19). Our results raise the possibility that the observed decrease in intimal thickening may be a specific consequence of suppressed growth factor production by ECs, and concomitant reduction of SMC proliferation.

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Site-Specific Oligonucleotide Binding Represses Transcription of the Human c-*myc* Gene in Vitro

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A 27-base-long DNA oligonucleotide was designed that binds to duplex DNA at a single site within the 5′ end of the human c-*myc* gene, 115 base pairs upstream from the transcription origin P1. On the basis of the physical properties of its bound complex, it was concluded that the oligonucleotide forms a colinear triplex with the duplex binding site. By means of an in vitro assay system, it was possible to show a correlation between triplex formation at −115 base pairs and repression of c-*myc* transcription. The possibility is discussed that triplex formation (site-specific RNA binding to a DNA duplex) could serve as the basis for an alternative program of gene control in vivo.

SEQUENCE-SPECIFIC RNA BINDING is important for the regulation of splicing (*1*), for the control of translation (*2*), and for protein export (*3*). Even though the possibility has been discussed for some time (*4*), there has been no evidence to suggest that RNA binding could also serve as a mechanism for the regulation of transcription initiation. Boles and Hogan have shown that at a position 115 bp upstream of the transcription start site P1 (270 bp upstream from P2) the 5′ end of the human c-*myc* gene assumes a DNA secondary structure that is in equilibrium between at least two alternative helix conformations in vitro (*5*). The conformational equilibrium at 115 bp appears to be coupled allosterically to the binding of small RNA molecules, which led to the proposal (*5*) that the bound complex may be similar to a colinear triplex of the sort deduced from fiber diffraction analysis of simple synthetic polynucleotide helices (*6*).

Our experimental design was based on the following observation: DNA oligonucleotides are easier to synthesize than RNA and data suggest that a third strand of DNA should bind to a duplex as stably as an RNA molecule with the same sequence (*7*). For these reasons we prepared a series of single-strand oligonucleotides that were designed to bind to the −115-bp site on *myc*, by means of colinear triplex formation. One of this series was found to bind tightly to the *myc* sequence of interest.

The *myc* helix element, which we have shown to engage in conformational equilibrium, is a G-rich polypurine sequence (Fig. 1). If a colinear triplex were to form on that duplex site, with structural features similar to that of the poly(dT)–poly(dA)–poly(dT) triplex that has been studied by fiber diffraction, then the third strand would be positioned within the major helix groove and would be bound to the underlying duplex by hydrogen bonding (*6*).

Fresco and colleagues have shown that a poly(dA)–poly(dA)–poly(dT) forms at neutral *p*H, with properties that are as expected for a colinear triplex (*8*). The pattern of base pairing that stabilizes this complex may be similar to the A46-A22-U13 triplet that occurs in yeast $tRNA^{Arg}$ (*9*). As Cantor and Schimmel noted, the A-A-U triplet is isomorphous with the G46-G22-C13 triplet that has been detected in the yeast tRNA Phe crystal structure (*9*). These triplets are based on A-A and G-G hydrogen bonding to the underlying Watson-Crick duplex, which is identical to the pattern of self

