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An Antisense Promoter of the Murine c-*myc* Gene Is Localized within Intron 2

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Previously we have demonstrated the existence of stable transcripts from the noncoding strand of a rearranged c-*myc* gene in murine plasmacytomas in which the oncogene has translocated to an immunoglobulin constant-region gene element (M. Dean, R. B. Kent, and G. E. Sonenshein, Nature [London] 305:443–446, 1983). The resulting RNAs are chimeric, containing c-*myc* antisense and immunoglobulin sense sequences. A normal unrearranged murine c-*myc* gene is transcribed in the antisense orientation throughout much of the gene; however, stable transcripts have not been detected. In this study, using Northern (RNA) blot, S1 nuclease, and primer extension analyses, we have mapped the 5′ end of the stable chimeric transcripts to a site 175 bp from the start of exon 3, within intron 2 of the c-*myc* gene. In vitro transcription assays with constructs containing this site and 400 bp upstream, in the antisense orientation, and nuclear extracts from plasmacytoma cells, as well as a number of cell lines with normal unrearranged c-*myc* genes, indicated that this promoter was functional. This finding was confirmed in transient transfection assays using the antisense promoter linked to the chloramphenicol acetyltransferase reporter gene. These results suggest that a normal promoter of antisense transcription is used following c-*myc* gene translocation.

Several years ago, we demonstrated expression of stable c-*myc* antisense transcripts in plasmacytomas with a translocation of the c-*myc* gene to an immunoglobulin (Ig) constant-region domain (5). In the murine MOPC 315 plasmacytoma, which normally expresses IgA, the c-*myc* gene has translocated to the excluded C-alpha heavy-chain allele in a head-to-head fashion. Northern (RNA) blot analysis of nuclear RNA from MOPC 315 variant line V-1, which has lost the productive alpha heavy-chain allele, indicated that transcription of these antisense c-*myc* RNAs initiated within the c-*myc* gene portion of the locus. These transcripts were read through the switch/recombination and the C-alpha regions in the sense direction and were terminated and polyadenylated at the normal site at the end of the CH_3 domain (21). Thus, the largest 7.5-kb nuclear RNA appeared to contain both c-*myc* and C-alpha sequences. In addition, multiple smaller RNAs, ranging in size from 1.5 to 3.4 kb, were localized in the cytoplasm.

The normal (untranslocated) c-*myc* gene has also been shown to be transcribed bidirectionally. Using nuclear run-on analysis, we and others have shown transcription of both strands of the c-*myc* gene in human, rodent, and bovine cells (2, 12, 20). In murine cells, the transcription of the sense and antisense strands occurs in an overlapping manner (12, 20) and is sensitive to α-amanitin, indicating that the transcription is mediated by RNA polymerase II (12). Despite intensive efforts, however, antisense transcripts have not been detected, either in the cytoplasm or nucleus, by using Northern blot, S1 nuclease, or primer extension analysis. To better characterize the c-*myc* transcription unit, we have used the stable nuclear c-*myc*–C-alpha chimeric transcripts to map a start site of c-*myc* antisense transcription to intron 2 and, furthermore, confirm its use in cells with normal as well as rearranged c-*myc* genes.

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MATERIALS AND METHODS

Cell lines and culture conditions. Murine plasmacytoma lines used include MOPC 315 variant V-1 (IgA) (22), MPC 11 (IgG2b), HOPC 1 (IgG2a), and S194 (IgA). Plasmacytoma cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. Culture conditions and goat anti-mouse Ig treatment of the murine WEHI 231 early B-cell lymphoma cell line were as described previously (19). The pre-B Ba/F3 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 10% conditioned medium from the myeloid (interleukin-3-producing) WEHI-3 cell line. B104, a rat neuroblastoma cell line, was grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

RNA extraction and analysis. Cytoplasmic and nuclear RNAs were isolated, and 20 μg was subjected to Northern blot analysis as described previously (5). Poly(A)-containing RNA was isolated according to Maniatis et al. (18). *Xba*I-*Sst*I, *Sst*I-*Pvu*II, and *Pvu*II-*Hin*dIII c-*myc* DNA fragments (shown in Fig. 1) were subcloned into M13 mp18 and mp19 and used as probes. These probes were labelled by primer extension with the M13 universal primer in the presence of [^{32}P]dCTP as described by Hu and Messing (9). For S1 nuclease analysis, the *Sst*I-*Pvu*II fragment of c-*myc* intron 2 was labelled with [^{32}P]ATP and T4 polynucleotide kinase (18) and used as a probe. Hybridization and digestion were performed as described by Yang et al. (27).

