element (enhancer A) present in the HLA-B2 gene (Biro *et al.*, 1983). (B) Sequence similarities: region A and region B of the TR promoter are compared to similar motifs in other genes (see text for details). Sequence similarity to region A is emphasized by bold type. Regions A and B are imperfect palindromes (illustrated by solid lines).

coupled to cyanogen bromide-activated Sepharose 4B. The column was then washed with 40 column volumes of loading buffer containing 40 μg/ml sonicated salmon sperm DNA, to remove nonspecific DNA-binding proteins. The remaining bound protein was then eluted from the column with 0.6 M KCl buffer in successive 1-ml fractions. These fractions were then analyzed by gel retardation analysis, employing fragment TR140 as probe (Figure 2A). The major TR-specific binding activity eluted in the second high-salt fraction (Figure 2A, lane 2) and at least five distinct DNA-protein complexes were resolved at lower protein concentrations. To determine the complexity of the affinity purified fractions, they were analyzed by SDS-PAGE and visualized by silver-staining. Four major proteins were observed, with apparent molecular weights of 235, 120, 82, and 62 kDa (Figure 2B). Multiple preparations of affinity purified samples derived from different batches of HeLa cell nuclear extract consistently yielded the 82 and 62 kDa proteins in apparently equimolar amounts (as determined by the relative intensity of both silver- and Coomassie Blue-staining and verified by amino acid analysis of the purified proteins—data not shown), while the recovery of the 235 and 120 kDa proteins was highly variable. DNA-affinity chromatography of the remaining DEAE fractions from the gradient did not result in significant enrichment of any other protein species.

82 kDa (TREF1) and 62 kDa (TREF2) proteins possess TR promoter-specific binding activity

Size-exclusion chromatography. The affinity purified sample described above (Figure 2B) was fractionated further by size-exclusion chromatography employing an FPLC filtration column (TSK G3000SW) and buffer containing 0.2 M KCl and 0.1% Triton X-100. Each fraction was assayed by gel retardation analysis using TR140 as probe (Figure 3A) and analyzed by SDS-PAGE (Figure 3B). The major TR140-binding activity is present in fractions 39 to 42 as five distinct DNA-protein complexes and corresponds to a protein peak containing the 82 kDa (TREF1) and the 62 kDa (TREF2) proteins. TREF1 and TREF2 behave as a heterodimer (TREF), since they always co-migrate, are present in apparently equimolar amounts, and elute together from the column before the 120 kDa protein species. It is noteworthy that a second peak of the TREF1 and TREF2 proteins, again in equimolar amounts, elutes in the high molecular weight range of the column, before the 235 kDa protein species (Figure 3B, fractions 32 and

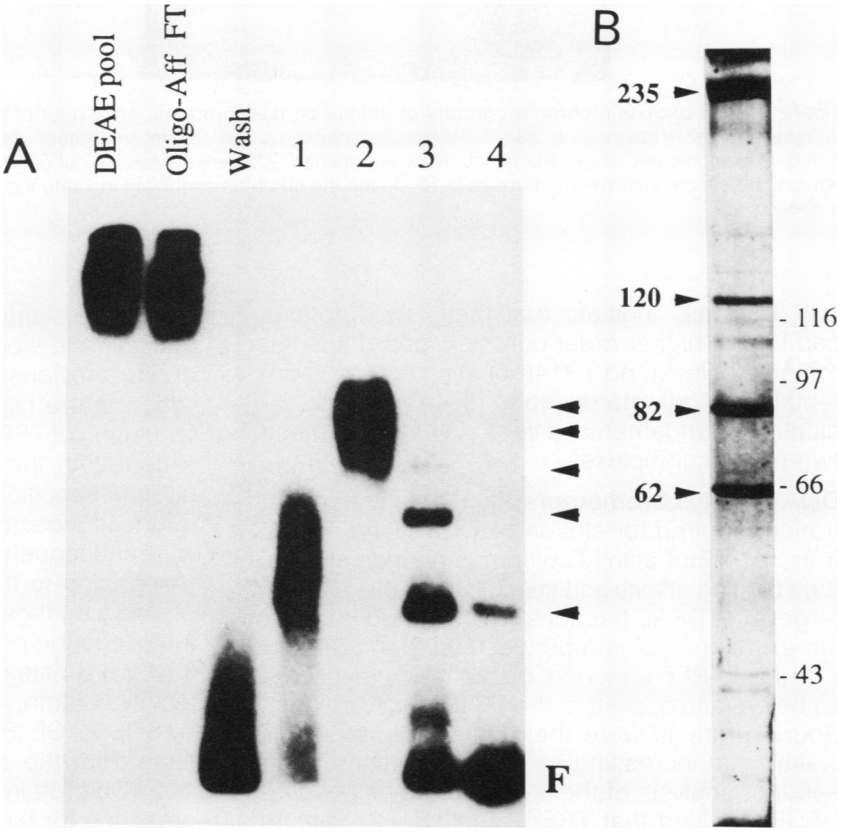


Figure 2. **DNA-affinity purification of pooled DEAE fractions exhibiting TR promoter binding activity.** The oligonucleotide-affinity column was made by coupling TR56 (Fig. 1A) to cyanogen bromide-activated Sepharose 4B. Bound proteins were eluted in 1-ml fractions with 0.6 M KCl. (A) gel retardation analysis of the affinity column fractions. The numbered lanes represent the consecutive 1-ml 0.6 M KCl fractions. The arrowheads indicate the five separate DNA-protein complexes observed. The three slowest complexes (lane 3) are not detected at the exposure shown but are easily seen on a longer exposure (and see Fig. 4C). F = free probe. (B) Silver-stained SDS-polyacrylamide gel of the proteins from the second high salt fraction (lane 2 in Figure 2A) of the affinity column. The numbers on the right refer to the positions of the molecular weight markers ($\times 10^{-3}$). Arrowheads on the left indicate the apparent molecular weights of the major proteins ($\times 10^{-3}$).

