Transcription of the Human c-Src Promoter Is Dependent on Sp1, a Novel Pyrimidine Binding Factor SPy, and Can Be Inhibited by Triplex-forming Oligonucleotides*

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The tyrosine kinase pp60c-src has been implicated in the regulation of numerous normal physiological processes as well as the development of several human cancers. However, the mechanisms regulating its expression have not been addressed. In the present study, we report the presence of two Sp1/Sp3 binding sites and three polypurine:polypyrimidine (Pu:Py)2 tracts in the c-Src promoter that are essential for controlling expression. We demonstrate that Sp1, but not Sp3, is capable of activating the c-Src promoter and that Sp3 is also capable of inhibiting Sp1-mediated transactivation. The presence of multiple Pu:Py tracts conferred S1 sensitivity on plasmids in vitro, suggesting they are capable of adopting non B-DNA conformations. These tracts specifically bind a nuclear factor we named SPy (Src pyrimidine binding factor), which demonstrates both novel double- and single-stranded binding specificities. Mutations eliminating SPy binding compromised Src transcriptional activity, especially in concert with additional mutations affecting Sp1 binding, suggesting the two factors may cooperate in regulating c-Src expression. Finally, we demonstrate that triplex-forming oligonucleotides designed to target both Sp1 and SPy binding sites can down-regulate c-Src expression in vitro, suggesting a potential therapeutic approach to controlling c-Src expression in diseases where aberrant expression or activity has been documented.

The human c-Src proto-oncogene gene encoding the tyrosine kinase, pp60c-src, was originally identified as a homologue of the transforming gene of Rous sarcoma virus (1). c-Src is the prototype of a large family of highly conserved genes (Yes, Fyn, Fgr, Lyn, Lck, Hck, Blk, and Yrk), whose products have been implicated in many signal transduction pathways and a wide variety of cellular processes (2). Most c-Src research over the last decade has focused on identifying the enzyme’s targets, the mechanisms of post-translational kinase activity, and interacting proteins. In addition, considerable progress has been made in elucidating the many signal transduction pathways of which c-Src and its family members appear to be a part (2, 3). For example, it has been shown that c-Src activation is required in growth factor mediated passage of fibroblasts from the G2 phase of the cell cycle to mitosis (4, 5), as well as a variety of other diverse signaling mechanisms including endothelin-dependent hypertrophic activation in cardiac myocytes (6). c-Src has also been implicated in the regulation of Ca2+ release through store-operated channels (7). Interestingly, c-Src knockout mice display a single major phenotype, osteopetrosis, identifying c-Src activation as an essential component of osteoclast function (8). This finding also supports the hypothesis of redundancy between Src family members (especially Yes and Fyn), which appear to share critical functions (9). In addition to these normal physiological functions, overexpression or activation of c-Src has been frequently linked to the development of human neoplasms, especially those of the colon (10–12) but also breast (13), lung (14), and pancreas (15). Most recently, activating mutations in the c-Src gene have been documented in a small percentage of advanced colon tumors (16).

Thus, while considerable information and knowledge has accumulated on pp60c-src, it is curious that virtually nothing is known about the transcriptional regulation of this important gene in normal or neoplastic cells. This is despite the fact that c-Src gene expression is developmentally regulated in the brain (17) and transcriptionally up-regulated during osteoclast activation (18) and in regenerating neurons (19). In addition, c-Src mRNA levels vary dramatically in both human cell lines and tissues, and we have recently shown that the frequently reported c-Src overexpression in many colon cancer cell lines results from transcriptional activation of the gene.1 To begin to address this hitherto unexplored facet of c-Src regulation, we have isolated and identified a human c-Src promoter (20). The promoter region has characteristics typical of the housekeeping class of genes, such as high GC content, lack of obvious TATA or CCAAT regulatory sequences, and multiple transcription start sites (20). Many of these start sites were mapped to three unusual polypurine:polypyrimidine (Pu:Py)2 tracts, identified in a region of the promoter essential for full transcriptional activity. In this current report we describe a detailed study of the basal c-Src promoter. We show that transcriptional regulation of the c-Src promoter is absolutely dependent on the Sp family of transcription factors but also on the presence of these Pu:Py tracts. In addition, we describe a novel factor (SPy) which displays both double-stranded and single-stranded binding specificity when interacting with these important Pu:Py tracts. Finally, we were able to design a series of synthetic

1 K. Bonham, unpublished observations.
2 The abbreviations used are: Pu, polypurine; Py, polypyrimidine; TFO, triplex-forming oligonucleotide; EMSA, electromobility shift assay; CAT, chloramphenicol acetyltransferase; Inr, initiator; PCR, polymerase chain reaction; bp, base pair(s).

