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A Nuclease-Hypersensitive Element of the Human c-*myc* Promoter Interacts with a Transcription Initiation Factor

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Transcription of the human c-*myc* oncogene is elaborately regulated, but the relevant molecular mechanisms are not yet understood. To begin to define elements and enzyme systems responsible for c-*myc* transcription in vitro, we partially purified a transcription factor essential for efficient and accurate in vitro initiation from the principal *myc* promoter, P2. DNA mobility shift assays located the factor binding domain at −142 to −115 with respect to the P1 promoter. This region contains pur/pyr sequences (predominantly purines in one strand), nuclease-hypersensitive sites (U. Siebenlist, L. Henninghausen, J. Battey, and P. Leder, Cell 37:381–391, 1984; C. Boles and M. Hogan, Biochemistry 26:367–376, 1987), and a triple-helix-forming element (M. Cooney, G. Czernuszewicz, E. Postel, S. Flint, and M. Hogan, Science 241:456–459, 1988). Methylation interference mapping established that the factor, termed PuF, directly contacts the repeated palindromic sequence GGGTGGG of the −142/−115 element. The interaction of PuF with this *cis*-acting element is necessary for P2 transcription in vitro, for (i) deletion of this 5′ region from the *myc* promoter greatly reduced transcription efficiency and (ii) a synthetic duplex oligonucleotide corresponding to the −142/−115 sequence completely repressed c-*myc* transcription in the presence of the partially purified factor. These observations lend support to the hypothesis that pur/pyr sequences perform important biological roles in the regulation of c-*myc* gene expression, most likely by serving as transcription factor binding sites.

Transcription of the human c-*myc* oncogene is subject to complex and, thus far, poorly understood regulatory mechanisms. Obvious difficulties stem from the facts that transcription of the gene is driven by at least three promoters, P0, P1, and P2 (3–5), which lack absolute polarity (4), and transcription is modulated at several different levels, including initiation (8, 21, 30, 40), elongation (4), and termination (5, 21). Our present knowledge of the role of *cis*-acting promoter elements and *trans*-acting factors in the initiation of transcription of the c-*myc* gene is limited to information obtained principally through measurements of steady-state RNA levels in transient transfection assays that use different cell lines (8, 21, 30) or expression in *Xenopus* oocytes (39). In both situations, the effect on RNA initiation is difficult to establish and only abundant factors are likely to be detected. While there is a consensus of opinion on the involvement of several different positively and negatively acting *cis* control elements and *trans*-acting factors in regulation of the expression of the human c-*myc* gene (8, 21, 44), there are conflicting reports as to which regions are essential for expression and whether a particular region exerts positive or negative control (see, for example, references 8, 21, 30, and 40).

A number of protein factors interacting with the c-*myc* gene have been described (21, 25, 31, 44). It has also been suggested that c-*myc* transcription may be regulated at the level of template structure (6, 10). Indeed, a colinear triplex formed between a site-specific oligonucleotide and c-*myc* duplex DNA at −115 base pairs (bp) (from P1) can repress c-*myc* transcription from the P2 promoter in vitro (10). This result suggested that the −115-bp region of the c-*myc* promoter, which is rich in pur/pyr sequences (sequences composed of purine and pyrimidine in separate strands) and is nuclease hypersensitive (6, 21, 44), might serve as a target for a specific transcription factor(s) needed for P2 expression. Consistent with this view, Hay et al. (21) have reported that this region exerts a modest stimulatory effect on P2 transcription (utilizing transient assays) and plays a much more important role in P1 promoter utilization.

To acquire some insight into the function of the −142/−115 and other promoter elements and of *trans*-acting factors in the initiation of c-*myc* RNA synthesis, we have fractionated HeLa cell nuclear extracts into components that support the accurate synthesis of human c-*myc* RNA in vitro. We describe here a partially purified HeLa cell transcription factor that is required for accurate c-*myc* transcription from the second promoter (P2). This factor, termed PuF, binds to the −142/−115 region of the promoter DNA by making contact with the GGGTGGG sequence motif. Our results strongly suggest a direct role for this structurally unusual DNA element in initiation of human c-*myc* transcription.

MATERIALS AND METHODS

Preparation and fractionation of nuclear extracts. Nuclear extracts were prepared by the procedure of Dignam et al. (12), with some modifications. Briefly, 20 liters of HeLa cells at a density of 5×10^5 cells per ml were harvested, washed twice with phosphate-buffered saline, and suspended in 3 volumes of 12 mM Tris hydrochloride, pH 7.9–1.2 mM EDTA–6 mM dithiothreitol. Following a 20-min incubation on ice, the cells were homogenized with a motor-driven pestle until cell lysis was complete. Nuclei were pelleted, washed once with the same buffer, and suspended in 1.5 volumes of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.9, also containing 20% (vol/vol) glycerol, 0.6 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Lysed nuclei were incubated for 1 h at 4°C with stirring, after which the chromatin was pelleted by centrifugation at 50,000 rpm for 3 h in a Beckman L8-70M ultracentrifuge in a Ti70 rotor. Supernatant from this spin was dialyzed for 6 to 8 h against 1,000 volumes

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of 0.1 M HM buffer (20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 20% glycerol, 0.1 M KCl). Dialysis buffer in this and subsequent procedures contained, in addition, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], and 1 μg each of pepstatin A, antipain, and leupeptin (all Sigma Chemical Co.) per ml. Dialyzed extracts were cleared by centrifugation for 10 min at 10,000 rpm in a Sorvall RC5B centrifuge, and small samples were frozen at −80°C. The remainder of freshly prepared extracts (200 mg, 2 mg/ml) was applied at 30 ml/h to a 40-ml Affigel-heparin-agarose (Bio-Rad Laboratories) column equilibrated in 0.1 M HM buffer. The column was washed with 3 volumes of 0.1 M HM and then eluted with 0.2 and 0.4 M HM (HM, containing 0.2 and 0.4 M KCl, respectively) to collect the H.2 and H.4 proteins (see Fig. 2A). All fractions were frozen in small samples at 80°C and used only once. Protein-containing fractions of the 0.2 and 0.4 M eluates were each pooled, dialyzed against 0.1 M HM containing the above protease inhibitors, and passed through parallel 30-ml DEAE-Sepharose CL6B (Pharmacia) columns at flow rates of 10 ml/h. The DEAE-Sepharose column to which the H.2 fraction was applied was washed with 3 volumes each of 0.1 and 0.2 M HM and eluted with 0.4 M HM (this contained the D.4 proteins). The column to which the H.4 fraction was applied was washed with 0.1 M HM and eluted with 0.25 M HM (this contained the RNA polymerase II-rich C1.25 fraction). Protein concentrations were measured by the method of Bradford (7). Protein yields from 20 liters of cells were as follows: nuclear extract, 200 mg, 2 mg/ml; D.4, 2 mg, 0.35 mg/ml; C1.25, 1 mg, 0.30 mg/ml.