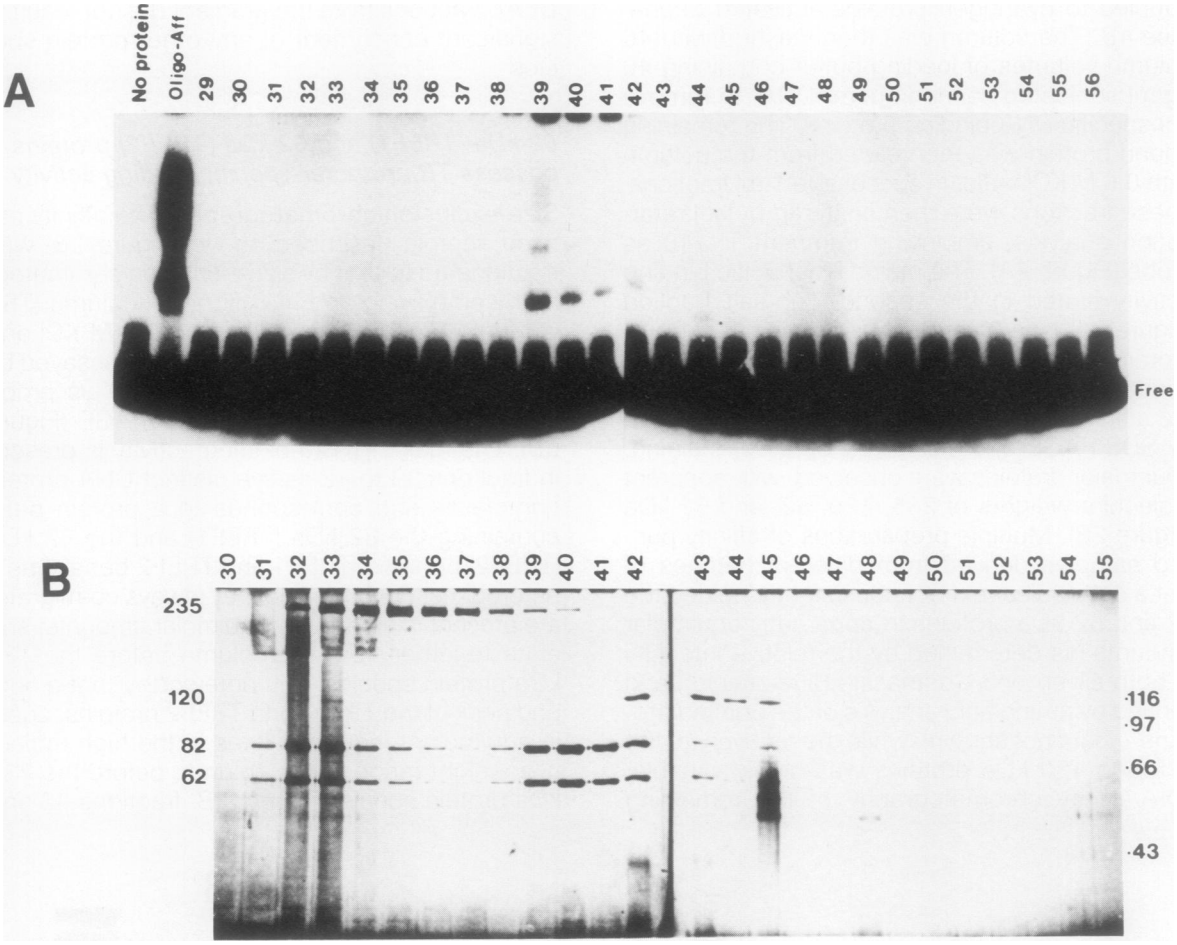


Figure 3. **Size exclusion chromatography of affinity purified proteins.** An aliquot of the affinity purified proteins (Fig. 2) was analyzed by gel filtration on a G3000SW GlasPac column (LKB). (A) gel retardation analysis of the column fractions. Fraction numbers are indicated above each lane. (B) silver-stained SDS-polyacrylamide gel of the column fractions. The numbers on the right indicate molecular weight markers ($\times 10^{-3}$), and the numbers on the left indicate the apparent molecular weights of the major proteins ($\times 10^{-3}$).

33). This may indicate that these two proteins can form a higher order complex, possibly a tetramer. However, no TR140-binding activity is detectable by gel retardation in these fractions, indicating a fundamental difference between these two protein complexes.

DNA-affinity chromatography. The salt concentration required for elution of TREF1 and TREF2 from the TR56-affinity column is an indication of their binding affinity and specificity for the TR DNA target site. DEAE fractions which eluted from the ion-exchange column between 0.22 and 0.27 M KCl (i.e., TREF-enriched fractions) were pooled as before and applied to the TR56-affinity column. Bound proteins were then eluted by sequential washing at increasing salt concentrations. Subsequent analysis of the eluted fractions by SDS-PAGE revealed that TREF1 and TREF2 co-elute from the column at 0.6 M KCl (Figure 4A), and maintain the stoichiometric relationship observed during previous fractionation steps. The observation that a high salt concentration is required for elution of TREF from the affinity column is consistent with the ability of TREF to bind in a sequence-specific manner to TR promoter sequences present in the TR56 ds oligonucleotide.

In subsequent analyses, DEAE fractions corresponding to those which had previously been pooled (i.e., those fractions which eluted from the ion exchange column between 0.22 and 0.27 M KCl), were instead applied individually to the TR56-affinity column, and the eluted proteins analyzed by SDS-PAGE (Figure 4B). TREF1 and TREF2 co-elute from the ion-exchange column before the 120 kDa protein, and are not dependent upon its presence for binding to the DNA-affinity column