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**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse fibroblast 10T1/2 and human colon adenocarcinoma SW480 and SW620 cell lines were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum at 37 °C, 5% CO2. The human c-Src promoter-CAT construct, containing the entire c-Src promoter region and a small portion of intron 1 sequence, was a gift of L. Lania (University of Naples “Federico II,” Naples, Italy).

**Oligonucleotides**—Table I lists the synthetic oligonucleotides that were used in these experiments. All of the TFOs used in this study were triplex-forming oligonucleotides (TFOs), which were able to specifically target the Pu-Py tracts in the c-Src promoter and inhibit transcription in vitro. In addition to expanding our understanding of c-Src gene regulation and genes controlled by similar sequence motifs, this work also suggests a potential therapeutic approach to down-regulating c-Src in diseases related to c-Src overexpression.

| Oligonucleotide                  | Sequence                                    |
|----------------------------------|---------------------------------------------|
| Sp1 (ds)                         | 5'-ATTTCGATCGGGCGGGGGGGGGGGCGA
gA                      | 5'-GATCGAATCGAGCGGGGGGGGGGGGGAG |
| Mut GC1 primer                  | 5'-GGAGTGGAGAAGGGGCTCTG                    |
| Mut GA2 primer                  | 5'-GCCCTTCTGAGCGGGGGGT                    |
| TC1 CT                         | 5'-GGCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC|
FIG. 1. Schematic of the human c-Src promoter region. A, the positions of the two Sp1 consensus binding sites (GC1 and GA2) and the three Pu:Py tracts (TC1, TC2, and TC3) are shown. The boundaries of the Src-CAT constructs 0.38Src-CAT and 0.20Src-CAT are also shown, as well as restriction sites used to generate fragments A and B used in EMSAs. B, wild-type and mutated base pairs for each nuclear factor binding site are listed. Mutated bases are shown in bold, and the conserved Sp1 binding sites (CTTCC and CTTTC) are underlined.

several individual clones.

Transient Expression of Reporter Gene—Supercoiled plasmids were prepared for transfections using an EndoFree Plasmid Maxi Kit (Qiagen). For SL2 cells, CAT expression vector 0.38Src-CAT (10.0 μg), p97b (10 μg), and pPacSp1 (0.1 μg) were typically co-precipitated with calcium phosphate and applied to 5 × 10^5 cells/plate in duplicate. For 10T1/2 and SW480 cells, CAT expression vector (3.0 μg) and pCH110 (10.0 μg) were mixed with 172 μl of OptiMEM (Life Technologies, Inc.) and 20 μl of Superfect (Qiagen). Following a 15-min incubation at room temp, 1200 μl of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and appropriate antibiotics were added to the DNA-Supercoiled complexes, and applied to duplicate in wells of a 6-well plate (1.5 × 10^5 10T1/2 cells or 3.75 × 10^5 SW480 cells/well). Cells were harvested 48 h later with the provided lysis buffer, and protein levels determined using Bio-Rad reagent. β-Galactosidase activity was determined as described previously (24), and CAT protein levels quantified using a CAT ELISA kit (Roche Molecular Biochemicals). Reported CAT levels are all corrected for transfection efficiency, and were repeated at least three times in duplicate. For triplex inhibition studies, 200–500 nMolar excess of TFO over reporter plasmid concentration was first preannealed to the plasmid in a 40-μl reaction containing 10 μg MgCl2, 90 μg TFO, and 90 μM Tris. The reactions were heated to 65°C for 10 min and left at room temperature for at least 2 h. The annealing reaction was then transfected in duplicate to six-well plates as described above.