Sequence-specific DNA affinity chromatography. Oligonucleotide coupling and DNA affinity chromatography were done essentially as described by Wu et al. (49). A 1-ml affinity column was prepared by coupling the 28-bp −142/−115 oligonucleotide (see Fig. 1C), synthesized with a TTCG overhang on the 5' end of the noncoding strand, to cyanogen bromide-activated Sepharose (Bio-Rad). The column was equilibrated with 0.1 M HM and loaded with 3 ml of D.4 (350 μg/ml) in 0.1 M HM containing protease inhibitors and 100 μg of sonicated salmon sperm DNA per ml as nonspecific carrier. Eluted proteins were recycled onto the column twice. Bound proteins were then eluted stepwise with 0.1 to 1.0 M HM buffers. Fractions, 100 μl, were collected and tested for sequence-specific binding to the 28-bp −142/−115 probe as described below. Fractions eluting between 0.2 and 0.4 M HM contained the sequence-specific binding activity; these were pooled and subjected to another round of affinity chromatography on a 0.5-ml column. Active fractions from the second column were frozen in aliquots at −80°C with the addition of 2% polyvinyl alcohol.

In vitro transcription. The template for runoff analysis was the 6.2-kilobase plasmid pMHX linearized with *Xba*I (10). Transcription reaction mixtures contained 3 μg of template DNA per ml, 13.4 mM HEPES, pH 7.9, 67 mM KCl, 6 mM $MgCl_2$, 0.01 mM EDTA, 14% (vol/vol) glycerol, 250 μM each unlabeled ATP, CTP, and UTP, 50 μM GTP, 5 μCi of [^{32}P]GTP (700 Ci/mmol; Dupont, NEN Research Products), 2% polyvinyl alcohol, and nuclear extract (10 μg), C1.25 (3 μg), and/or D.4 (5 μg) (as indicated) in a total volume of 30 μl. Reactions were incubated for 30 min at 30°C and terminated by the addition of 100 μl of "stop buffer" (3.5 M urea, 0.5% sodium dodecyl sulfate, 100 mM LiCl, 10 mM EDTA, 100 μg of tRNA per ml). Following organic extractions and ethanol precipitation, RNA was denatured by glyoxalation (34) and electrophoresed for 3 h in 1.4% agarose gels cast in 10 mM sodium phosphate, pH 7.0–0.1 mM EDTA. Gels were rinsed with H_2O, dried, and exposed onto Kodak X-ray film.

In vitro transcription reactions for RNase protection analysis were performed as described above, with the exception that supercoiled pRV101 was used as the template, all four ribonucleotide triphosphates were present at 500 mM, the labeled nucleotide was omitted, and reactions were scaled up fivefold. pRV101 (wild type) was derived from pHmex1-neo in which a *Hin*dIII-*Pvu*II fragment was inserted in front of the neomycin gene (24). This construct contains approximately 2.3 kilobase pairs of the 5'-flanking region of human c-*myc* and most of the first exon. The *Sma*I site at −101 with respect to P1 has been changed to an *Eco*RV site by the addition of a linker. Uni- and bidirectional Bal 31 deletions were constructed and sequenced by standard procedures (1). Each deletion contains an *Eco*RV linker at the join. Transcription reactions were terminated with 25 U of DNase I (Promega) for 15 min at 37°C, followed by protease K (100 μg/ml in 0.5% sodium dodecyl sulfate; Boehringer Mannheim Biochemicals) treatment for 15 min at 37°C, organic extraction, and ethanol precipitation. RNA was hybridized to a 670-nucleotide human c-*myc* exon 1 probe prepared by linearization of plasmid pT7Hmexl (24) with *Hin*dIII and subsequent transcription with T7 polymerase (Bethesda Research Laboratories) in the presence of 100 μCi of [^{32}P]CTP (Dupont, NEN) by the method of Ansubel et al. (1). In vitro synthesized RNA and 10^5 Cerenkov cpm RNA probe were hybridized at 45°C overnight and then treated with RNase A (100 μg/ml; Sigma) and RNase T_1 (2 μg/ml; Sigma), followed by protease treatment, organic extraction, and precipitation, as described before (1), and analyzed on a 6% sequencing gel. In vivo HeLa cell RNA was prepared as described previously (18).

Gel electrophoresis mobility shift assays. Approximately 1 ng of ^{32}P-end-labeled DNA restriction fragments (10^4 Cerenkov cpm) or double-stranded oligonucleotides (10^5 cpm) was incubated for 20 min at 20°C with 3.5 μg of D.4 protein, unless otherwise noted, in the presence of either 0.1 μg of sonicated salmon sperm DNA or 1 μg of poly(dA-dT) (Boehringer Mannheim Biochemicals), as nonspecific carrier DNA, and 2% polyvinyl alcohol in a total volume of 12 μl. Free and bound DNA samples were separated on 1.5-mm-thick 4% acrylamide gels run and prerun in high-ionic-strength buffer (90 mM Tris borate, pH. 8.3, 2.5 mM EDTA). The gels were transferred onto filter paper, dried, and exposed to X-ray film. In band shift competition experiments (45), competitor DNA was added with the probe DNA and the binding assay was otherwise performed as described above. Probe DNA and competing oligonucleotides were quantitated by UV spectroscopy.

Methylation interference assay. The 5' end-labeled, 28-bp, −142/−115 single-stranded oligonucleotide probe and its unlabeled complementary strand were renatured and partially methylated (7 min) by the procedure of Maxam and Gilbert (32). The reaction was stopped by the addition of sodium acetate (pH 5)–β-mercaptoethanol to final concentrations of 1.5 and 1 M, respectively. Methylated DNA was precipitated twice, suspended in TE, and used in fivefold-scaled-up gel-binding reactions described above. Following electrophoresis, the gel was wrapped in cellophane and exposed to Kodak XAR film at 4°C overnight. The complexed and free fragments were excised, and the gel slices were cast into 1% agarose gels, electrophoresed onto a DEAE membrane, and recovered as described before (1). The dried pellet was suspended in 100 μl of 1 M piperidine,

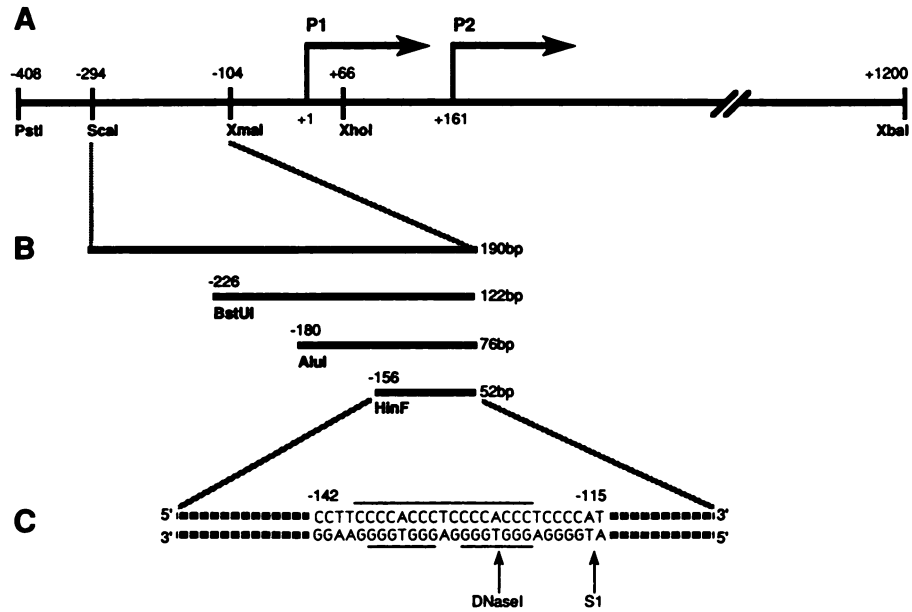


FIG. 1. Region of the human c-*myc* gene relevant to our studies. (A) Restriction map of the −408/+1,200 segment used in transcriptional and DNA-binding studies. (B) Restriction fragments showing sequence-specific binding of the transcriptionally active D.4 chromatographic fraction. (C) Sequence of the −142/−115 region used for sequence-specific binding, competitions, and methylation interference footprinting. Underlined bases indicate residues involved in protein binding; sequences overlined are bases involved in triplex formation in vitro (from Cooney et al. [10]). A DNase I-hypersensitive site at position −125 (44) and a major S1 cleavage site at −115 are also indicated (6).

and the bases were cleaved for 30 min at 90°C. Following lyophilization, the pellets were counted in a scintillation counter and approximately equal numbers of counts were analyzed in a 16% sequencing gel.