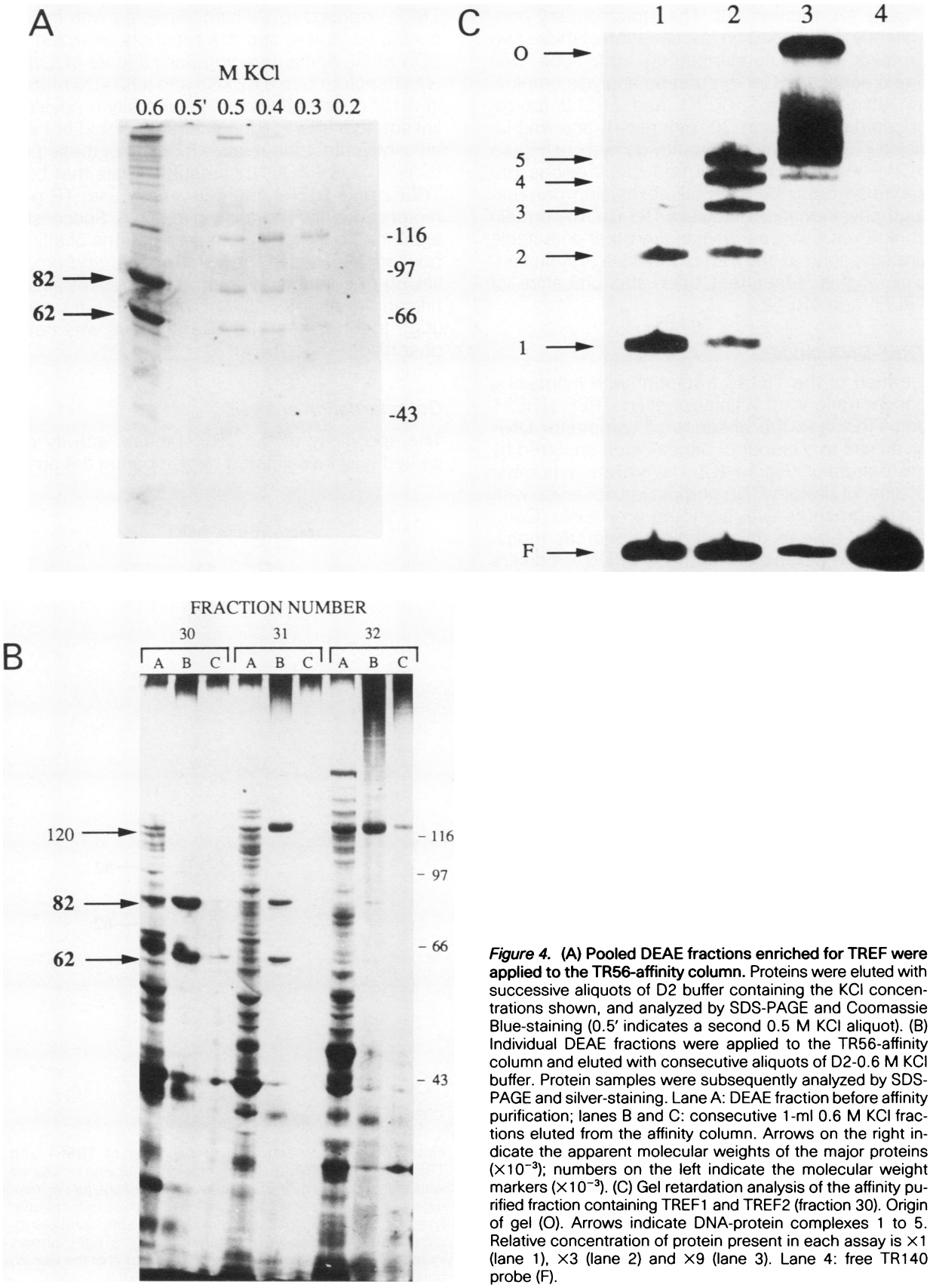


Figure 4. **(A) Pooled DEAE fractions enriched for TREF were applied to the TR56-affinity column.** Proteins were eluted with successive aliquots of D2 buffer containing the KCl concentrations shown, and analyzed by SDS-PAGE and Coomassie Blue-staining (0.5′ indicates a second 0.5 M KCl aliquot). (B) Individual DEAE fractions were applied to the TR56-affinity column and eluted with consecutive aliquots of D2-0.6 M KCl buffer. Protein samples were subsequently analyzed by SDS-PAGE and silver-staining. Lane A: DEAE fraction before affinity purification; lanes B and C: consecutive 1-ml 0.6 M KCl fractions eluted from the affinity column. Arrows on the right indicate the apparent molecular weights of the major proteins ($\times 10^{-3}$); numbers on the left indicate the molecular weight markers ($\times 10^{-3}$). (C) Gel retardation analysis of the affinity purified fraction containing TREF1 and TREF2 (fraction 30). Origin of gel (O). Arrows indicate DNA-protein complexes 1 to 5. Relative concentration of protein present in each assay is ×1 (lane 1), ×3 (lane 2) and ×9 (lane 3). Lane 4: free TR140 probe (F).

(Figure 4B, fraction 30). The stoichiometry consistently maintained on fractionation of these two proteins under nondenaturing conditions was again observed. Gel retardation analysis employing affinity purified TREF1 and TREF2 (corresponding to fraction 30, Figure 4B) and end-labeled TR140 was subsequently carried out (Figure 4C). These results confirmed the previous observation obtained from size-exclusion chromatography: formation of the five TR140 DNA-protein complexes is independent of any other detectable proteins such as the 120 or 235 kDa protein species and is dependent upon the presence of TREF1 and TREF2.

TREF-DNA ladder

Titration of the TR140 fragment with increasing concentrations of affinity purified TREF (TREF1 and TREF2) in the absence of competitor DNA gives rise to a ladder of bands when analyzed by gel retardation (Figure 4C). The maximum number of distinct protein-DNA complexes observed with this fragment is five, as noted previously. Comparison of the relative gel electrophoretic mobilities of the complexes to those of DNA markers of known size revealed that the apparent molecular weight increments between the complexes were regular, i.e., complexes 1, 2, 3, 4, and 5 have apparent molecular weights of n, 2n, 3n, 4n, and 5n, respectively. When the shorter DNA probe TR56 is used instead of TR140, saturation is reached at 3n (data not shown). The simplest interpretation of this result is that n is equivalent to one mole of TREF and that complexes 1, 2, 3, 4, and 5, contain 1, 2, 3, 4, and 5 moles of TREF bound per mole of DNA. However, TREF protein-TR DNA complexes with apparent molecular weights greater than n are unstable compared to the specific complex n (see below), since they are competed away readily by nonspecific competitor DNA (unpublished observations). This type of "stacking" behavior has also been described for the *lac* repressor, which yields a ladder of eight complexes when titrated with a 203 bp target operator fragment (Fried and Crothers, 1981).

UV-photoactivated crosslinking of TREF1 and TREF2 to TR promoter DNA

To verify the DNA-binding specificity of TREF1 and TREF2 and to determine whether one or both of the TREF proteins actually contacts the target DNA, the following UV-crosslinking experiment was carried out, employing TR46 as probe. The ds oligonucleotide TR46 (Figure 1A) contains 30 bp of TR sequence (−84 to −55), and unlike TR56, contains region A but not region B. Uniformly ^{32}P-labeled TR46 was incubated with affinity purified TREF, exposed to UV-light, digested with micrococcal nuclease, and the products analyzed by SDS-PAGE and autoradiography (Figure 5). Label was transferred to a 62 kDa and a 82 kDa protein in a UV-dependent manner, and since covalent linkage to a short oligonucleotide should not significantly affect the relative mobility of these proteins in SDS-PAGE, this result reveals that both TREF1 and TREF2 interact directly with TR promoter sequences containing region A. Successive experiments with different preparations of affinity purified TREF gave the same result, and crosslinking of contaminating proteins present at significant levels in certain of these TREF fractions (e.g., the 120 and 235 kDa species) was never observed.