RESULTS

The c-Src Promoter Binds the Sp Family of Transcription Factors—The isolation and identification of the c-Src promoter has been previously reported (20, 22). Construction of a series of 5′ promoter-CAT deletion plasmids identified a 180-bp region located between 0.38Src-CAT and 0.20Src-CAT (Fig. 1A) that was required for full promoter activity in mouse fibroblast and several human cell lines (20).1 We therefore began our studies by examining the ability of nuclear extracts from both human and mouse 10T1/2 cell lines to form specific complexes with DNA probes derived from this promoter region. Digestion of the promoter with Nar I and Bss HII generated two fragments spanning the 180 bp of interest (Fig. 1A), which, when used as probes, formed three DNA-protein complexes (Fig. 2, A and B). For the relative intensity of the complexes was observed to be very similar between human and mouse cells (data not shown). Due to the high GC content (82%) of this region of the promoter, it was not surprising to find two strong consensus binding sites (GC1 and GA2 at positions −608 to −617 and −471 to −481, respectively) for the transcription factor Sp1 (G/T(C/G)/A/G(C/A)(C/T)(G/A)(G/A)(C/T)) (25) (Fig. 1B). To determine if the bandshifted complexes were indeed Sp family-related, a series of competition experiments were carried out. The DNA-protein complexes formed with both fragments A and B were successfully competed with an excess of double-stranded oligonucleotide containing a canonical high affinity site for the Sp1 transcription factor (Fig. 2, A and B). However, an oligonucleotide containing the GC-rich AP2 transcription factor consensus binding site failed to compete (Fig. 2, A and B).

Since the Sp family comprises several similar proteins (Sp1, Sp3, and Sp4), each capable of binding the same consensus site (26), we performed EMSAs using antibodies specific to the different Sp family members. Using both fragments A and B, the addition of anti-Sp1 and anti-Sp3 antibodies to the band-shift reaction resulted in the loss of one band with anti-Sp1, two bands with anti-Sp3, or all three bands with both antibodies (Fig. 2, C and D). Purified recombinant Sp1 formed a complex that co-migrated with the band recognized by anti-Sp1 (Fig. 2C, right lane). The apparent decrease in intensity of the remaining bands with anti-Sp1 was not a consistent observation. It therefore appears that these three complexes can be accounted for by just two Sp family members. To confirm that the regions GC1 and GA2 were truly responsible for complex formation, the sites were mutated as described under “Experimental Procedures.” EMSAs using the mutated fragments showed the complete loss of complex formation with Sp1 and Sp3 (results not shown). It was therefore concluded that these two sites in the c-Src promoter were responsible for interacting specifically with Sp family members. Occasionally we observed a slower migrating complex that disappeared with anti-Sp3 antibody (Fig. 2, band noted by asterisk). We are uncertain as to the identity of this factor; however, it could be the result of either Sp3 dimers or Sp factors binding to other low affinity Sp1 sites present within each fragment.

Sp1, but Not Sp3, Is Capable of Transactivating the c-Src Promoter—Our next objective was to determine if Sp family members were able to influence c-Src promoter activity. However, the vast majority of mammalian cells contain endogenous Sp1 and Sp3, making transfection experiments using exogenous Sp1 and Sp3 difficult to interpret. Therefore, to determine the effects of Sp family members on c-Src promoter activity, transfection experiments were performed in the Drosophila SL2 cell line, which lacks endogenous Sp1. Transfection of 0.38Src-CAT wild-type constructs into SL2 cells alone showed no detectable CAT expression. However, when co-transfected with the Sp1 expression vector, pPacSp1, the c-Src promoter was highly activated in a dose-dependent manner (Fig. 3A and results not shown). The construct 0.20Src-CAT, lacking the
GC1 and GA2 binding sites (but containing several weaker Sp1 motifs), was transactivated by Sp1 to less than 10% of the level of 0.38Src-CAT (Fig. 3A). Since protein/DNA interactions at GC1 and GA2 consist of Sp1 as well as Sp3 members, we investigated the role of Sp3 on c-Src transcription. In SL2 cells, Sp3 alone was unable to transactivate c-Src under any condition tested. However, when increasing concentrations of Sp3 were expressed with constant amounts of 0.38Src-CAT and pPacSp1, Sp3 could repress Sp1-dependent transactivation of the c-Src promoter in a dose-dependent manner (Fig. 3B). It therefore appears that Sp3 can negatively effect Sp1-mediated transactivation of the c-Src gene. We concluded that c-Src promoter activity was Sp1, but not Sp3 dependent in this experimental system.