Primer extension. Primer extension analysis was performed by a modification of a Promega protocol. Six micrograms of poly(A) nuclear RNA was lyophilized and resuspended in 10 μl containing 50 mM NaCl, 5 mM $MgCl_2$, 340 mM Tris-HCl (pH 8.3), and 100 ng of primer (shown in Fig. 3). The mixture was heated to 60°C for 10 min and then cooled to 42°C over a 60-min period. Two microliters 0.1 M dithiothreitol (DTT), 20 μCi of [^{35}S]dATP (>600 Ci/mmol), and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase was then added to the mixture (RT mix). To each of four tubes, labelled G, A, T, and C, 2 μl of

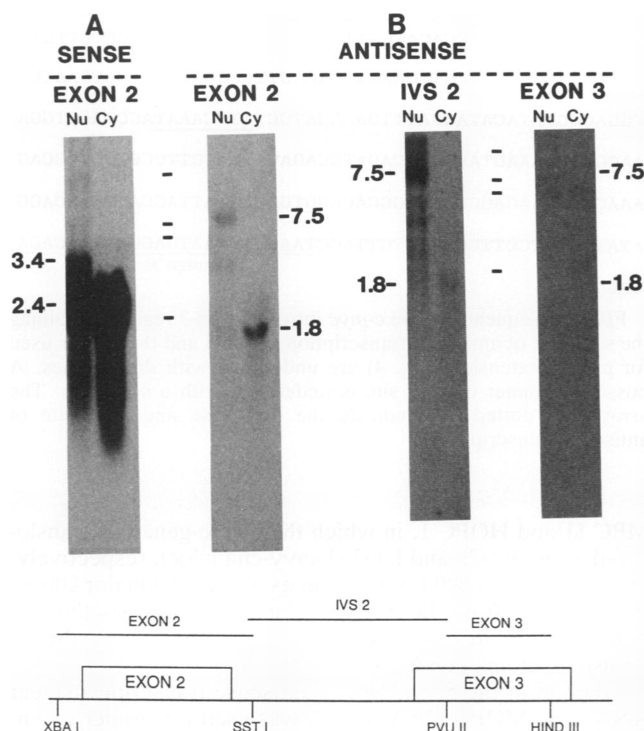

FIG. 1. c-*myc* sense and antisense transcripts in the MOPC 315 variant V-1, as shown by Northern blot analysis using 20 μg of either nuclear (Nu) or cytoplasmic (Cy) RNA isolated from MOPC 315 V-1 cells. Duplicate lanes were hybridized with single-stranded probes detecting either sense or antisense sequences of exon 2 or antisense sequences of intron 2 (IVS2) or exon 3, as indicated. Dashed lines indicate positions of 18S and 28S rRNAs and their precursors. Sizes are indicated in kilobases. Below is a map of the probes used.

nucleotide mix (G mix, 50 μM ddGTP; A mix, 3.6 μM ddATP; T mix, 200 μM ddTTP; C mix, 100 μM ddCTP; all mixes contained 250 μM deoxynucleoside triphosphate [dNTP] without dATP), and 3 μl of RT mix were added, respectively, and the tubes were incubated at 42°C. After 25 min, 1 μl of 2 mM dNTP was added, and the mixture was incubated for a further 25 min. RNase A (1 μl of a 200-μg/ml solution in 10 mM EDTA) was then added, and the mixture was incubated for 30 min at 42°C. Reactions were stopped by addition of 4 μl of sequencing loading buffer, and products were separated by electrophoresis on a 8% acrylamide sequencing gel. DNA sequencing was performed by using a New England Biolabs sequencing kit.