RESULTS

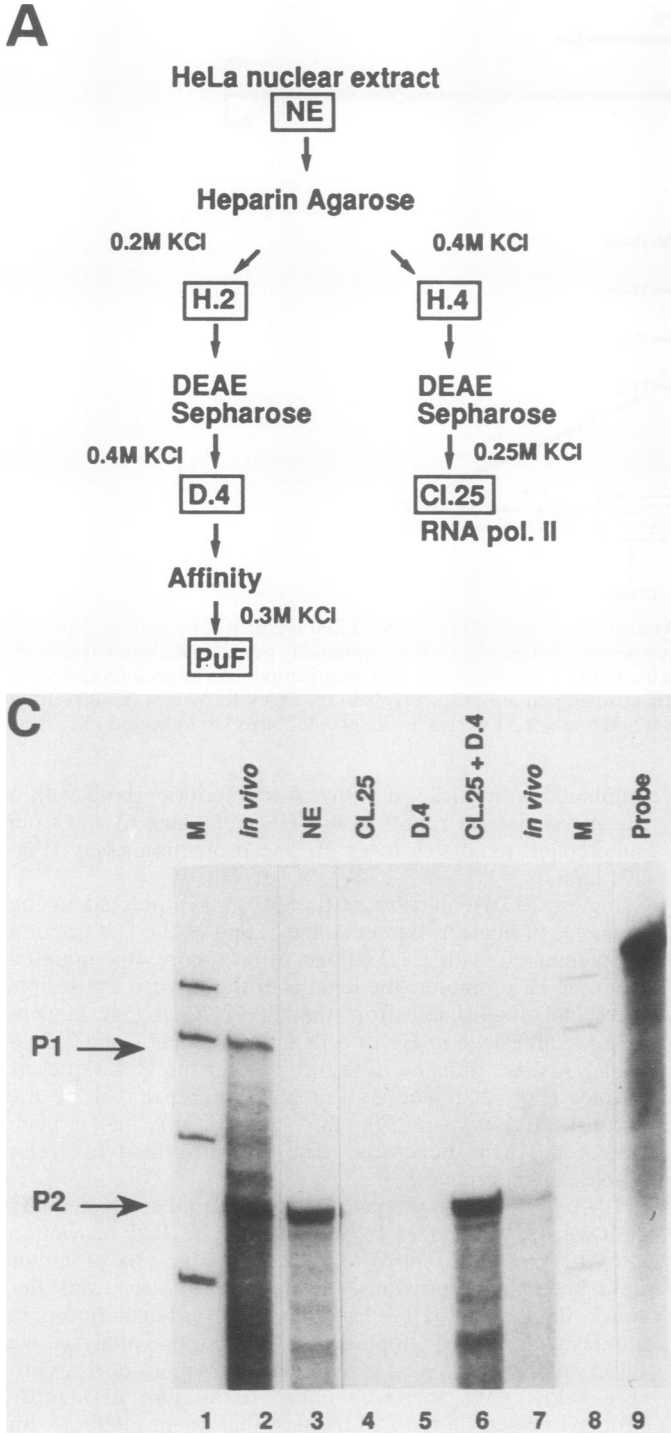
Partial purification of a c-*myc* transcription factor that correctly initiates RNA synthesis in vitro. We have shown previously that HeLa cell nuclear extracts support the efficient synthesis of an α-amanitin-sensitive, 0.97-kilobase runoff transcript from a human c-*myc* DNA template that was cleaved downstream from the in vivo initiation sites (in the first intron *Xba*I site; Fig. 1A). From the size of this runoff transcript, and from S1 nuclease-protected fragments, it was concluded that transcription in vitro is initiated at the major cap site, P2, of the human c-*myc* gene (10).

To identify and characterize the HeLa cell nuclear factor(s) responsible for this accurate expression of c-*myc* in vitro, standard chromatographic procedures were used to separate HeLa cell transcription components (13). Nuclear extracts prepared from growing cells were first chromatographed on heparin-agarose columns. The two transcriptionally active fractions recovered, the 0.2 M (H.2) and the 0.4 M (H.4) KCl eluates, were chromatographed further on DEAE-Sepharose (Fig. 2A). The 0.25 M KCl DEAE column fraction (C1.25) of H.4 contained the bulk of RNA polymerase II activity, as was expected from previous studies (13) and confirmed by Western blotting (immunoblotting; not shown). The C1.25 fraction was by itself inactive in c-*myc* transcription (Fig. 2B and C, lanes 4). The H.2 heparin-agarose fraction, which contained significant *myc* transcriptional activity when added to the C1.25 fraction, was fractionated further on a separate DEAE-CL6B column (Fig. 2A). The 0.4 M KCl fraction (D.4) contained the c-*myc* P2 transcription factor, subsequently termed PuF; the D.4 fraction was also inactive alone (Fig. 2B and C, lanes 5), but when combined with C1.25, it restored transcription both with a linear template in runoff assays (Fig. 2B, lane 6) and from supercoiled templates in an RNase protection assay (Fig. 2C, lane 6).

Figure 2C also illustrates that RNA synthesized in the presence of nuclear extracts (lane 3) and of the D.4 fraction supplemented with C1.25 (lane 6) was correctly initiated from the P2 promoter: the lengths of the in vitro transcripts were indistinguishable from that of P2 RNA (342 nucleotides) synthesized in HeLa cells in vivo (lanes 2 and 7). No P1 RNA was produced in vitro by either the HeLa nuclear extract (Fig. 2C, lane 3; see also reference 10) or the reconstituted system (Fig. 2C, lane 6), nor has it been observed when the c-*myc* gene is transcribed in HeLa whole-cell extracts (9).

DNA protein interactions. (i) Gel electrophoresis mobility shift assays. Sequences from −408 (at the *Pst*I restriction site) to +66 (at the *Xho*I site) of the human c-*myc* promoter have been shown previously to be important for transcription both in vivo (8, 21, 30) and in vitro (10). Furthermore, in studies with deleted templates for transcription analysis, we observed that this region is required for transcription in vitro (see below). We therefore chose to attempt to identify proteins present in the D.4 fraction that might interact with these sequences by using the gel electrophoresis mobility shift assay (19, 20).