The GC1 and GA2 Sites Are Critical for c-Src Expression—We next investigated the relative contribution of the various Sp1 binding sites to c-Src transactivation by constructing a number of mutant 0.38Src-CAT vectors as described under "Experimental Procedures." The mutant constructs were then co-transfected with pPacSp1 into SL2 cells. Individual mutations in GC1 and GA2 reduced promoter activity to about 60% of wild-type (Fig. 3A). Mutations in both GC1 and GA2 together further reduced transactivation to about 30% of wild-type. It therefore appeared that these two sites could account for the bulk of Sp1-mediated transactivation in this system. Since our interest is in the transcriptional regulation of c-Src in human cells, the effects of these mutations were next examined in a human cell line. When the wild-type and mutated 0.38Src-CAT constructs were transfected into human adenocarcinoma (SW480) cells, similar results to the SL2 system were observed (Fig. 3C). Together, these results show that Sp1, through its interaction at two sites, is required for full c-Src transcriptional activation.

The Pu:Py Tracts Confer S1 Sensitivity and Are Also Required for Optimal Transcriptional Activity—In addition to the small Pu:Py tract overlapping the Sp1-GA2 binding site, the c-Src promoter contains three other longer Pu:Py sequences located between regions −515 and −391 (Fig. 1). Pu:Py sequences have the ability adopt non-BDNA conformations known as H-DNA, a structure consisting of triple-stranded and single-stranded regions of DNA (27). H-DNA motifs can be assayed by their hyperreactivity to S1 nuclease (28). It was concluded from these results that the presence of Pu:Py tracts within the c-Src promoter could confer a non-BDNA (probably H-DNA) structure containing a single-stranded region. To further investigate the significance of these Pu:Py tracts, we engineered a series of Pu:Py deletion mutants in the basic 0.38Src-CAT vector, as described under “Experimental Procedures.”
dures," and tested their activity following transfection. Interestingly, we found individual deletions in TC1, TC2, and TC3 reduced CAT activity to 35%, 55%, and 65% compared with wild-type, respectively, even in the presence of the Sp1 binding sites GC1 and GA2 (Fig. 4C). We concluded that these Pu:Py tracts were also essential for full activity of the c-Src promoter.

**A Novel Nuclear Factor Interacts with the c-Src Pu:Py Tracts**—We next investigated potential protein/DNA interactions at these Pu:Py sites by EMSAs using nuclear extracts from both human and mouse 10T1/2 cells. A major complex was observed using 32P-labeled synthetic double-stranded oligonucleotides to double-stranded TC1 and TC2, but not with TC3 (Fig. 5A). Examination of the sequences of each Pu:Py tract revealed that the sequence CTTCC was present twice in TC1 and once in TC2, but was absent in TC3 (Fig. 1B). A single base pair substitution in both of these sites in TC1 (CTTCC to CTTTC) completely abolished the binding of this factor (Fig. 5A), while maintaining the integrity of the pyrimidine tract. A similar mutation in the single site in TC2 also prevented any complex formation (results not shown). Competition experiments demonstrated that unlabeled double-stranded TC1 and TC2 oligonucleotides could effectively compete away the binding of this factor, while the TC1mut or Sp1 double-stranded oligonucleotides failed to do so (Fig. 5B). The nonspecific band in Fig. 5B was an inconsistent observation with certain nuclear extracts, and could be competed away by dissimilar oligonucleotides such as Sp1 (data not shown).

Since our S1 experiments had demonstrated that this region of the c-Src promoter is capable of adopting structures that are partially single-stranded, we also examined potential complex formation using 32P-labeled single-stranded probes in EMSAs. A robust bandshift was observed with probes derived from the pyrimidine strand of TC1 and TC2, while probes from the TC3 pyrimidine strand appeared to produce a bandshift consisting of more than one complex. Probes, on the other hand, derived from the corresponding purine strands showed significantly weaker interactions (Fig. 6A). For these reasons we have named this factor SPy (Src pyrimidine binding factor). SPy was effectively competed away using a molar excess of unlabeled pyrimidine strands, including unlabeled TC1mut CT (Fig. 6B). Most significantly, SPy was also competed away using excess unlabeled double-stranded TC1 and TC2 but not the mutated double-stranded form of TC1 (Fig. 6B). The reciprocal competitions also held true, and the complex formed with 32P-labeled double-stranded TC1 was competed away with unlabeled sin-
gle-stranded pyrimidine oligonucleotides but not purine oligonucleotides (Fig. 6B). The most logical conclusion from these experiments is that Sp3 is a factor with a double-stranded binding specificity dependent on a CTTCC motif, which is also capable of binding single-stranded pyrimidine strands with a more relaxed sequence requirement.