In vitro transcription. Nuclear extract preparations and in vitro transcription reactions were performed by a modification of the procedure of Dignam et al. (6). Cells were washed in phosphate-buffered saline, resuspended in 1 ml of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml. Following incubation on ice for 15 min, Nonidet P-40 was added to a final concentration of 0.2%. Nuclei were pelleted by centrifugation for 2 min at 1,000 rpm in a microfuge, resuspended in 1 packed nuclear volume of buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml), and incubated on ice for 30 min. One packed nuclear volume of buffer D (buffer C with no KCl) was added, and the mixture was incubated for 30 min. Nuclear debris was removed by centrifugation for 5 min in a microfuge. In vitro transcription reaction mixtures included 12.5 μl of nuclear extract adjusted to a final concentrations of 25 mM HEPES (pH 7.9), 1 mM DTT, 6 mM $MgCl_2$, 9% glycerol, 100 mM KCl, 600 μM NTP, and 0.5 μg of template DNA in a 25-μl volume. Following incubation for 45 min at 30°C, 2 μl of 1.25-mg/ml DNase I in 15 mM $CaCl_2$ was added, and the mixture was incubated for a further 30 min at 37°C. To stop the reaction, 175 μl of a solution containing 220 μg of proteinase K per ml, 0.5% sodium dodecyl sulfate, 0.3 M sodium acetate, and 2.5 mM EDTA was added, and the mixture incubated for 30 min at 37°C. Proteins were removed by phenol extraction, and the RNA was precipitated with ethanol and subjected to S1 nuclease analysis.

Transfections and CAT assays. The *Sst*I-*Pvu*II DNA fragment (shown in Fig. 5) was subcloned into the pBLCAT3 vector (17). Transient transfections of Ba/F3 cells were performed according to Lieber et al. (16) with 15 μg of DNA. Forty-eight hours after transfection, cells were washed with Puck's saline, resuspended in 250 mM Tris-HCl (pH 8.0), and lysed by freezing and thawing. Cell extracts were normalized for total protein content and assayed for chloramphenicol acetyltransferase (CAT) activity (7).

RESULTS

Mapping of the 5′ end of c-*myc* antisense–C-alpha sense transcripts. In previous work, we demonstrated that c-*myc*–C-alpha chimeric transcripts in the MOPC 315 plasmacytoma hybridized to a double-stranded c-*myc* probe that contained all of exon 2, intron 2, and exon 3 (5). To verify the orientation of these transcripts and begin mapping the 5′ ends of the c-*myc* antisense RNA, cytoplasmic and nuclear RNAs were isolated from the MOPC 315 V-1 line and subjected to Northern blot analysis using single-stranded M13 probes containing c-*myc* exon 2 sequences (Fig. 1A). The probe detecting antisense transcripts hybridized to a 7.5-kb nuclear RNA species which correlated with the largest RNA species detected by C-alpha and c-*myc* probes previously (5). In the cytoplasm, hybridization to a 1.8-kb RNA species was the most prominent band observed. In contrast, a probe detecting exon 2 sense sequences hybridized with bands of 3.4 and 2.4 kb in the nuclear RNA. A 2.4-kb RNA species, as well as additional lower-molecular-weight species seen better on a lighter exposure, were observed in the cytoplasmic RNA. These species ranged in size from 1 to 2.5 kb and are apparently due to the use of multiple cryptic start sites of transcription within intron 1 as a result of the loss of the normal P1 and P2 promoters in the translocated c-*myc* gene (3, 11).

To delineate the 5′ end of the antisense transcripts more specifically, single-stranded subclones of intron 2 (IVS2) and exon 3, shown in Fig. 1, were used. The 7.5-kb nuclear RNA species hybridized to the probe detecting antisense sequences in intron 2 but not with the exon 3 probe (Fig. 1B). The low level of hybridization seen with the exon 3 probe appeared to colocalize with the rRNA bands and is thus likely due to cross-hybridization. Hybridization to the 1.8-kb RNA band in the cytoplasm was also observed with the intron 2 but not exon 3 probe. These results suggest that the 5′ end of these transcripts lies within intron 2 or the beginning of exon 3 and that the 7.5-kb RNA is spliced and translocated to the cytoplasm.