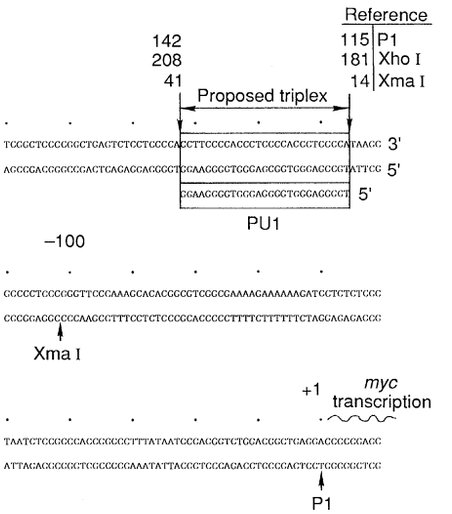

Fig. 1. A region of the human c-*myc* gene, −170 to +10 bp relative to the transcription start site P1 (*19*). The boxed segment is in conformational equilibrium (*5*) and is equivalent to the short duplex fragment described in Fig. 2. The sequence of the 27-base oligonucleotide PU1 has been presented in 3′ to 5′ alignment to emphasize its orientation relative to the duplex binding site in a colinear triplex model. To facilitate analysis of the footprinting assay (Fig. 3), the end points of the proposed triplex binding site have been numbered relative to several downstream reference points in the human c-*myc* gene (the transcription start site P1 and nearby Xma I and Xho I cleavage sites).

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association that has been detected for A and G derivatives by crystallography (*10*).

On the basis of this analogy with tRNA triplet formation and the A-A-U triplex, a colinear triplex at the 115-bp site in *myc* might be stable when G is placed in opposition to a GC base pair or when A is in opposition to AT. Using that preliminary estimate of sequence specificity, we synthesized (*11*) the DNA oligonucleotide PU1 (5′-TGGGGAGGGTGGGGAGGGTGGG-GAAGG-3′).

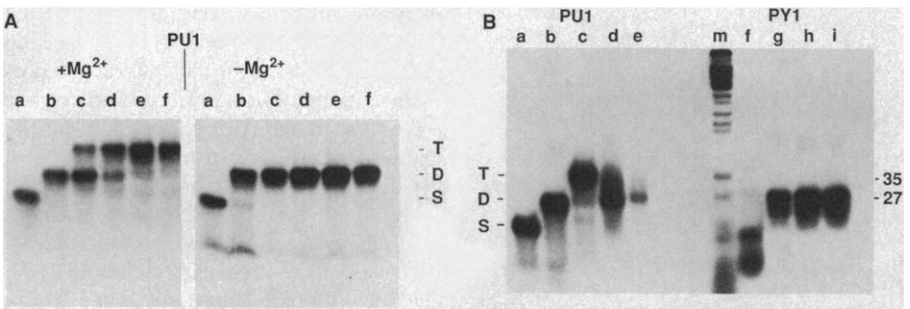

Fig. 2. (**A**) (**Left**) PU1 binding to the *myc* duplex was measured in the Mg^{2+}-containing buffer, as described in (*13*). The ^{32}P-labeled PY1 concentration was held constant throughout at 1 n*M*. Unlabeled PU1 concentration associated with lanes a through f was as follows: 0, 0.012, 0.12, 1.2, 12, and 24 μ*M*, respectively. (**Right**) To determine if triplex formation is dependent on divalent ion binding, EDTA was added to each titrated sample to a final total concentration of 38 m*M*, thereby chelating all of the available Mg^{2+}. Binding was then analyzed at 4°C, but in an electrophoresis buffer containing EDTA (90 m*M* tris-borate, *p*H 8.0, 5 m*M* EDTA). Samples marked a through f are as in the left panel, except that the oligonucleotide concentration in these samples had been reduced 25% by dilution with EDTA. (**B**) The experiment in (A) was reversed so that ^{32}P-labeled PU1 was titrated with cold PY1, then assayed at 4°C in the Mg^{2+}-containing buffer system. Labeled PU1 concentration was held constant throughout at 1 n*M*. PY1 concentration associated with lanes f through i was as follows: 0, 0.01, 0.5, and 1.0 μ*M*, respectively. To generate markers, the titration described in (A) (left) was repeated by selecting a PU1 concentration that resulted in species that are predominantly single strands (S), duplex (D), or triplex (T). PU1 concentration in marker lanes a through d was as follows: 0, 0.012, 24, and 0.012 μ*M*, respectively. Lane e was the same as b and d, but was underloaded to emphasize duplex mobility. Lane m is a duplex length standard (the ^{32}P-labeled Hpa II digest of pBR322) with characteristic 27-bp and 35-bp bands.