**Sp3 and Sp1 Act Together in the Transactivation of the c-Src Promoter**—To determine the consequence of mutations that abolish Sp3 double-stranded binding, 0.38Src-CAT vectors containing the Sp3 mutations in TC1 and TC2 were generated as described under “Experimental Procedures.” In addition, each Sp3 mutation was introduced into 0.38Src-CAT constructs already containing the Sp1 mutations at GC1 and GA2, producing 15 possible combinations of mutants. The constructs were then transfected into mouse 10T1/2 cells and reporter CAT levels assayed as described under “Experimental Procedures” (Fig. 7). The Sp3 mutation in TC1 alone produced a 25% decrease in activity compared with wild-type, while the comparable TC2 mutations reduced CAT levels to about 50%. Interestingly, mutations in the TC1 Sp3 and the Sp1 GC1 sites together inhibited CAT activity significantly more than either of the individual mutations (Fig. 7). In addition, a similar cooperative effect was observed between the TC1, TC2, and GA2 triple mutant. Any one or two of these mutations showed CAT activity of 70–75%; however, the combination of all three mutations resulted in promoter activity near 40%. This cooperativity was not observed with any other combinations of Sp1 and Sp3 mutants. Similar findings were observed when transfection of the mutants was performed in human SW480 cells. We conclude that full c-Src transcriptional activity is dependent on Sp3’s ability to bind its double-stranded targets in TC1 and TC2 in coordination with the binding of Sp1 at GC1 and/or GA2, and that these Sp3-Sp1 interactions can regulate transcription cooperatively.

**Inhibition of c-Src Promoter Activity Using Triplex-forming Oligonucleotides**—We have previously reported on the design of a series of purine antiparallel (Aap)-based TFOs targeted to each of the c-Src Pu:Py sequences (22). It was shown that all four TFOs could specifically interact with their respective targets with nanomolar affinities. In this study we describe the ability of these TFOs to down-regulate c-Src promoter activity. Each of the TFOs was pre-annealed to 0.38Src-CAT, and the constructs transfected into 10T1/2 cells. An oligonucleotide incapable of binding to the promoter was also included as a control, as well as a reporter construct lacking the TC2 TFO target sequence (0.38Src-CAT ΔTC2), incapable of binding TC2Aap. All four TFOs (TC1Aap, TC1.1Aap, TC2Aap, and TC3Aap) could inhibit c-Src promoter activity (Fig. 8); however, TC1.1Aap (targeted to the GA2 binding site) and TC2Aap were most effective. These results further demonstrate the importance of these Pu:Py tracts in c-Src regulation and suggest that a TFO-based methodology may be a realistic and very specific approach to inhibiting c-Src overexpression.

**DISCUSSION**

Although the activation and/or overexpression of pp60c-src has been implicated in many cellular processes, the mechanisms concerning transcriptional regulation of the c-Src gene are completely unknown. Our approach, therefore, has been to focus on c-Src transcriptional regulation by initially examining the basal promoter in detail. Through EMSA analysis, two Sp1/Sp3 binding sites, GC1 and GA2, were identified in a region of the promoter previously determined to be required for transactivation. Mutational analysis confirmed that these sites are responsible for Sp1/Sp3 binding, and transfection studies in Drosophila, mouse, and human cells demonstrated that these regions are critical for promoter activity. It was also observed that mutations in both GC1 and GA2 together resulted in greater than 75% decrease in promoter activity, suggesting that these sites may be acting cooperatively. Taken together, these data strongly implicate these two Sp1 binding sites in the regulation of the c-Src promoter. Sp3 was shown to negate Sp1 transactivation of the c-Src promoter, as has been observed in a number of other systems (29). While it is clear that Sp3 can act through direct competition for the same binding site, it also appears to contain an active repression domain (30, 31). Interestingly, there are also an increasing number of reports that Sp3 can transactivate genes using the identical SL2 cell line and expression vectors described in this report (32–34). To date, it appears that the ability of Sp3 to either activate or repress transcription is purely a matter of promoter context and is thus difficult to predict. The observation that Sp3 does not activate c-Src transcription and inhibits Sp1-mediated
transactivation suggests the Sp1:Sp3 ratio within a cell could influence c-Src expression levels.