Gel retardation analysis

The specificity of the TREF-binding activity observed was investigated by comparing the ability

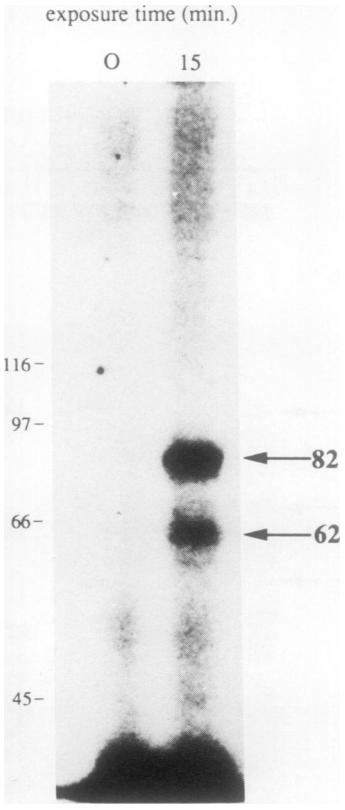


***Figure 5.* UV-photoactivated crosslinking of TREF1 and TREF2 to TR46 DNA.** Uniformly labeled TR46 was incubated with affinity purified TREF, exposed to UV-light for 15 min, digested with micrococcal nuclease, and the products analyzed by SDS-PAGE and autoradiography. Left: numbers indicate the molecular weight markers ($\times 10^{-3}$); right: arrows indicate the apparent molecular weights ($\times 10^{-3}$) of the labeled bands observed.

of a number of DNA sequences to compete for DNA-TREF complex (i.e., complex 1, Figure 4C) formation. Competition gel retardation studies were carried out in which labeled TR56 was incubated with either pre-affinity TREF fractions (i.e., pooled TREF-enriched DEAE fractions; Figure 6A) or post-affinity purified TREF (i.e., TR56-affinity purified DEAE fractions; Figure 6B) in the presence of increasing concentrations of unlabeled competitor DNA. As previously observed when employing the TR140 fragment as probe, TR56 gave rise to DNA-protein complexes of identical mobility when incubated with DEAE fractions both prior and subsequent to affinity purification. Furthermore, comparative competition analysis yielded qualitatively similar results in both cases. The TR-specific oligonucleotides TR56 and TR46 (Figure 1A) were able to successfully compete for the formation of the TR56-TREF complex, whereas unrelated sequences such as the oligonucleotide EN-A (Figure 1), and Msp 1-digested pBR322 (pBR) were unable to compete even at competitor-to-probe molar ratios (EN-A) or mass ratios (pBR) of 80 to 250. Unlike TR56, the ds oligonucleotide TR46 contains only 30 bp of TR promoter sequence encompassing region A only. These results suggest, therefore, that DNA containing TR region A alone is sufficient for binding of TREF. Single-stranded TR56 (TR56-SS) also failed to compete for binding to the double-stranded oligonucleotide under these conditions. Interestingly, among the various TR-unrelated ds oligonucleotides tested are a few which are able to compete, at least partially, for TR56-TREF binding in this assay.

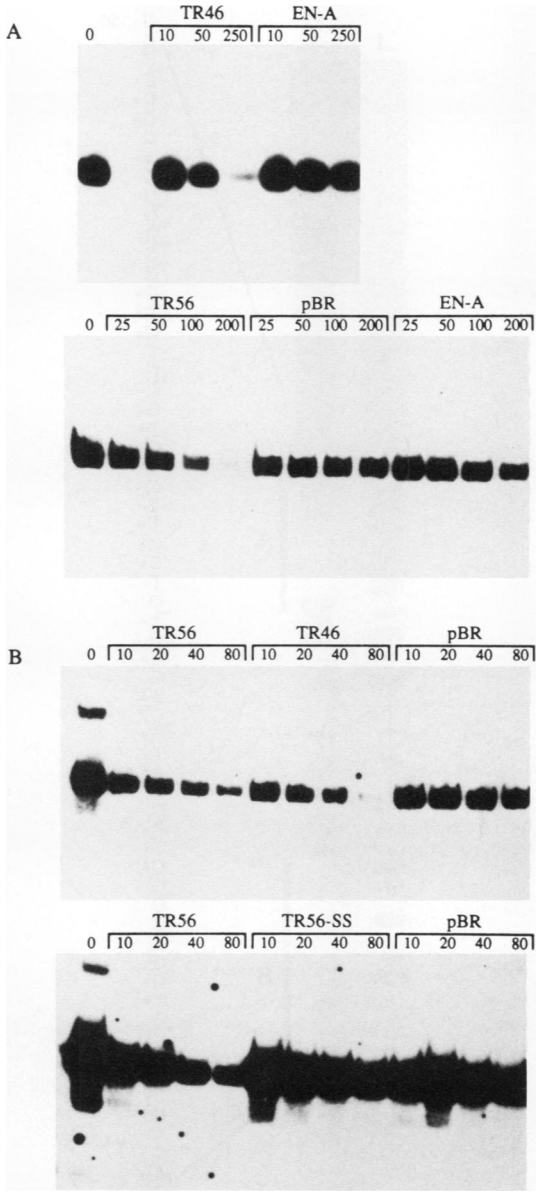


Figure 6. **Sequence specificity of TREF-binding activity.** Competition gel retardation assays involving labeled TR56 incubated with either pre-affinity (A) or post-affinity (B) TREF are shown. Poly(dI-dC) was present at a concentration of 5 μg/ml in the pre-affinity assays (A) only. Numbers refer to the molar ratio of competitor to probe for the ds (TR56, TR46, and EN-A) and ss (TR56-SS) oligonucleotides and to the mass ratio of Msp-1 digested pBR322 (pBR) to probe.

Footprinting analysis

To further characterize the interactions of the TREF proteins with the TR promoter sequences, DNase 1 protection assays were performed. The footprint obtained on incubation of the TR140 promoter fragment with pre-affinity TREF fractions (i.e., pooled TREF-enriched DEAE fractions) is shown in Figure 7. A unique pattern of protected sequences (indicated by dots) and enhanced cleavage sites (indicated by arrows) that span more than 60 bp of the TR promoter are observed. The 5′ flanking promoter sequence displayed is homologous to the oligonucleotides TR56 and TR46, and more specifically, contains a strongly protected sequence flanked by hypersensitive sites that encompasses region A (−80 and −62) of the TR promoter (Figure 1, A and B). This is the same region that we have previously shown to be footprinted by crude HeLa cell nuclear extract and to have sequence similarity to a motif found in a similar position in the human DHFR gene (Miskimins *et al.*, 1986). This region is also striking in that it encompasses the transcriptional control element defined in vivo by Casey *et al.* (1988) as being between −77 and −70. Furthermore, it shows a high degree of sequence similarity to a number of functionally important elements from other promoter and enhancer regions (Figure 1B, and see Discussion). Region A is also