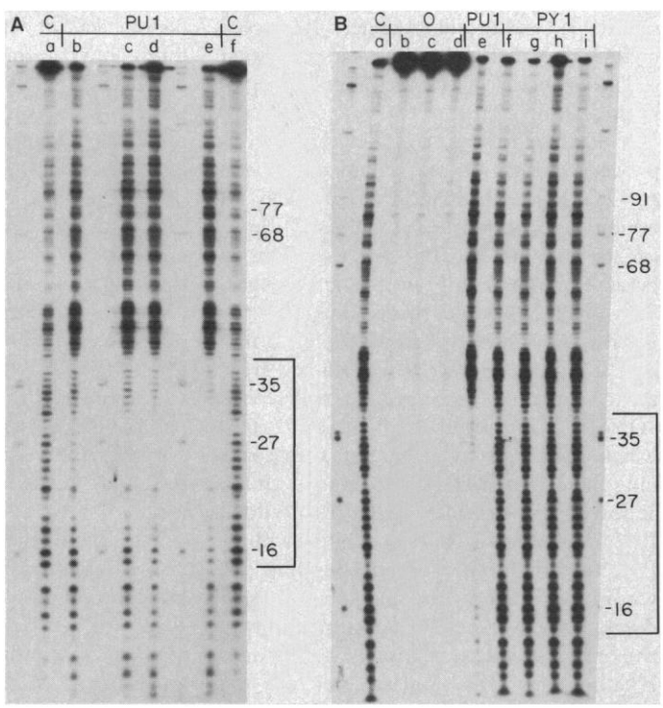

Fig. 3. To assay for triplex formation by DNase I footprinting, a single Sca I–Xma I c-*myc* DNA fragment, 190 bp in length, was ^{32}P-labeled (*22*), titrated with PU1 or PY1 (in 20 m*M* tris-HCl, *p*H 7.2, 10 m*M* $MgCl_2$), and preincubated at 20°C for 45 min. DNase I was then added to 2 U/ml. The reaction was quenched after 8 min by adding 15 m*M* EDTA and calf thymus DNA to 0.2 mg/ml. The digestion products were then desalted by ethanol precipitation, resuspended in formamide, heated to 60°C for 3 min, and applied to a standard (10% polyacrylamide: 0.5% bisacrylamide: 8*M* urea) sequencing gel. (**A**) PU1 footprinting. Lanes b through e correspond to increasing PU1: 1, 2, 4, and 8 μ*M*, respectively. Lanes a and f are a control digest with no added oligomer. The other lanes on the gel are length standards (the Hpa II digest of pBR322, labeled with [α-^{32}P]dCTP and Klenow polymerase). Several of those marker bands have been identified to the right. The well-defined footprint spans a 25-base segment, beginning 16 bases from the labeled Xma I terminus (identified by a bracket). (**B**) PY1 footprinting. Lanes f through i are a titration with PY1 added to a final concentration of 0, 8, 10, and 20 μ*M* PY1, respectively. Lane e corresponds to a titration with PU1 at 8 μ*M* and has been included for direct comparison with the PY1 titration. Lane a is a control DNase I digest with no added oligomer. Lanes b through d are undigested DNA controls. The other lanes are length standards as described in (A).

The relation of PU1 to the underlying *myc* duplex in a colinear triplex is shown in Fig. 1. Coincidentally, the preliminary binding predictions suggest that the sequence of PU1 could be made identical to the purine-rich strand of the duplex binding site for reasons pertaining to triplet formation, rather than Watson-Crick base pairing. Because of that degeneracy, we could test the colinear triplex model against alternative binding models based on Watson-Crick pairing of the oligonucleotide. One specific alternative is a D-loop complex, as has been detected in supercoiled DNA molecules (*12*).