The −408/+66 472-bp fragment was first cleaved with restriction enzymes *Sca*I and *Xma*I into three subfragments of 114, 190, and 169 bp (Fig. 1A), which were then tested for D.4 protein binding in the mobility shift assay. A protein titration (Fig. 3) showed that neither the −408/−294 114-mer nor the −104/+66 169-bp fragment bound to proteins present in the D.4 fraction. By contrast, the −294/−104 190-bp fragment formed two complexes with the transcriptionally active D.4 fraction. At low D.4 protein concentration, only the faster-migrating complex (C1) was present, whereas at



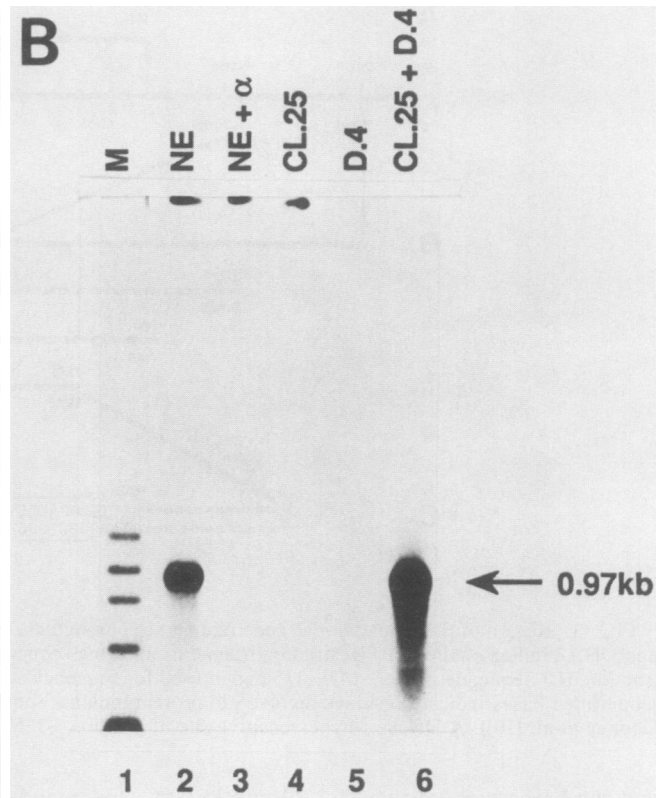


FIG. 2. Reconstruction of c-*myc* transcription with partially purified protein fractions. (A) Protein fractionation scheme. (B) Electrophoretic analysis of glyoxal-denatured runoff RNAs from the *Xba*I-cleaved pMHX template synthesized in vitro. Lanes 2 and 3 contain nuclear extracts (10 μg) without and with 2 μg of added α-amanitin per ml; lane 4, C1.25 alone (3 μg); lane 5, D.4 alone (5 μg); and lane 6, C1.25 and D.4 together (see Materials and Methods for details). Lane 1 contains end-labeled marker DNAs generated by *Hin*dIII digestion of ϕX174 DNA. Arrowhead on the right points to the 0.97-kilobase c-*myc* transcript. kb, Kilobase. (C) RNase protection analysis of RNA synthesized from supercoiled RV101 c-*myc* templates by nuclear extract (lane 3, 50 μg), by DEAE-Sepharose column fraction C1.25 alone (lane 4, 15 μg), D.4 alone (lane 5, 25 μg), and C1.25 and D.4 combined (lane 6). Refer to Materials and Methods for further details. Lanes 2 and 7 represent 20 and 5 μg of HeLa cell RNA synthesized in vivo, and lanes 1 and 8 are *Hpa*III-cleaved pBR322 size markers. Lane 9 contains the 670-nucleotide human c-*myc* exon 1 antisense RNA probe alone. Arrows on the left indicate the protected RNA species expected from the P1 (512 nucleotides) and P2 (342 nucleotides) promoters.

higher protein concentrations a more slowly migrating complex (C2) was also formed (Fig. 3, lanes 5 to 8) and eventually was the only complex observed (see, for example, Fig. 4). D.4 protein concentrations were adjusted for the formation of the more slowly migrating complex in subsequent mobility shift assays.

Dissection of the 190-bp fragment into smaller overlapping probes helped to locate a D.4 binding site to the sequence −156/−104, within a 52-bp fragment (Fig. 1B; results not shown). This region of the c-*myc* promoter contains tandemly repeated pur/pyr sequences and a DNase I-hypersensitive site (III_1) in the −125 region (43), as well as several S1 cleavage sites at around −115 (6). Furthermore, we have suggested previously that the −115 region of the c-*myc* promoter may be associated with a *trans*-acting factor, for the formation of a colinear triplex of *myc* promoter DNA with a single-stranded antisense oligonucleotide corresponding to the −142/−115 sequence repressed c-*myc* RNA synthesis in vitro (10). Indeed, a double-stranded version of this pur/pyr oligonucleotide formed a strong complex with D.4 proteins (see, for example, Fig. 4D, lane 1).

To determine whether D.4 binding to the 28-bp, double-stranded synthetic oligonucleotide containing the nuclease-hypersensitive pur/pyr sequences was specific and whether D.4 binding to the c-*myc* promoter was mediated by the