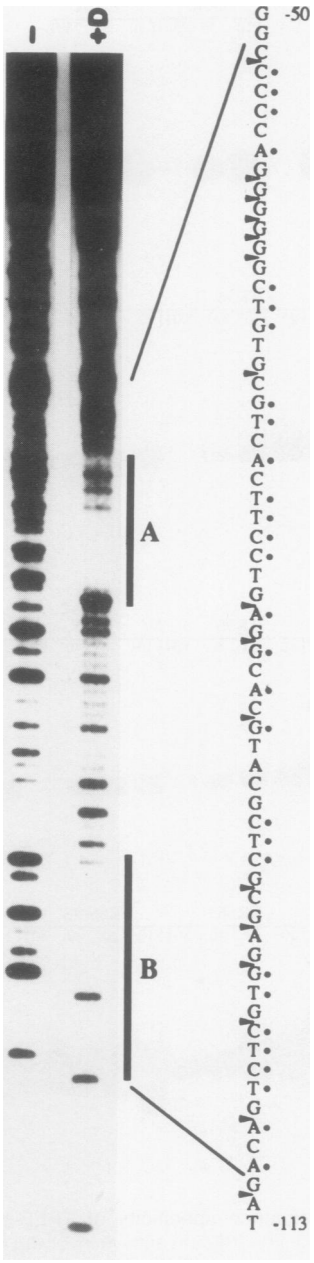


Figure 7. **DNase 1 footprinting analysis.** DEAE fractions (+D) enriched for TREF were analyzed by DNase 1 footprinting. The TR140 fragment (−114 to +26), labeled at the 3′ end of the template strand was employed as probe. The control without protein is labeled (−). Bars A and B show the locations of regions A and B (Fig. 1) in the TR sequence. Arrowheads indicate sites where cleavage is enhanced and dots indicate sites of diminished cleavage. The sequence shown is that of the template strand and therefore complementary to that shown in Figure 1.

a partial palindrome, and the axis of symmetry falls at the centre of this strongly protected sequence. Interestingly, the −77/−70 transcriptional control element described above corresponds to one-half of this palindrome. A second protected region located upstream of −100 in the TR promoter fragment corresponds to region B (Figure 1A), also a partial palindrome that shares some sequence similarity to region A as well as to other transcriptional control elements (Figure 1B).

Similar qualitative results were also obtained from DNase 1 analysis employing TR56-affinity purified TREF (data not shown). None of the remaining DEAE fractions from elsewhere in the gradient (i.e., those not corresponding to the TREF-enriched fractions assayed) were productive in DNase 1 footprinting analysis of the TR promoter.

Helical twist map

A map of the helical periodicity within the TR promoter was constructed by the method of Tullius and Dombrowski (1985). The TR promoter fragment TR140 (−114 to +26) was end-labeled, adsorbed to calcium phosphate crystals (Rhodes and Klug, 1980) and then treated with iron-EDTA to nick the DNA backbone. The DNA fragments were separated on a sequencing gel (Figure 8, bottom) revealing the helical periodicity in this sequence. The periodicity of the 5 upstream helical turns (turns 1–5, Figure 8) is ~10.6, a value close to that expected for B DNA. However, the DNA between twists 5 and 6 appears to have an unusual structure. This particular helical turn is unique in that it contains a highly GC-rich sequence of 16 bp, 2 bases of which are extremely sensitive to cleavage by the hydroxl radical. The molecular basis for this observation is unknown at present, but it is known that highly GC-rich regions such as this one are able to adopt non-B configurations (McCall *et al.*, 1985). Of interest here is that this unusual structure is found within the promoter sequences that are protected by the TREF proteins (downstream of region A at sequences −50 to −63, Figure 7).

The protected regions (shaded areas, Figure 8) and the enhanced cleavage sites (arrows, Figure 8) observed by DNase1 footprinting (Figure 7) were superimposed on the helical twist map. It is apparent that the protected nucleotides are spaced across at least 5 contiguous helical turns. The enhanced cleavage sites are in general found between the areas of protection, some spaced approximately one helical turn apart, while others are only about a half turn apart. Interaction of TREF with the TR promoter therefore results in alteration of the local DNA conformation over multiple helical turns, resulting in the differential exposure of nucleotides to DNase nicking.

Discussion

The studies described in this paper represent a first step toward elucidating the molecular mech-