The colinear triplex model makes several useful predictions that can be used as the basis for a binding assay. Binding should induce only a modest change in the overall dimensions of the duplex binding site because the third strand wraps about the helix, buried in the major groove (*6*). However, in a triplex the average separation between phosphate oxygens decreases significantly when compared to the duplex. As a result, multivalent cations bind tightly to, and selectively stabilize, the triplex (*7*).

We developed an electrophoresis assay (*13*) that takes advantage of the reduction of DNA charge that is likely to accompany triplex formation. We reasoned that if a triplex has the same dimensions as a duplex, but a significantly reduced phosphate charge, it will migrate more slowly than a duplex through a gel matrix. The outcome of such a titration experiment is shown in Fig. 2, as assayed on a 10% polyacrylamide gel, under conditions that should favor triplex formation.

The Watson-Crick complement to PU1 (PY1) was ^{32}P end-labeled with polynucleotide kinase, then titrated with unlabeled PU1. The titration produced two distinct species (Fig. 2A, left). At low concentration the duplex formed, and then bound to a second equivalent of PU1 to form a complex that migrated more slowly. The titration midpoint for the second binding event was near 0.4 μm (strand equivalents), corresponding to an apparent bimolecular dissociation constant (K_d) of $4 \pm 2 \times 10^{-7} M$ (*14*).

One prediction of the colinear triplex model is that binding should be specifically stabilized by multivalent ions. To test this prediction, we repeated the binding analysis at the same ionic strength, but in the absence of Mg^{2+} (Fig. 2A, right). Triplex formation could not be detected in the divalent-free assay system, under conditions where it is driven to completion in the

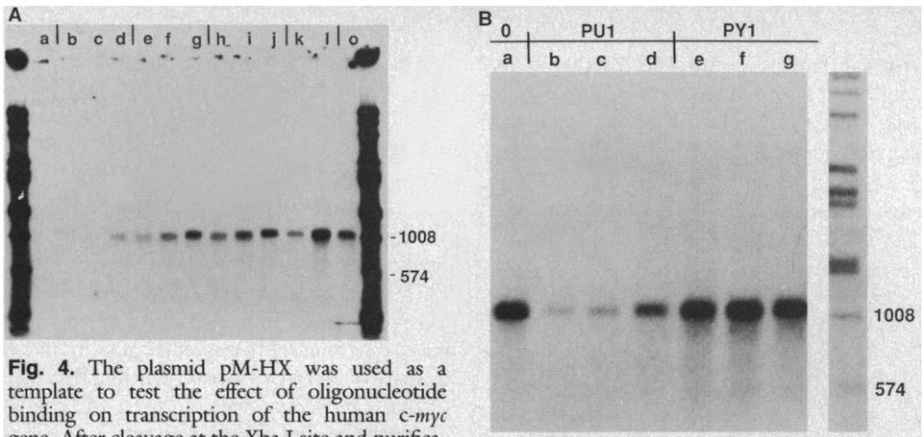

Fig. 4. The plasmid pM-HX was used as a template to test the effect of oligonucleotide binding on transcription of the human c-*myc* gene. After cleavage at the Xba I site and purification, DNA was incubated in reaction mixtures containing: 33 μg/ml DNA template, 16% by volume of HeLa extract (2 mg/ml protein), 13.4% glycerol in 13.4 m*M* Hepes (*p*H 7.9), 67 m*M* KCl, 6 m*M* $MgCl_2$, 10 μ*M* EDTA, 250 μ*M* each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and uridine triphosphate (UTP), 25 μ*M* of guanosine triphosphate (GTP) (all unlabeled), and 5 μCi of [α-^{32}P]rGTP. Oligonucleotides were added to the reaction mixture from a concentrated stock solution. Transcription was allowed to proceed at 30°C for 30 min, then RNA was purified by organic extraction and ethanol precipitation. The RNA was then denatured with glyoxal and analyzed directly on a 1.4% agarose gel relative to the Sma I digest of Ad2 DNA. (**A**) PU1 titrations. The lanes differ only in the concentration of PU1 in the transcription assay. Lanes a through l are 330, 170, 66, 33, 17, 8, 3.3, 0.17, 0.080, 0.030, 0.017, and 0.003 n*M* of added PU1. Lane o is a control with no added oligonucleotide. There is a twofold difference in the amount of 1-kb RNA in lanes l and o, although they are similar enzymatic reactions. That variance is indicative of the error associated with this assay system. (**B**) PY1 titrations. The lanes differ only in the identity and concentration of the oligonucleotide in the transcription assay. Lanes e through g are: 160, 100, and 66 n*M* PY1. Lanes a through d are: 0, 100, 66, and 33 n*M* PU1.