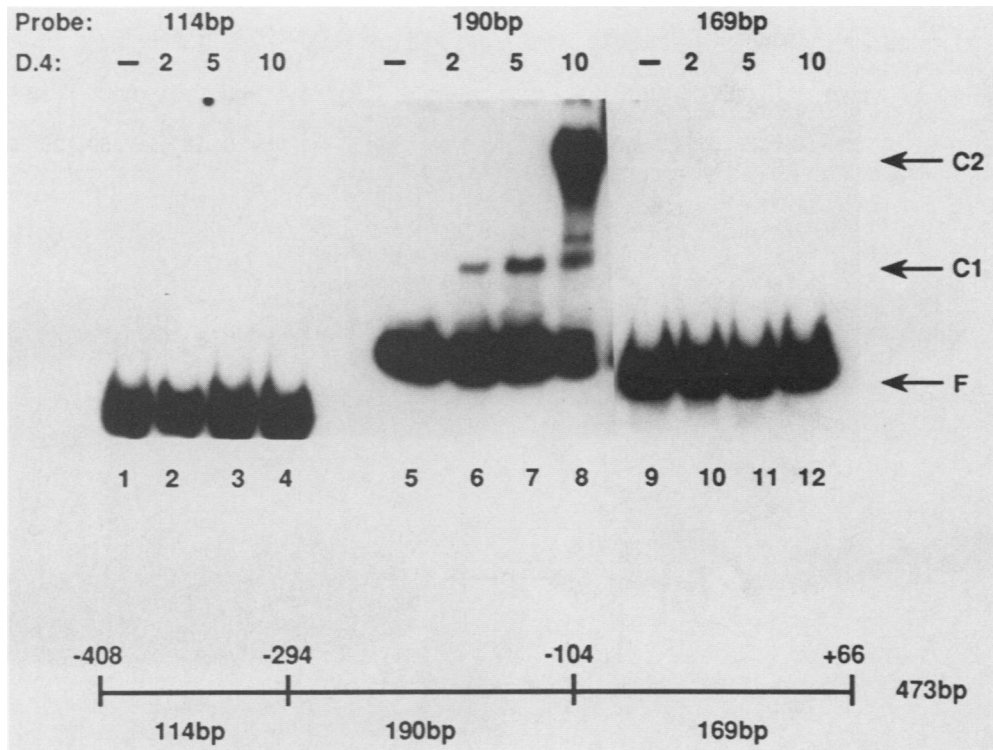
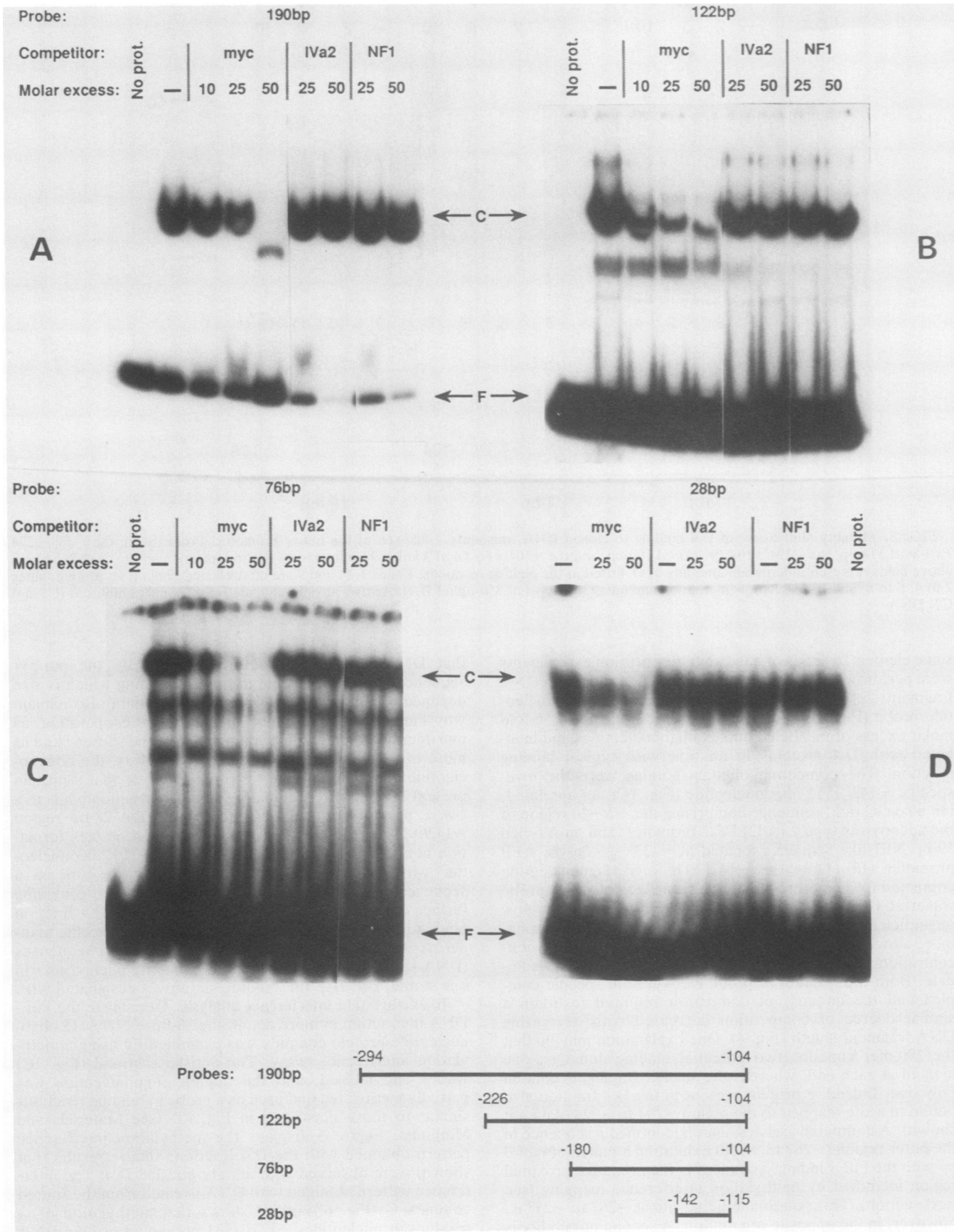


FIG. 3. Mobility shift assay of D.4 binding to cloned DNA fragments −408/+66 of the c-*myc* promoter. Probes were the −408/−294 *PstI/ScaI* 114-bp, the −294/−104 *PstI/XmaI* 190-bp, and the −104/+66 *XmaI/XhoI* 169-bp fragments (bottom panel; also Fig. 1A). Numbers above lanes represent microgram amounts of D.4 used in the binding reactions. Lanes 1, 5, and 9 are controls and contain no protein; lanes 2 to 4, 6 to 8, and 10 to 12 show increasing amounts (2 to 10 μl of 350-μg/ml D.4). Arrows at right indicate free (F) and complexed (C1 and C2) DNA.

same element, a series of band shift competition experiments were performed (45). End-labeled 190-, 122-, and 76-bp DNA fragments, as well as the 28-bp duplex pur/pyr oligonucleotide probe (Fig. 1B and C), were mixed with up to 50-fold molar excess of unlabeled duplex oligonucleotides and incubated with D.4 proteins in an otherwise typical binding reaction. The competing oligonucleotides were the *myc*-specific −142/−115 oligonucleotide (Fig. 1C); an unrelated, but also GC rich, sequence comprising the −2/−30 region of the adenovirus type 2 (Ad2) IVa2 promoter; and an AT-rich 39-bp NF1-like sequence located at −133/−96 on the AFP promoter (16). Only the −142/−115 *myc* oligonucleotide competed for D.4 protein binding, regardless of which probe was used (Fig. 4A to D). A 50-fold molar excess of the *myc* oligonucleotides virtually eliminated formation of the strong, more slowly migrating C2 complex, whereas the same concentration of IVa2 and NF1 oligonucleotides failed to compete. Both the fraction of probe recovered in specific complex and the quantity of competitor required to attain a similar degree of competition decreased with decreasing DNA fragment length (Fig. 4). One explanation may be that the 190-mer contains two potential binding domains, one located at each end, whereas the smaller fragments contain only one. Indeed, a protein present in the D.4 fraction also bound to a site mapped to the region −294 to −226 (data not shown). A computer-assisted search identified a sequence in the latter region (−267 to −258) exhibiting a match of 6 of 7 bp with the PuF binding sequences in the promoter-proximal region identified by methylation interference mapping (see next section). This potential binding site at −267 to −258 is, however, in the opposite orientation. A second possibility is that DNA secondary structure assumed by the pur/pyr sequences also plays a role in protein binding which is size dependent. Either of these possibilities would also explain why binding to the 190-mer appears to be cooperative, as one does not see a gradual competition of the shifted band as more *myc* competitor is added. Nevertheless, the oligonucleotide competition results strongly suggest that a protein present in the transcriptionally active D.4 fraction binds to a c-*myc* promoter element located within the 28-bp region −142/−115 in the human c-*myc* promoter. Complex formation between D.4 proteins and the −142/−115 oligonucleotide was retained after two rounds of purification on a sequence-specific DNA affinity column (Fig. 5), providing further evidence that a protein present in the D.4 fraction binds to the −142/−115 region in a sequence-specific manner. As this region of the c-*myc* contains pur/pyr sequences (DNA segments composed of predominantly oligopurines in one strand; Fig. 1C), this binding factor was designated PuF.