To further define the 5′ ends of these transcripts, the same intron 2 probe was used for S1 nuclease analysis. The

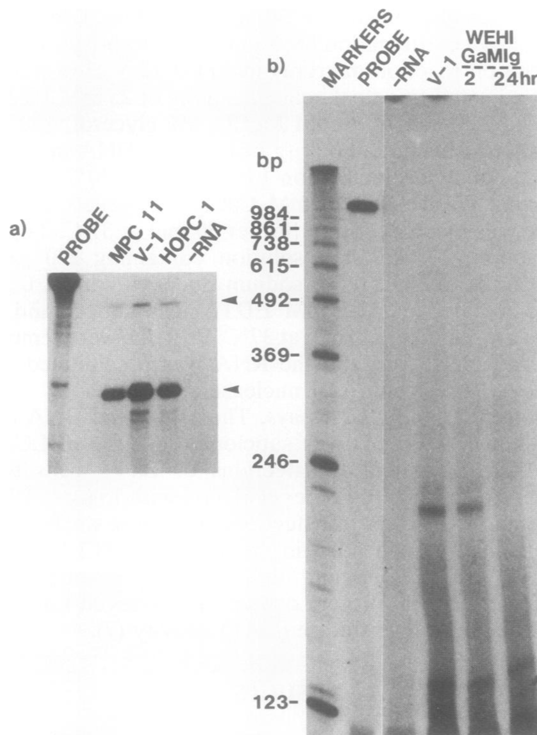

FIG. 2. Mapping of the 5′ ends of the c-*myc*–C-alpha chimeric transcripts. S1 nuclease analysis was performed by using 10 μg of nuclear RNA isolated from MOPC 315 V-1 (V-1), MPC 11, or HOPC 1 cells or 10 μg of cytoplasmic RNA isolated from WEHI 231 cells treated with goat anti-mouse Ig (GaMIg) for 2 or 24 h. The IVS2 DNA (Fig. 1), labeled at both ends, was used as a probe. An identical reaction with no RNA added (−RNA) was included as a control. The 123-bp ladder (Bethesda Research Laboratories) was added, as shown in panel b, to determine the size of the protected fragments (indicated by arrows).

1,100-bp probe was labelled at both ends, and therefore two fragments were expected to be protected: a 200-bp fragment representing protection of the DNA encoding the 3′ end of exon 3 due to hybridization to c-*myc* mRNA, and a fragment of unknown size corresponding to the protection of DNA encoding the 5′ end of the antisense RNA. Nuclear RNA from MOPC 315 V-1 cells was hybridized to the probe and digested with S1 nuclease (Fig. 2). Protection of a 200-bp fragment and a 600-bp fragment was observed. To confirm the identity of the 200-bp protected fragment, RNA isolated from the WEHI 231 early B-cell lymphoma cell line, which has unrearranged c-*myc* genes, was similarly analyzed (Fig. 2). In particular, RNA was extracted from WEHI 231 cells treated with goat anti-mouse Ig for times at which c-*myc* mRNA was stimulated (2 h) or depressed below control levels (24 h) (15). As can be seen, a band at 200 bp was protected with RNA from 2-h-treated cells but not from 24-h-treated cells, confirming that this band corresponds to protection due to the c-*myc* mRNA. Thus, the antisense transcripts protected a 600-bp fragment, indicating that their 5′ end is approximately 150 to 200 bp from the beginning of exon 3 within intron 2.

To ensure that this start site was not a peculiar phenomenon of the MOPC 315 V-1 cells, two other plasmacytomas with translocations of the c-*myc* gene to different Ig loci were similarly analyzed (Fig. 2a). Nuclear RNA isolated from

EXON 3
CTTCTCCACAGACACCACATCAATTTCTTCCTCATCTTCTTGCTCTTCTT

CTGTAGAGGGAGGAAGTGGAAGATCACAGTTAGCCACGCCTCCTGAATCC

CGGACGCTATACATAGGATTTGACCGATGCTATCCAAATACCAAGTTGGA

AAGGGAAGAAAGTAAGAATCAGATCCAGACACCATCTTCCCTTCTCCCAG

AAAGGCAGAACAGGGTTAGGGCACAGGTGAGAAAATTACCATGTACCAGG

ATATGGTATCCTTGCCAATTCTTACCTAAGATTTAATGAGGCTTCAGACA
PRIMER A
GAGCCAAA

FIG. 3. Sequence of the c-*myc* intron 2/exon 3 region containing the start site of antisense transcription. Exon 3 and the primer used for primer extension (Fig. 4) are underlined with dotted lines. A possible octamer binding site is underlined with a solid line. The arrow and dotted line indicate the first base and start site of antisense transcription.