presence of 5 m*M* Mg^{2+}.

In a third titration (Fig. 2B, right) we determined whether the Watson-Crick complement of PU1 can form a triplex. PU1 was ^{32}P end-labeled and then titrated with its unlabeled Watson-Crick complement. Again, titration produced the duplex, but binding of a second PY1 equivalent to form a triplex could not be detected at any oligonucleotide concentration that had been tested, up to 1 μ*M*, at which point the complementary titration produced 73% triplex (*14*).

The general sequence dependence for third-strand complex formation that we detected by the gel method is as predicted from the colinear triplex model presented in Fig. 1. Our result is inconsistent with models that are based on Watson-Crick base pairing on the *myc* binding site, such as D-loop formation (*12*). Our reasons for rejecting a looped structure as an alternative explanation for these binding data are as follows:

1) Because of the twofold symmetry of the duplex binding site, both PU1 and its Watson-Crick complement would have formed a looped triplex, each with the same pattern of H-bonding. Failure to see triplex formation with the Watson-Crick complement of PU1 is strong evidence against a D-loop model.

2) The triplexes we detected are formed on a 27-bp DNA duplex. We are unaware of any experimental data to suggest that a D-loop structure could form on a duplex that is this small. The available data suggest that even a large DNA duplex must be highly supercoiled and equilibrated near the duplex melting temperature before it can form such a D-loop with a complementary single-strand DNA fragment (*12*).

In addition to steric considerations, the B to A helix transition that would accompany colinear triplex formation (*6*) is likely to inhibit deoxyribonuclease I (DNase I) cleavage throughout the binding site. This is because DNase I appears to cleave the A form of a DNA duplex more slowly than the B form (*15*). Therefore, stable triplex formation at the −115-bp site in a cloned fragment of the *myc* gene should result in a "DNase I footprint" analogous to that which occurs at protein binding sites, spanning the 27 bp of DNA in contact with the added oligonucleotide. At 2 μ*M* of added PU1, where the 27-bp *myc* fragment binds PU1 to saturation, the corresponding site within the 5′ end of the human c-*myc* gene developed a well-defined footprint, indicative of binding site saturation (Fig. 3A, lanes b through e). The similarity between the two kinds of binding assay serves as a check for internal consistency.

To accommodate the temperature dependence of enzyme activity, DNase I cleavage was performed at 20°C rather than at 4°C. Because of that temperature difference, the quantitative similarity between the two binding assays suggests that triplex formation has only a modest temperature dependence (*13*).

Footprinting could not detect PU1 binding at any other site within the 190 bases of sequence that have been assayed (Fig. 3A), even though there are several closely related polypurine segments within those flanking sequences. Such site-specific protection provides a useful qualitative measure of the (high) apparent sequence specificity of the triplex binding interaction.

Consistent with the previous band shift analysis, the Watson-Crick complement of PU1 (PY1) had no effect on the distribution of DNase I cleavage within the −115-bp *myc* site (compare lanes f and i in Fig. 3B, which correspond to 0 μ*M* and 20 μ*M* of added PY1, respectively).