(ii) Methylation interference analysis. To analyze the PuF-DNA interaction in more detail, the 28-bp −142/−115 oligonucleotide-protein complex was examined by using a methylation interference assay. The antisense strand (Fig. 1C) was 5′-end labeled, and the duplex oligonucleotide was partially methylated and used as a probe in binding reactions similar to those illustrated in Fig. 4D (see Materials and Methods). Figure 6 displays the methylation interference pattern obtained with the D.4 fraction; similar results (not shown) were observed with affinity-purified D.4. The interference pattern obtained with DNA isolated from the shifted complex C (Fig. 6, lane 3) shows that methylation of G residues at nucleotides −137/−131 prevented protein-DNA


Probe:
190bp
122bp
Competitor:
No prot.
myc
IVa2
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10
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Probe:
76bp
28bp
Competitor:
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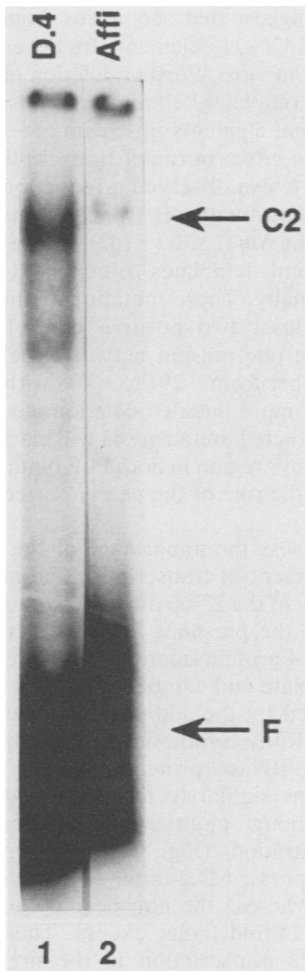

FIG. 5. Binding of D.4 proteins before and after affinity purification to the 28-bp, −142/−115 c-*myc*-specific, double-stranded oligonucleotide probe. Lane 1, 3.5 μg of D.4; lane 2, 10 ng of A.3, obtained from D.4 after two rounds of affinity purification.

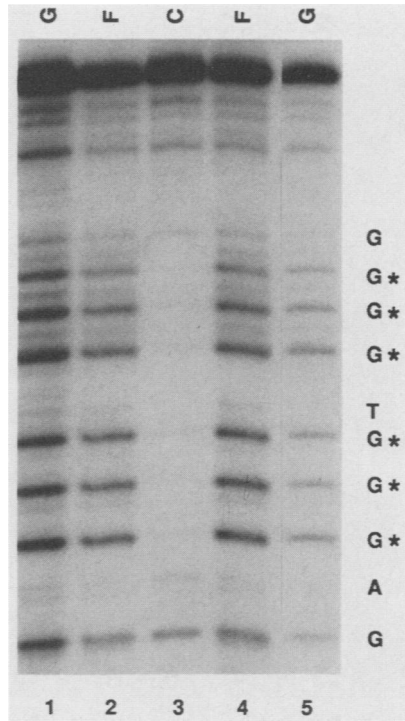

FIG. 6. Methylation interference analysis of protein-DNA complex formed between the 28-bp, −142/−115 c-*myc*-specific oligonucleotide and D.4. Preparative binding reaction was as described in Materials and Methods. Free probe (F; lanes 2 and 4) DNA from the specific complex (C; lane 3), and G-specific cleavage products of the probe labeled on the 5′ end of the antisense strand (32) (lanes 1 and 5) were separated on a 16% sequencing gel as described in Materials and Methods. The nucleotide sequence on the right indicates the positions where methylation interfered with complex formation (starred residues).

complex formation, suggesting that contact with PuF is made over the GGGTGGG palindromic motif at nucleotides −137/−131. A similar contact pattern was also observed with the repeated sequence at −128/−122 (data not shown). Both of these binding sites can be seen as underlined segments of the oligonucleotide sequence shown in Fig. 1C. Most interestingly, the boundaries of the PuF contact region are virtually identical to the −138/−120 region of c-*myc* DNA that is involved in transcriptional repression potentially through triplex formation (10; also see Fig. 1C, overlined sequence).

The −142 to −115 *cis* element is necessary for transcription in vitro. To address the role of the −142/−115 *cis* element in in vitro transcription, we have tested several 5′ deletion mutant templates for transcriptional efficiency. Figure 7 shows that deletion of the region from −118 to −17 did not affect P2 transcription. By contrast, transcription was reduced fivefold when sequences −160 to −101 were removed.

FIG. 4. Mobility shift and oligonucleotide competition assay of D.4 binding to subclones of the −294/−104 190-bp *Sca*I/*Xma*I fragment. Probes were the (A) 190-bp fragment, (B) 122-bp −226/−104 *Bst*UI/*Xma*I fragment, (C) 76-bp −180/−104 *Alu*I/*Xma*I fragment, and (D) 28-bp −142/−115 double-stranded oligonucleotide. See bottom panel and Fig. 1B and C for fragment orientation. D.4, 10 μl (3.5 μg), was used in all reactions, except in controls (no protein added). Competing double-stranded oligonucleotides were a 28-bp −142/−115 *myc*-specific sequence (5′-CCTTCCCCACCCTCCCCACCCTCCCCAT-3′), a 31-bp Ad2 IVa2 −30/−2 promoter sequence (5′-ACGAAGGCTCGCGTCC AGGCCAGCACGAAGG-3′), and a 39-bp NF1-like sequence (CCTCTGTTAATTATTGGCAAATTGCCTAACTTCAACGTG-3′). Amounts of nucleotides used, expressed in reference to the probe concentrations as molar excess, are indicated above each lane. Specific complexes (C) and free DNA probe (F) are marked with arrows. Gel exposures were adjusted to show approximately equal intensity of the *myc*-specific complex (C), and therefore the intensity of free probe bands (F) appears to be variable. Lanes 6 to 9 of panel A containing nonspecific competitor appear to bind more probe than those that do not contain or contain *myc* competitor. Since this phenomenon is entirely reproducible, we attribute it to an enhancement of protein binding in the presence of higher nonspecific DNA concentration in these reactions. The origin of the faster-migrating bands in panel A, lane 5; panel B, lanes 2 to 4; and panel C is unknown, but they appear to represent nonspecific complexes. Their absence from some of the lanes suggests that an interaction takes place between specific and nonspecific proteins. There are two *myc*-specific bands in panel C: the slower-migrating complex represents binding to the 76-bp fragment and below it the complex binding to the 68-bp left-hand fragment (see text), with which the 76-bp fragment is contaminated despite two rounds of gel purification.