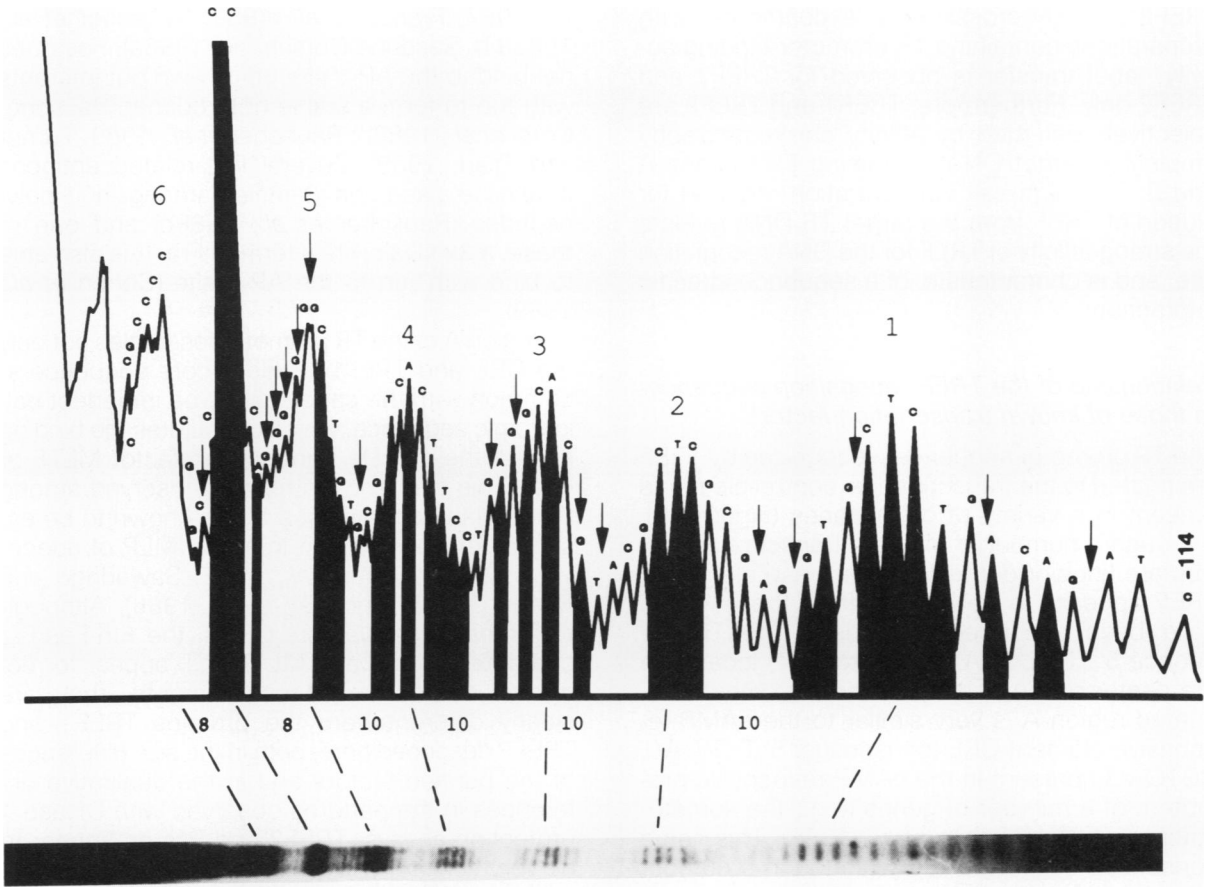


Figure 8. **Helical twist map.** The helical periodicity of the TR promoter sequences was analyzed by the method of Tullius and Dombrowski (1985). An autoradiograph of the sequencing gel is shown at the bottom. A densitometer scan of the same film is shown at the top. Helical turns are arbitrarily labeled 1–6. Arrows indicate sites of enhanced cleavage from footprint analysis and shaded areas indicate regions of diminished cleavage (see Figure 7).

anisms involved in transcriptional regulation of the transferrin receptor gene. To this end, we have purified and characterized two putative transcription factors, TREF1 and TREF2, which interact specifically with the transcriptional control element of the transferrin receptor promoter under conditions in which they appear to be associated as a heterodimer.

TREF interacts specifically with TR promoter elements

TREF-binding activity is specific for TR promoter sequences, as revealed by both DNase 1 footprinting analysis and by competition gel retardation experiments. Region A of the TR promoter with which the TREF proteins interact, is known to be essential for transcription both in vivo (Casey *et al.,* 1988) and in vitro (M. R. Roberts, unpublished data), whereas region B appears to be dispensable when assayed under the same conditions. Upon TREF binding to the TR promoter fragment TR140, region A is revealed by DNase 1 footprinting analysis as a strongly protected sequence flanked by hypersensitive sites. Although region B is also observed as a second protected sequence, gel retardation studies show that a ds oligonucleotide containing 30 bp of TR sequence encompassing region A only competes for TREF binding as effectively as a longer oligonucleotide encompassing both region A and region B. Taken together, these studies suggest that sequences within region A alone may be sufficient for TREF recognition (and see below).

Several lines of evidence indicate that TREF1 and TREF2 are responsible for the TR promoter-binding specificity detected by both DNase 1 footprinting and retardation assays. When protein preparations derived from a number of different purification steps involving ion exchange, DNA-affinity, and size-exclusion chromatography are analyzed, the TR promoter-specific binding activity observed is dependent upon, and consistently co-purifies with, the two proteins TREF1 and

TREF2. In UV-crosslinking experiments with preparations containing TR promoter-binding activity, label transfer is observed for TREF1 and TREF2 only. Furthermore, TREF1 and TREF2 are selectively enriched by affinity chromatography employing target DNA containing TR regions A and B. The high salt concentration required for elution of TREF from the target TR DNA reflects the strong affinity of TREF for the DNA recognition site, and is characteristic of a sequence-specific interaction.

Relationship of the TREF recognition sequence to those of known transcription factors

The TR promoter sequences recognized by TREF are related to the transcriptional control elements present in a variety of other genes (Figure 1B). Although a number of different transcription factors are known to interact with these elements, TREF appears to be distinct from those which have been described previously. The TR sequence 5′-TGACGCA-3′ (−73 to −67) located in the center of the partial palindrome within footprinted region A is very similar to the cAMP-responsive element CRE (consensus: 5′-T G/T A/T CGTCA-3′) present in the cAMP-responsive promoters of a number of genes (e.g., the somatostatin gene, Yamamoto *et al.,* 1988). This same consensus sequence is also found in a number of other cellular and viral promoters such as the E1A-inducible early genes of adenovirus type 5 (Lee *et al.,* 1987a; Hurst and Jones, 1987), c-fos (Gilman *et al.,* 1986) and Hsp 70 (Wu *et al.,* 1986). A factor of 43 kDa, ATF, which interacts with the CREs of E1a-inducible promoters of adenovirus type 5 has been purified from HeLa cells (Hurst and Jones, 1987) and is probably identical to the previously characterized 43 kDa phosphoprotein CREB isolated from rat brain, which binds with high affinity to the CRE of the somatostatin gene.

Region A of the TR promoter is also very similar to the conserved TPA-responsive element TRE (consensus: 5′-T G/T AGTCA G/C-3′) which is present in the promoters of a number of phorbol ester-(TPA-)inducible genes (e.g., stromolysin, human metallothionein IIA, P motif of the SV40 enhancer; Angel *et al.,* 1987). The TRE is capable of conferring TPA inducibility upon heterologous promoters and is recognized by a TPA-regulated DNA-binding activity known as AP-1. Affinity-purified preparations of AP-1 have been shown to be rather heterogeneous, containing several polypeptides in the 35–50 kDa range (Lee *et al.,* 1987b). The transcription factor AP-1 consists, at least in part, of the 62 kDa phosphoprotein Fos as a protein complex with the 39 kDa protein Jun (Bohmann *et al.,* 1987; Bos *et al.,* 1988; Distel *et al.,* 1987; Franza *et al.,* 1988; Rauscher *et al.,* 1988a,b; Sassone-Corsi *et al.,* 1988a). Fos does not bind to the AP-1 site on its own but interacts with Jun to form a stable heterodimer (Sassone-Corsi *et al.,* 1988b; Rauscher *et al.,* 1989; Turner and Tjian, 1989). Several Fos-related antigens (Fra) have also been identified among AP-1 polypeptides (Rauscher *et al.,* 1988b) and one of these, a 38 kDa protein termed Fra-1, is also able to bind with Jun to the AP-1 site (Cohen *et al.,* 1989).

Region A of the TR promoter resembles not only the CRE and TRE, but has a core sequence in common with the canonical 12 bp imperfect palindromic sequence, which constitutes the binding site for the 46 kDa transcription factor MLTF or USF. This sequence is highly conserved among the human adenoviruses and is known to be essential for transcription from the MLP of adenovirus type 2 (Hen *et al.,* 1982; Sawadago and Roeder, 1985; Chodosh *et al.,* 1986). Although the transcription factors CREB, the Jun-Fos/Fra complex of AP-1, and MLTF/USF appear to recognize related sequence elements, they are clearly different from the proteins TREF1 and TREF2 described here, both in the size differences of the purified factors and in the qualitative differences in the patterns observed with DNase 1 protection assays. TREF2 and Fos do appear to be very similar in size, however, although Fos is known to yield a highly heterogeneously sized protein population upon affinity purification, in direct contrast to the discrete 62 kDa protein band present in purified preparations of TREF2. The relationship, if any, between these two proteins remains to be determined.