MPC 11 and HOPC 1, in which the c-*myc* gene has translocated to the IgG2b and IgG2a heavy-chain loci, respectively, both protected a 600-bp fragment as well as the major 200-bp band derived from the mRNA. This result suggests that the putative start site of transcription is common to a number of mouse myeloma cell lines.

To confirm the 5′ end of the antisense transcript, nuclear RNA from MOPC 315 V-1 cells was used for primer extension analysis. Since the sequence of intron 2 of c-*myc* is not in the literature, we sequenced this region and used the information to synthesize a 26-bp primer that should hybridize approximately 50 bp downstream of the putative start site (Fig. 3). The unlabelled primer was hybridized to 6 μg of poly(A)-containing nuclear RNA from MOPC 315 V-1 cells and then extended, using M-MLV reverse transcriptase in the presence of [^{35}S]ATP and dideoxynucleotides to sequence the RNA as well. The products of the reaction were then separated by electrophoresis on a gel next to a DNA sequencing reaction in which the same primer was used. Unfortunately, the concentration of the dideoxynucleotides in this reaction was too low for incorporation with this enzyme. Thus, the primed DNA extended correctly and the sequencing of the RNA was unsuccessful. As can be seen in Fig. 4, all lanes of the primer extension reaction extended to the same length, which corresponded to the place that had been mapped by S1 nuclease analysis. The same results were obtained with use of a labelled primer and cold nucleotides (data not shown). Thus, the start site of transcription is mapped to 175 bp upstream of the 5′ end of exon 3, as illustrated in Fig. 3.

Antisense transcription of normal and translocated c-*myc* genes starts at the same site. To test for promoter usage, a DNA fragment containing 400 bp upstream and 135 bp downstream of the start site was subcloned upstream of the CAT reporter gene (ASD3CAT) (Fig. 5) and used to direct RNA synthesis in in vitro transcription reactions. Nuclear extract was prepared from S194 cells, a murine plasmacytoma cell line with a translocated c-*myc* gene. Linearized ASD3CAT plasmid was incubated with the S194 nuclear extract in the presence of unlabelled nucleotides for 30 min at 30°C to allow transcription to take place. The resulting RNA was isolated and subjected to S1 nuclease analysis using a probe which was labelled at the *Eco*RI site, 250 bp within the CAT gene of the ASD3CAT plasmid, and included all of the c-*myc* sequences (Fig. 5). The RNA protected a 420-bp fragment of the DNA probe (Fig. 5, lane 2). This mapped the start site of transcription to the same point seen