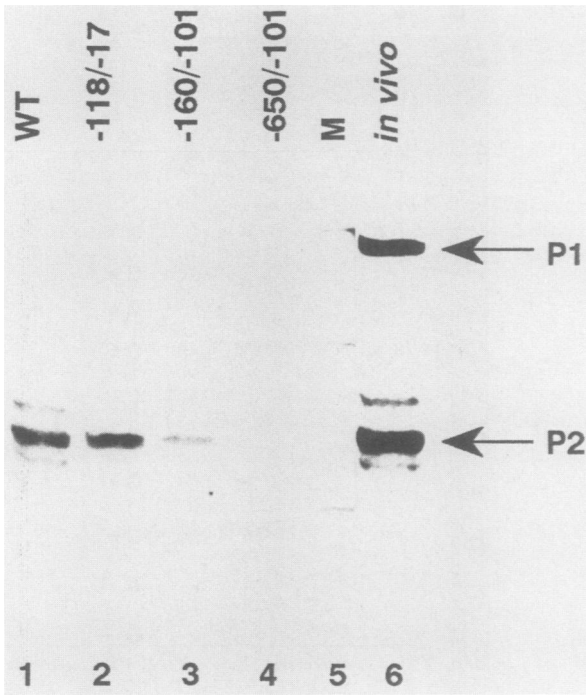

FIG. 7. In vitro transcription with promoter deletion mutants. Bal 31 deletion mutants (see Materials and Methods) were transcribed with 50-μg nuclear extracts (as for Fig. 2C) and analyzed by the RNase protection assay. Deleted sequences relative to P1 are indicated above each lane. Lane 1, RV101 (wild-type) template; lanes 2 to 4, mutant templates; lane 5, *Hpa*II-cleaved pBR322 size markers; lane 6, 10 μg of HeLa cell RNA synthesized in vivo. Arrows on the right indicate the protected RNA species expected from the P1 (512 nucleotides) and P2 (342 nucleotides) promoters. These experiments were repeated several times, and the percent transcription was determined by excising and counting the P2-specific bands in a liquid scintillation counter.

These results suggest that sequences from −160 to −118, including the −142/−115 element, are necessary for efficient P2 transcription in vitro. Further deletion of sequences from −101 to −650 completely eliminated transcription, indicating that additional elements upstream of −160 are required for P2 activity in vitro. In runoff transcription analyses (not shown), we have also observed a reduction in transcription efficiency by using templates truncated at the *Sca*I site at −294 (Fig. 1), the *Sma*I site (−101), or the *Xho*I site (+66). On the other hand, templates truncated at −408 (*Pst*I site) functioned normally. These mutational studies suggest that there exist at least two positive control regions for P2 activity in vitro: one present between nucleotides −118 to −160 and the other from −294 to −408 with reference to the P1 promoter. A more detailed deletion analysis in conjunction with site-directed mutagenesis will clarify the role of the −142/−115 pur/pyr region in both PuF binding and transcription, as well as the role of the nearly perfect repeat element at −267/−258.

To further assess the importance of the −142/−115 protein-binding element in transcription initiation in vitro, we tested the ability of the 28-bp duplex oligonucleotide to block transcription in the presence of the D.4 fraction. In such experiments, D.4 protein fractions were preincubated with a mixture of template and competitor DNAs before transcription was initiated by the addition of RNA polymerase and triphosphates. RNA synthesized in these reactions was analyzed in an RNase protection assay. The pattern of competitions was similar to that observed in the mobility shift assays, although more sensitive to competing oligonucleotide concentrations (Fig. 8). The 28-bp −142/−115 oligonucleotide repressed D.4-initiated transcription at 10-fold molar excess, whereas the nonspecific competitors had no effect, even at 25-fold molar excess. These findings argue strongly that P2 transcription in the presence of D.4 is

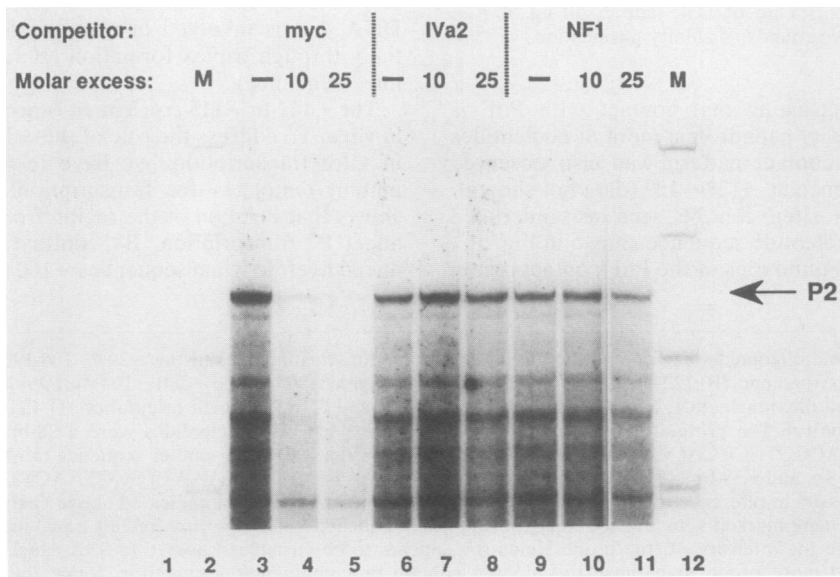

FIG. 8. Repression of D.4-initiated transcription by the 28-bp, −142/−115 *myc*-specific oligonucleotide. Lanes 3 to 11 each contain 3.5 μg of D.4 preincubated with a mixture of template DNA and oligonucleotide competitors in standard 12-μl binding reactions as described in the legend to Fig. 4. After 15 min of incubation at 20°C, transcription was initiated by the addition of C1.25 and triphosphates, as described in Materials and Methods. Lane 1 is control, C1.25 only (3 μg); lanes 3 to 11 all received D.4 and various amounts of oligonucleotide competitors as shown at the top. Oligonucleotides are described in the legend to Fig. 4. Lanes 2 and 12 show *Hpa*II-cleaved pBR322 used for size markers. Arrow on the right indicates the 342-nucleotide P2 transcript.

dependent on sequence-specific protein binding to the pur/pyr element.

DISCUSSION

To identify promoter elements and *trans*-acting factors necessary for c-*myc* expression, we have developed a partially reconstituted in vitro transcription system in which the human c-*myc* gene is efficiently and accurately transcribed. We present evidence that the 0.4 M KCl DEAE-Sepharose fraction (D.4) contains at least one factor, termed PuF, which, when combined with RNA polymerase II-containing fractions, can direct accurate and efficient RNA synthesis from the second promoter (P2) of the human c-*myc* gene (Fig. 2). It seems likely that the D.4 fraction contains factors in addition to PuF required for c-*myc* transcription in vitro, for PuF purified by sequence-specific DNA affinity chromatography failed to direct c-*myc* transcription but retained its ability to bind to DNA specifically (Fig. 5). It is also possible, although less likely, that PuF lost its transcriptional activity or was present at limiting concentrations following affinity purification. In addition to the D.4 fraction, at least one other activity present in the H.4 fraction (Fig. 2A) stimulated c-*myc* transcription (data not shown). These observations suggest that full in vitro reconstitution of c-*myc* transcription, by further characterization of this factor and PuF as well as analysis of the roles played by general transcription factors (41, 42), is a reasonable possibility.

Gel electrophoresis mobility shift DNA-binding assays demonstrated that PuF interacts with a 28-bp DNA segment corresponding to the region of the c-*myc* promoter −142/−115 (Fig. 4), an unusual DNA segment that contains tandemly repeated polypurine/polypyrimidine elements (Fig. 1C). Methylation interference footprinting (Fig. 6) established that PuF makes contact with the GGGTGGG palindromic repeat motif. Two lines of evidence suggest that this region of the c-*myc* promoter plays a functional role in P2 transcription in vitro. First, analysis of the efficiency with which deleted c-*myc* templates are transcribed demonstrated that the region between −160 and −118 contains a sequence that is an important element of the P2 promoter: deletion of this region reduced transcription fivefold, whereas deletion of sequences from −117 to −17 had little effect (Fig. 7). These findings are consistent with the previous report by Hay et al. (21) that the −101 to −293 domain exerts a stimulatory effect, albeit small, on P2 transcription in vivo. A more detailed mutational analysis of the −142/−115 element with successive microdeletions as well as base substitution mutants, which will address the precise sequence requirements for transcription as well as protein binding, is in progress. In addition, the role of the −142/−115 sequence in in vitro transcription from P2 was confirmed in transcription competition experiments: a 10-fold molar excess of the duplex oligonucleotide comprising the −142/−115 sequence suppressed transcription mediated by D.4 proteins, whereas unrelated oligonucleotides had no effect even at greater concentrations (Fig. 8). Together, these findings argue strongly that the sequence-specific DNA-binding protein PuF, present in the D.4 fraction, is required for c-*myc* transcription initiation in vitro and that therefore the −142/−115 sequence contains a positive transcriptional element.