Implications for TREF-mediated transcriptional regulation

The sequence GGAAGTGAC present within footprinted region A of the TR promoter shares eight out of nine nucleotides with element B of the human U1 snRNA gene (Figure 1B). This sequence encompasses the eight bp transcriptional control element (TCE, Figure 1B) of the TR gene and is also known to be an essential element for U1 gene transcription. In the U1 gene, element B is not only essential for transcription, but appears to function as a TATA box element in positioning the site of transcription initiation a fixed distance downstream (Murphy *et al.,* 1987; Gunderson *et al.,* 1988). While this manuscript was in preparation, we were intrigued to learn that another laboratory had purified and identified two proteins which were shown to bind element B of the human U1 snRNA gene (Knuth, Gunderson, and Burgess, personal communication). Comparative

immunological and amino acid sequence analysis subsequently indicated that these proteins were identical to TREF1 and TREF2 (M. R. Roberts and M. K. Knuth, unpublished observations). The snRNAs encoded by the snRNA genes form part of the ribonucleoprotein complexes that are involved in mRNA processing. Not surprisingly, the snRNA genes are ubiquitously expressed, and transcription occurs at a high level in actively proliferating cells. It is known that the level of transferrin receptor mRNA is also increased in rapidly dividing cells and in response to mitogenic stimulation (Pauza *et al.,* 1984; Depper *et al.,* 1985; Miskimins *et al.,* 1986), and like the snRNA genes, expression is found in almost all cell types. It is tempting to speculate that the TREF proteins may play a role in the transcriptional regulation of genes which perform essential functions associated with the proliferative status of the cell.

The sequence from −80 to −70 within footprinted region A is identical to an 11 bp sequence found in element 1 of the adenovirus type 5 E1A enhancer (Figure 1B) and includes the transcriptional control element described above. This enhancer element specifically regulates E1A transcription within transfected cells (Hearing and Shenk, 1986) and is identical to a sequence in the polyoma virus enhancer which plays a key role in the cell-type specificity displayed by polyoma virus variants for differentiated, as compared to undifferentiated, murine cells. It is possible that multiple factors (e.g., CREB, AP-1) including the TREF complex compete for binding within the transcriptional control element of the TR promoter and also with similar elements in other genes, perhaps in response to various phases of cell growth and differentiation, thereby modulating the transcriptional activity of target genes.

TREF1 and TREF2 behave as a heterodimer

Under the non-denaturing conditions employed, TREF1 and TREF2 always co-fractionate on DEAE (Figures 2B and 4B), size-exclusion (Figure 3B), and DNA-affinity (Figure 4A) chromatography and consistently maintain a stoichiometric relationship. When subjected to size-exclusion chromatography, in which proteins are eluted in order of decreasing molecular size, affinity-purified TREF exhibits fractionation behavior consistent with that of a protein with an apparent molecular weight > 120 kDa (Figure 3B). These data show that the native form of TREF possessing DNA-binding activity exists as a complex contributed to equally by TREF1 and TREF2, behaving as a heterodimer that is maintained in the absence of target DNA. It is intriguing that upon size-exclusion chromatography, a second peak of TREF eluting in a higher molecular weight range compared to the first does not exhibit TR DNA-binding activity. This suggests that TREF1 and TREF2 are able to associate in at least two forms which differ in their ability to bind TR DNA. DNA-affinity chromatography of the TREF proteins demonstrates that TREF1 and TREF2 dissociate from target DNA in the same salt fraction (Figure 4A), and in doing so they maintain the stoichiometric relationship observed for these proteins upon size-exclusion chromatography. Although further studies are required to determine the exact mechanism by which TREF binds to target DNA, the data are consistent with a model whereby TREF1 and TREF2 interact with TR region A as a heterodimer. Since UV crosslinking studies indicate that both TREF1 and TREF2 are able to contact target DNA directly (Figure 5), it is unlikely that one of the proteins is simply acting in an allosteric manner to promote direct high-affinity binding of the other. In the case of the Fos-Jun heterodimeric complex for example, it appears that an effective DNA-binding domain is contributed by both proteins. The CCAAT-binding proteins also appear to be heteromeric, and like Fos-Jun and TREF, are maintained as a complex in the absence of a DNA-binding site (Chodosh *et al.,* 1988).

TREF modifies DNA structure upon binding

When the footprinting data are superimposed on the helical twist map of the promoter fragment, it is apparent that the protected nucleotides are spaced 1 turn of the helix apart. Some of the enhanced cleavage sites are also spaced ~10 nucleotides apart, while others are spaced at half turn intervals (Figure 8). This type of protection is somewhat reminiscent of several other DNA-binding proteins; e.g., Sawadogo and Roeder (1985) have shown that TFIID, a TATA box-binding factor, alters the DNase I cleavage pattern over ~80 bp of the adenovirus major late promoter. They observed a pattern of unprotected or enhanced cleavage sites spaced about every 10 bp and interpreted this as an indication that the DNA was wrapped around the protein. Our data also suggest that the DNA structure is modified over multiple helical turns by the TREF protein complex but are insufficient at present to determine the cause of this structural alteration.

The helical twist map of the promoter region also revealed an unusual structure having an atypical periodicity and containing nucleotides that are hypersensitive to hydroxyl radical cleavage when adsorbed to calcium phosphate crystals (Figure 8). This structure lies in a highly GC-rich region of the promoter and could be due to a non-B form of DNA. One possibility is that this

GC-rich region is in an A-like configuration and that the hypersensitive nucleotides are part of the bend formed at the A-B junction (Selsing *et al.*, 1979).

Future aims

In this report, we have described the purification and characterization of a novel protein complex, TREF, which interacts specifically with the transcriptional control element of the TR gene. The functional relationship between the TREF complex and the TR gene is currently under investigation both in vivo and in vitro. Molecular cloning of TREF1 and TREF2 will greatly facilitate investigation of the mechanisms involved both in the inter-protein and DNA-protein interactions which govern regulation of the transferrin receptor gene. To this end, we have recently isolated and sequenced a full length cDNA encoding TREF1 and have identified a helix-turn-helix motif in the C-terminus of the protein which may be involved in protein-DNA interaction (M. R. Roberts, manuscript in preparation).