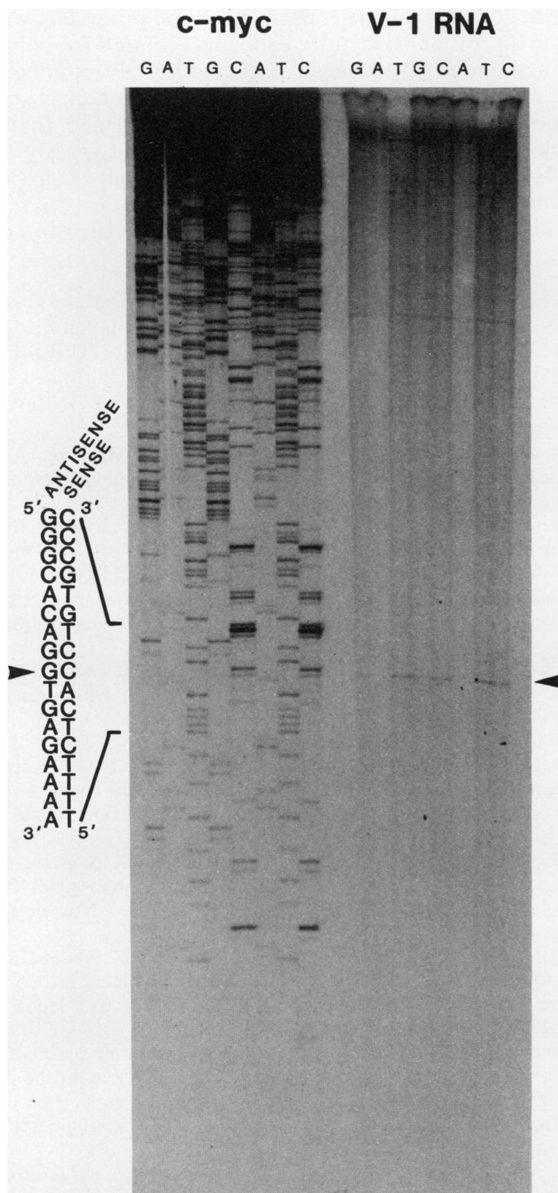

FIG. 4. Primer extension analysis of c-*myc*–C-alpha chimeric transcripts. The primer shown in Fig. 3 was hybridized with 6 μg of poly(A)-containing nuclear RNA isolated from MOPC 315 V-1 cells and extended with M-MLV reverse transcriptase in the presence of dideoxynucleotides (see Materials and Methods) (V-1 RNA). DNA sequencing reactions using the same primer with single-stranded IVS2 DNA were electrophoresed in the adjacent lanes (c-myc). Arrowheads indicate the position determined for base 1 in the antisense transcripts.

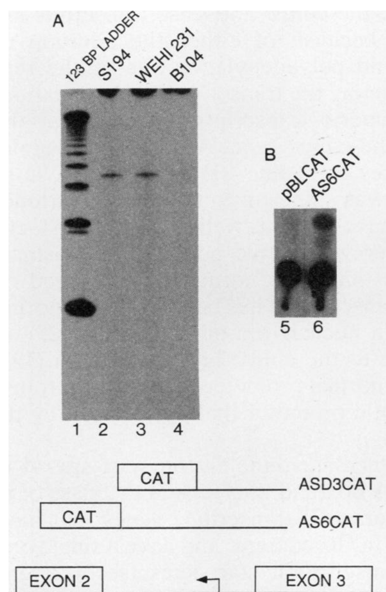

FIG. 5. Functional analysis of the c-*myc* antisense promoter. (A) In vitro transcription analysis was performed with nuclear extracts from mouse myeloma S194, WEHI 231, and B104 neuroblastoma cells and linearized ASD3CAT plasmid as the template DNA as described in Materials and Methods. The resulting RNA was subjected to S1 nuclease analysis using a 820-bp probe labelled at the *Eco*RI site, 250 bp within the CAT gene, and including all of the c-*myc* sequences in the ASD3CAT plasmid. (B) BaF/3 pre-B cells were transfected with 10 μg of the AS6CAT or the parental pBLCAT plasmid. Resulting cellular extracts were analyzed for CAT activity.

with the V-1 RNA above, and it demonstrated that these 535 bp within the c-*myc* gene represented at least the minimal promoter in an in vitro assay.

Since plasmacytomas contain plasmacytoma-specific nuclear factors that can bind to the c-*myc* promoter and affect its transcription (10), there was the possibility that this antisense promoter may be functional only in plasmacytomas. Thus, the activity of the promoter was tested by using nuclear extracts from cells which have normal unrearranged c-*myc* genes in the transcription assay. As can be seen in Fig. 5 (lanes 3 and 4), when nuclear extracts from the murine early B-cell lymphoma WEHI 231 or the rat neuroblastoma B104 cells were used, a fragment of the same size as with S194 extracts was protected. A similar fragment was protected when nuclear extracts from HeLa (a human cervical carcinoma cell line) and the murine early pre-B cell line Ba/F3 were used (data not shown). Thus, factors required for activity of this promoter are present in cells with unrearranged c-*myc* genes, and this promoter is functional in a variety of cell types as well as species.