The −142/−115 pur/pyr element of c-*myc* contains a number of potential protein-binding elements. Hay et al. (21) have noted the existence of closely clustered nuclear protein-binding sites in the −151/−101 region that contains several related sequences (Fig. 1C, for example). This region is within the −293/−101 domain these authors found to have had a twofold-stimulatory effect on P2 transcription in vivo (21). It is possible that PuF and the HeLa cell factor(s) detected by these authors by exonuclease footprinting experiments are related proteins.

The sequence GGGAGGG, located just upstream of the P2 promoter TATA box, although present in the opposite orientation, is also embedded in the −142/−115 pur/pyr element. The GGGAGGG sequence, conserved between humans and mice, is the binding site of the mouse exon 1 factor ME1a (31). It remains to be determined whether this motif also binds PuF or other proteins present in the D.4 fraction. However, our methylation interference data indicate that PuF makes contact with the GGGTGGG palindrome, which, while overlapping the GGGAGGG sequence, is located on the opposite strand. Furthermore, our genetic studies suggest that the ME1a-binding GGGAGGG element is not required for in vitro transcription from P2, for mutants that contain this element but which are deleted in the pur/pyr region are inefficiently transcribed. The CCCTCCC sequence motif also occurs in tandem repeats in the S1-sensitive −100 region of collagen promoters (17, 33).

The c-*myc* −142/−115 element also contains three copies of the TCCCCA motif (Fig. 1C), a consensus sequence for the enhancer-binding protein AP2 (22). However, PuF and AP2 differ in biochemical properties and sequence recognition (22, 36), suggesting that these two proteins are unrelated. The CACCCTCCCCA element of the c-*myc* −142/−115 sequence, to which PuF binds (Fig. 6), is a perfect match to a sequence occupying positions −53 to −45 of the Ad2 IVa2 promoter. This fact, combined with the stimulation of IVa2 transcription by PuF-containing fractions and the ability of synthetic or natural duplex DNA fragments containing the c-*myc* or IVa2 pur/pyr sequences to cross-compete in band shift assays (data not shown), provides strong evidence that PuF plays a role in initiation of transcription from the Ad2 IVa2 promoter, which lacks a TATA element (2). A sequence identical to the −100 consensus CACCC element of β-globin promoters (11, 29, 38) is also embedded in the IVa2 and c-*myc* pur/pyr elements and is also present at −25 to −14 of the Ad2 ML promoter. Both the β-globin (11, 14, 29, 38) and ML (26, 50) CACCC elements are associated with nuclease hypersensitivity and influence the efficiency of transcription from their respective promoters. PuF-containing fractions also stimulate transcription from the ML and human β-globin promoters (data not presented). We have not yet established whether PuF indeed binds to these ML and β-globin promoter elements or excluded the possibility that factors other than PuF present in the D.4 fraction are responsible for this stimulation of transcription. Thus, additional experiments will be required to establish the relationship of PuF to a putative CACCC element-binding factor.

Oligopurine/oligopyrimidine sequences (sequences with a purine bias in one strand) occur predominantly in the 5′ region of eucaryotic genes and have therefore been suggested to play biologically significant roles (reviewed in references 47 and 48). These sequences are capable of adopting different types of unorthodox DNA structures, for example, non-B DNA conformations, slipped helices, and triple-stranded structures that are unusually sensitive to the nucleases DNase I and S1 (15, 27, 47, 48). The pur/pyr element of the human c-*myc* promoter DNA that lies between nucleotides −101 and −151 is characterized by pyrimidines in the sense strand and purines in the antisense strand, with direct repeats of tri- and tetra-C/G pairs inter-

spersed with alternating T/A and A/T base pairs (Fig. 1C shows part of the sequence). Siebenlist et al. (43), who have previously mapped a DNase I-hypersensitive site (site III_1) to the −125 region of the human c-*myc* promoter, have been able to show that this site is involved in transcriptional regulation in the promyelocytic leukemia cell line HL60 (43). Boles and Hogan (6) have shown, using high-resolution mapping techniques, that the −142/−115 region contains two intense and several secondary S1 nuclease cleavage sites. Moreover, our previous observation that a single-stranded oligonucleotide represses c-*myc* transcription in vitro when bound in a triplex with this pur/pyr region (10) also suggested a role for this unusual sequence in the regulation of c-*myc* transcription. A correlation between the role of S1-sensitive sites and gene expression in transient transfection assays of the EGF receptor (23) and the chicken and mouse collagen promoter (33) has also been demonstrated. The data presented here, showing that the pur/pyr nuclease-hypersensitive element in the −142/−115 region of the c-*myc* promoter DNA is the site of binding of a positive transcription initiation factor, make this a direct and compelling correlation. Nevertheless, it should be noted that the specific cause-and-effect relationship of this coupling between nuclease hypersensitivity and transcriptional control, while remarkable, remains to be established.

A particularly striking aspect of these results is our finding that the PuF-binding sequence deduced from mobility shift and methylation interference assays (Fig. 4 and 6) and the site of the colinear triplex formed between c-*myc* DNA and the single-stranded, purine-rich, antisense oligonucleotide (10) appear virtually identical (Fig. 1C, underlined and overlined bases). Thus, the repressor activity of the single-stranded oligonucleotide (thus far seen only in vitro) can, as predicted (10), be explained by its ability to interfere with binding of the positive factor PuF. DNA sequences engaged in a triplex structure in which the third strand occupies the major groove of the duplex, as appears to be the case with the triplex containing the c-*myc* −142/−115 sequence (M. Cooney and M. Hogan, submitted for publication), can no longer accommodate proteins that interact with DNA via the major groove. The results of methylation interference mapping of PuF contacts within the c-*myc* pur/pyr element (Fig. 6) and our consistent failure to observe protection of this element from digestion by DNase I, which is considered to be specific for the minor groove (46), by D.4 proteins suggest that PuF is indeed a major groove binding protein.

Three-stranded structures have been shown to be associated with repressor molecules (35) and inhibited transcriptional activity of *Escherichia coli* RNA polymerase in vitro (37). The results we present and our previous observations (10) also suggest that triple-stranded structures are inactive transcriptional templates. Whether such structures, which might be formed as a result of intramolecular Hoogstein base pairing or by binding of an RNA or ribonucleoprotein in an intermolecular triplex (6, 10), play a role in regulation of c-*myc* transcription in vivo is not known. However, previous observations on the expression of c-*myc* from mutated promoters have suggested that the region containing the pur/pyr element exerts negative, as well as positive, control over the c-*myc* gene (8, 21, 30, 43). Experiments to investigate the potential negative regulatory function of this unusual sequence are in progress.

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