If a D-loop triplex were formed by binding PY1, the ^{32}P-labeled strand that has been monitored in Fig. 3 would have become a single-strand loop that spans the 27-base oligonucleotide binding sequence. It is difficult to explain how such a major change of DNA secondary structure could be accompanied by no change in the pattern of DNase I cleavage. Therefore, we believe that the DNase I cleavage data constitute additional evidence against a D-loop model for the PU1-*myc* triplex.

As an estimate of the biological potential of this triplex formation, we tested whether oligonucleotide binding can modulate RNA transcription from the *myc* gene in vitro.

A discrete RNA transcript, 1 kb in length, was synthesized from the c-*myc* DNA template (Fig. 4), in an assay system that included a HeLa cell nuclear extract as the source of RNA polymerase and the necessary cofactors (*16*). We used nuclease S1 mapping methods (*17*) to confirm that this RNA transcript was initiated at the P2 start site (*18*), which is the principal transcription start site of the human c-*myc* gene (*19*). We found that RNA synthesis in this assay was completely inhibited by 2 μg of α-amanitin per milliliter (*18*), which confirms that RNA synthesis from P2 is under the control of RNA polymerase II in our assay system (*16*).

We found that *myc* transcription was greatly inhibited upon addition of nanomolar concentrations of the oligonucleotide PU1 (Fig. 4A, lanes a through e). Under the same assay conditions, the Watson-Crick complement of PU1 had no measurable effect at concentrations as high as 160 n*M* (Fig. 4B, lanes e through g).

RNA molecules with a length other than 1 kb are not detected in these titrations (Fig. 4). This suggests that the PU1 binding effect results from inhibition of transcription initiation at P2, rather than a shift to the P1 origin (which would have produced a 150-

base RNA length increase) or inhibition of the elongation step (producing transcripts shorter than 1 kb).

For the PU1 titration, the apparent midpoint for half-maximal transcription repression is 4 ± 2 n*M*, which should approximate the K_d for triplex formation in this assay (*20*). The apparent K_d is approximately 100-fold lower than the corresponding value measured by footprinting or band shift analysis (Figs. 2 and 3). However, because the physical binding data were accumulated in a buffer that had not been optimized systematically (and is much simpler than the nuclear extracts, which almost certainly contain a variety of small polycations), it is likely that the K_d inferred from transcription inhibition may more clearly approximate PU1 binding affinity in a cellular context.

Although we cannot rule out all other possibilities, our physical and transcriptional data are consistant with the idea that triple-strand complex formation at the −115-bp binding site will repress transcription initiation from the human c-*myc* gene in vitro.

Considerable work remains before the structure and thermodynamics of this class of site-specific binding interaction can be fully understood. However, the PU1-*myc* complex that we have described is a useful starting point for physical and biochemical studies.

The −100 to −300 region of the human c-*myc* gene is important for transcription control in vitro (*19*). Also, this region is required for transcription from P1, and to a lesser extent from P2, in transient in vivo assays (*21*).

We propose that, although the −115-bp site may be required for activation of the c-*myc* gene (perhaps as a protein binding site), it cannot function in that way while bound to form a triplex.

It remains to be proved if triplex formation occurs in vivo. However, in some instances third-strand binding could serve as an alternative to protein binding as the physical basis for the regulation of transcription.

For technical reasons, we studied the process of using DNA oligonucleotides. However, if third-strand binding is used as a regulatory element in the cell, it is more likely that such interactions are based on endogenous RNA molecules. To stabilize such RNA with respect to hydrolysis, and as a mechanism to neutralize the unfavorable negative charge density of the triplex, it is likely that RNA molecules of this kind would exist in the cell as a protein complex. Such an RNA-protein complex might be a member of the class of small nuclear ribonucleoproteins (*1*) that, with the exception of the splicing machinery, remain largely undefined.