Methods

Protein purification

HeLa S3 cells were grown in suspension in Dulbecco's modified Eagles medium containing 10% fetal calf serum. Nuclear extracts were made essentially by the method of Dignam *et al.* (1983) with the following modifications. Packed cell pellets were resuspended in 5 volumes of buffer A consisting of (in mM) 10 HEPES pH 7.9, 1.5 $MgCl_2$, 10 KCl, and 0.5 dithiothreitol and kept on ice for 10 min. The cells were pelleted by spinning at 1000 rpm in an IEC 253 rotor at 4°C, resuspended in 2 packed cell volumes of buffer A and lysed using a glass homogeniser (Kontes, pestle B). Lysis was monitored by phase contrast microscopy. After centrifugation of the suspension for 10 minutes at 2000 rpm in an IEC 253 rotor, the pellet was resuspended in 75 ml buffer C consisting of 20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride per 50 g wet cells. The suspension was rocked gently for 30 min at 4°C and then centrifuged for 30 min at 14 500 rpm in a Sorvall SS34 rotor. The supernatant was dialyzed for several hours in buffer D2 consisting of 20 mM HEPES pH 7.9, 0.25 M sucrose, 50 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol. The nuclear extract was adjusted to 0.3 M KCl and applied to a DEAE-Sephacel column (40 ml bed volume) equilibrated with the same buffer. The column flow through was dialyzed against buffer D2, clarified by centrifugation, and fractionated by FPLC on a TSK DEAE-5PW column (8 × 75 mm, LKB). Bound proteins were eluted from the column with a 0.05–0.6 M KCl gradient over 50 min at a flow rate of 1 ml/min. One-milliliter fractions were collected and analyzed for TR promoter-specific binding activity as described in the text.

The DNA-affinity column was made by coupling the oligonucleotide TR56 (Figure 1A) to cyanogen bromide-activated Sepharose 4B (Pharmacia) employing a procedure kindly provided by Dr. Carl Wu. The protein sample was applied to the column (1 ml bed volume), allowed to flow through, and then reapplied. The column was then washed with 40 column volumes of buffer D2 containing 40 μg/ml salmon sperm DNA. The bound proteins were then eluted from the column with 1 ml aliquots of buffer D2 containing 0.6 M KCl and dialysed for 3 h against buffer D2 (0.05 M KCl). These affinity purified fractions were then analyzed for protein content and binding activity as described in the text. The "active" fractions were frozen in liquid nitrogen and stored at −70°C.

Gel filtration was carried out by FPLC on a TSK G3000SW GlasPac column (LKB) employing buffer D2 containing 0.2 M KCl and 0.1% Triton X-100. The column was run at a flow rate of 0.1 ml/min and fractions of 0.2 ml were collected.

DNA templates and recombinant plasmids

Single-stranded synthetic oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Complementary strands were then annealed in 0.1 M Tris-Cl (pH 8.0), 0.1 M NaCl and ds oligonucleotide purified by native polyacrylamide gel electrophoresis. The concentration of each purified ds oligonucleotide was routinely determined by both UV spectroscopy and EtBr fluorescence. The ds oligonucleotide TR56 was end-labeled by incubation with either T4 polynucleotide kinase in the presence of [^{32}P]ATP or Klenow fragment in the presence of [^{32}P]dGTP, followed by gel filtration over a 1 ml Sephadex G-25 (Pharmacia) spin column.

The DNA fragment TR140 (containing TR promoter sequences −114 to +26) was prepared by digesting pTRCAT16 (Miskimins *et al.*, 1986) with *Taq* 1 and end-labeling with Klenow fragment in the presence of [^{32}P]dCTP. The DNA was recovered by ethanol precipitation, digested with *Ava* 1, and the 140 bp fragment purified by agarose gel electrophoresis and electroelution.

Gel retardation assay

Protein-DNA complexes were resolved on low ionic strength polyacrylamide gels by a procedure similar to that of Fried and Crothers (1981). Protein samples were incubated with 1 ng of end-labeled double-stranded DNA (~10^4 cpm) in a final volume of 20 μl. Incubations were carried out at 25°C for 15–30 min in binding buffer containing 10 mM Hepes-NaOH pH 8.0, 60 mM KCl, 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 5% polyethylene glycol (PEG), 1 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin (BSA). Where described, 1 μg of poly(dI-dC) (Pharmacia) was included in the binding reaction as non-specific competitor DNA. In competition assays, unlabeled competitor DNA templates were included in the binding reactions at the concentrations stated. Samples were then applied to gels composed of 3.5% acrylamide, 10 mM Hepes-NaOH pH 8.0, and 0.5 mM EDTA. Gels were electrophoresed at 200 V at room temperature employing 10 mM Hepes-NaOH pH 8.0, 0.5 mM EDTA as running buffer. Following electrophoresis, the gels were dried and autoradiographed.

DNase 1 footprinting

DNase 1 footprints employing the TR140 fragment described above were performed exactly as described previously by Miskimins *et al.* (1986).

UV-photoactivated cross-linking

The coding strand of oligonucleotide TR46 was annealed to a 12-nucleotide primer complementary to the 3′ end, and used as a template for Klenow fragment in the presence of 100 μM dATP, dGTP, dTTP, and 4 μM [^{32}P]dCTP. The uniformly labeled probe was then purified by phenol-chloroform extraction, followed by gel filtration over a 1 ml Sephadex G-25 (Pharmacia) spin column. Affinity-purified protein samples were incubated with 5 ng of probe in 30 μl of binding buffer (as described for gel retardation assays) for 15 min at 25°C. The mixture (in an Eppendorf tube) was then placed on ice and irradiated under a Sylvania UV lamp (Blacklite F15T8-BL; intensity 0.2 μW/cm^2)

at a distance of 10 cm from the UV source. After irradiation, samples were digested at 37°C for 30 min in the presence of 2 U of micrococcal nuclease (Worthington) and 5 mM $CaCl_2$. Samples were then subjected to SDS-PAGE as described above, and the gel was dried and autoradiographed.

Helical twist map

A helical twist map of the TR promoter was made by a method similar to that of Tullius and Dombrowski (1985). Forty microliters of a suspension of calcium phosphate crystals (made by mixing equal volumes of 80 mM K_2HPO_4 and 50 mM $CaCl_2$) was mixed with 40 μl of a solution containing 1–2 ng of end-labeled TR140 promoter fragment, 13.3 mM HEPES pH 7.9, 13.3% glycerol, 67 mM KCl, 0.13 mM EDTA, 1 mM dithiothreitol, 4% polyethylene glycol (molecular weight ~8000), 8 mM $MgCl_2$, 10 μg/ml pGEM plasmid DNA, and 40 μg/ml poly(dI-dC). After a 1-h incubation at room temperature, 20 μl of a freshly prepared solution of 4 mM EDTA, 8 mM ferrous ammonium sulfate was added. Enough EDTA to clear the solution was added after a further 5 min incubation, and the nucleic acids were ethanol precipitated. The pellet was rinsed with 70% ethanol, dried, and dissolved in 98% formamide, 0.1% bromophenol blue. The DNA fragments were then separated on a 7% acrylamide, 8.3 M urea sequencing gel.

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