The antisense promoter functions in cells with normal c-*myc* genes. To test whether this promoter is active in cells with unrearranged c-*myc* genes, a vector which included the entire *Sst*I-*Pvu*II DNA fragment upstream of the CAT gene was constructed (AS6CAT; Fig. 5). AS6CAT or the parental pBLCAT3 plasmid DNA was transfected into Ba/F3 cells. After 48 h, extracts were prepared, normalized for protein content, and assayed for CAT activity. As can be seen in Fig. 5 (lanes 5 and 6), there was a four- to fivefold increase in CAT activity over the background level of the parental vector when the AS6CAT plasmid was used. The transcription start site was confirmed by S1 nuclease analysis of RNA from NIH 3T3 cells stably transfected with the ASD3CAT plasmid (data not shown). Thus, the antisense promoter functions in cells with unrearranged c-*myc* genes.

DISCUSSION

We have identified a promoter within intron 2 of the c-*myc* gene that directs transcription in the antisense direction. In plasmacytomas, in which the c-*myc* gene has translocated to

the Ig locus, the c-*myc* antisense transcripts are stabilized, presumably because of either the addition of the 3′ Ig sequences and polyadenylation site or the removal of sequences targeting the transcripts for degradation. The 5′ end of these chimeric transcripts mapped near the 3′ end of intron 2 of the c-*myc* gene. A construct containing this site and sequences extending 400 bp upstream, in the antisense orientation, was sufficient to initiate transcription in in vitro transcription reactions as well as when transfected into cells. The promoter was active not only in plasmacytomas but also in cells that have normal unrearranged c-*myc* genes. Antisense transcription has been shown to normally occur in this region in nuclear run-on assays (20, 25) and does not increase due to the c-*myc*-Ig translocation (12), suggesting that this is a normal promoter for antisense transcription and is not a cryptic promoter that is activated by the translocation.

The sequence surrounding the start site of transcription has no TATA box and thus falls in the class of promoters of RNA polymerase II-transcribed genes that have no TATA box, are low in GC content, and have a single or a few tightly clustered transcription start sites (see reference 24 for a discussion of transcriptional initiation in eukaryotes). The sequence ATCCAAAT (underlined in Fig. 4), resembling the octamer binding site ATGCAAAT (23), is located 70 bp upstream of the start site of transcription. It is intriguing that octamer binding sites are also found in the promoter for antisense transcription of the N-*myc* gene. The N-*myc* antisense promoter, however, differs in that it falls into the other class of TATA box-deficient promoters. It is GC rich, containing many Sp1 binding sites (8), and transcription starts at many sites spread over a fairly large area (13). Furthermore, the antisense transcription of the N-*myc* gene is coordinately regulated with the sense transcription and results in a stable transcript (13), suggesting a possible function different from that of the antisense transcription of the c-*myc* gene.

While first observed in prokaryotes and viruses, antisense transcription has been found in a growing number of eukaryotic genes as well. Studies in prokaryotes and viruses as well as with artificial antisense RNA in eukaryotes have demonstrated that antisense transcripts are able to regulate the processes of DNA replication, transcription, translation, mRNA processing, and mRNA stability. This specific regulation can result in up to 100% reduction in the target gene's expression (reviewed in reference 26). The antisense transcription of the c-*myc* gene, however, does not result in a stable transcript, and therefore its function is perplexing. Similarly, the other nuclear oncogenes c-*fos*, c-*myb*, and L-*myc* all have antisense transcription but no stable transcripts (1, 14). All of these genes are early response genes, and their expression is rapidly induced when cells are stimulated to proliferate. It is possible that in order to achieve the rapid induction of transcription, the gene must be in an open conformation allowing transcription factors access to its promoter. In most cell types and under a variety of conditions, the c-*myc* antisense transcription is constitutively expressed (12, 20), consistent with this hypothesis. Recently, however, we have demonstrated that in the interleukin-3-dependent pre-B cell line Ba/F3, removal of the interleukin-3 results in a 10- to 20-fold increase in the antisense transcription of c-*myc* (4). During this time, there is no change in the sense transcription or mRNA half-life yet there is a decrease in the mRNA level. This finding suggests the possibility that the antisense transcription, or its labile transcript, may be targeting the sense transcripts for immediate degradation. Having established the promoter, we are now in a position to identify elements required for constitutive and induced antisense transcription. Presuming that these are not within the coding region of the gene, constructs for homologous recombination can be designed that will knock out the antisense transcription without grossly altering the gene structure. These constructs should help define its function within the cell and may shed light on the role of antisense transcription in other systems as well.

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