If, among that heterogeneous family of RNA-protein particles, there are members possessing an RNA component that binds to the human c-*myc* gene by triplex formation, the binding and transcriptional analyses described here provide tools that can be used to identify them.

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11. Oligonucleotides PU1 and PY1 were prepared by the phosphoramidite method on an automated synthesizer (Applied Biosystems, Inc.). Portions (1 μg) of each oligomer were 5′ end-labeled with [α-^{32}P]ATP and T4 polynucleotide kinase, deproteinized, and purified by means of Sephadex G25 chromatography.
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13. To assay for triplex formation, ^{32}P-labeled PY1 was titrated with unlabeled PU1 (in 20 m*M* tris-HCl, *p*H 7.2, 10 m*M* $MgCl_2$, 10% sucrose), then loaded on a 10% polyacrylamide: 0.5% bisacrylamide gel [containing 90 m*M* tris-borate, *p*H 8.0, and 5 m*M* $MgCl_2$ (no EDTA)]. Electrophoresis was performed at 10 V/cm for 2 hours at 4°C. Under those conditions, kinetic analysis showed that triplex lifetime was longer than the duration of the gel assay (that is, identical binding distributions were detected after 1 or 2 hours of electrophoresis). However, when the assay was performed at 37°C, we detected approximately 30% dissociation of the triplex species during the 2 hours of electrophoresis. The apparent midpoint of the duplex to triplex titration was found to be approximately 1.5×10^{-7}*M* at 37°C. However, because of the kinetic artifact, we have not evaluated the 37°C band-shift data in more detail.
14. Binding data as in Fig. 2A have been quantified by densitometry to yield the duplex/triplex ratio (D/T) as a function of the inverse of the total added PU1 strand concentration. If triplex formation approximates a simple bimolecular process, then $D/T = K_d \times 1/[PU1]$, where K_d is the dissociation constant. The data of Fig. 2 have been fit to this relation to yield an apparent $K_d = 4 \pm 2 \times 10^{-7}$*M*.
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20. We define *f* as equal to the transcription rate at some added oligonucleotide concentration [*S*], divided by that in the absence of added oligonucleotide. If transcription initiation is completely repressed by triplex formation, then transcription data should be simply related to the fraction of *myc* promoter sites that remains as a duplex. Specifically, $(1/f) - 1 = [S]/K_d$, where K_d is the apparent bimolecular dissociation constant for the triplex to duplex equilibrium. A regression of the PU1 titration data in Fig. 4 yields $K_d = 4 \pm 2 \times 10^{-9}$*M*.
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22. To prepare DNA for footprinting, the plasmid pM-HX, containing the first *myc* exon and 2 kb of 5′ flanking sequence (*5*), was cleaved with Xma I and Sca I, 3′ end-labeled at its Xma I site with [α-^{32}P]dCTP and Klenow polymerase, then purified by means of Sepharose 4B chromatography.

23. We thank J. R. Fresco for advice and discussion in evaluating the oligomer sequences that might bind to the *myc* DNA target sequence. Supported by grants from the National Cancer Institute (M.H.) and the National Institutes of Health (S.J.F.).

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Large Microtubule-Associated Protein of *T. brucei* Has Tandemly Repeated, Near-Identical Sequences

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The parasitic protozoon *Trypanosoma brucei* contains a highly organized membrane skeleton, consisting of a dense array of parallel, singlet microtubules that are laterally interconnected and that are also in tight contact with the overlying cell membrane. A high molecular weight, heat-stable protein from this membrane skeleton was isolated that is localized along the microtubules. Protease digestion experiments and sequencing of a cloned gene segment showed that most of the protein is built up by more than 50 nearly identical tandem repeats with a periodicity of 38 amino acids.

MICROTUBULE-ASSOCIATED PROteins (MAPs) from many different organisms and tissues have been extensively studied (*1–5*), and their role in such cellular functions as differentiation and intracellular transport are beginning to be understood. The cytoskeleton of

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