The most intriguing aspect of the c-Src promoter is the presence, size, and strategic location of three perfect Pu:Py tracts. Many of the multiple transcription start sites mapped in the gene arise either within or close to these tracts (20). Similar Pu:Py sequences are critical components of other promoters and often display the ability to adopt H-DNA structures composed of an intramolecular triplex and single-stranded DNA (27). The S1 sensitivity observed in the TC2-TC3 region of the c-Src promoter suggests that, under appropriate conditions, this area is capable of adopting an H-DNA conformation. Furthermore, deletion of the individual Pu:Py tracts had a significant impact on promoter activity. This is a notable finding, considering the GC1 and GA2 Sp1 binding sites were both intact in these constructs, demonstrating that these Pu:Py tracts are also essential for full promoter activity. However, since internal deletions disrupt the normal architecture and spacing within a promoter, we decided to examine the ability of these individual tracts to bind sequence-specific nuclear factors. A factor with novel double- and single-stranded DNA binding characteristics was identified. This factor, which we named SpY, recognizes and binds specifically to the double-stranded CTTCC motif shared by TC1 and TC2, as well as to the single-stranded pyrimidine tracts of all three Pu:Py tracts. We believe this factor shares both double- and single-stranded binding properties, based on the following reasons. First, the relative mobility and diffuse appearance of this complex following electrophoresis was very similar with both double and single-stranded probes. Second, SpY bound to the TC1 double-stranded probe could be competed for with pyrimidine (including the mutant forms) but not purine single-stranded oligonucleotides. Conversely, SpY bound to single-stranded pyrimidine probes and could be competed for with wild-type double-stranded TC1 or TC2 but, importantly, not the mutated forms. Competition experiments using excess double-stranded TC2 unlabeled oligonucleotide successfully compete for SpY binding; however, bands migrating at a higher molecular weight seem to appear. This phenomenon, we feel, is due to the abundance of other pyrimidine binding factors with a variety of affinities within the cell, which, after the titration of SpY with unlabeled competitor, can bind to the labeled probe with lower affinity. Third, a subtle difference in the sequence requirements for SpY binding for single- versus double-stranded probes was observed. A strong requirement for the core CTTCC motif when binding to double-stranded probes was noted, and binding was abolished when this motif was mutated to CTTTC. Binding and competition experiments demonstrated SpY was also able to bind single-stranded TC3 and the mutated single-stranded pyrimidine versions of TC1 and 2. Interestingly, the resulting mutated binding motif of TC1 and 2, CTTTCC, is present in the wild-type TC3 sequence. This provides a rationale to explain why SpY binds TC1 and TC2 in both single- and double-stranded forms but only binds the single-stranded form of TC3.

Transfection of CAT vectors containing SpY mutations alone or in combination with Sp1 mutations confirmed that SpY binding appears to be critical for full promoter activity and is therefore a positive regulatory factor. Interestingly, the most dramatic reduction in promoter activity involving a SpY mutation was seen when a construct containing both a TC1 and GC1 mutation was assayed. Here activity was reduced by 70%, suggesting these two individual sites also act in a cooperative or additive manner, similar to the results seen with the GC1 and GA2 Sp1 sites. In contrast, no single additional mutation decreased the activity of the vector with a TC2 SpY mutation. Taken together, these results confirm the individual importance of the GC1, GA2, TC1, and TC2 sites and suggest a complex series of interactions might occur, which will require further experiments to elucidate. In addition, it is important to note the SpY mutations described here interfered only with this factor’s ability to interact with double-stranded targets. Further mutations that destroy SpY’s ability to interact with TC1, TC2, and TC3 in a single-stranded manner could also prove to be most informative. The association of transcription sites with the Pu:Py tracts (35) suggests they may be acting as initiator (Inr) elements, possibly similar to the atypical Inrs detailed in the majority of ribosomal protein (36) as well as other promoters (37). These Inrs are characterized as a 12-20-bp Pu:Py tract often buried within a GC-rich sequence. Based on the location of the Pu:Py tracts and the unusual binding characteristics of SpY, we propose the following working model; SpY initially binds to its specific double-stranded target sequence in TC1 and 2 and is either involved in local DNA melting or stabilization of the resulting “bubble” by virtue of its specific pyrimidine-strand binding properties. The subsequent entry of the RNA polymerase (possibly aided by SpY) and transcription from the opposite purine-rich strand is activated by Sp1 and possibly other factors (including SpY). Thus, SpY may represent a novel Inr binding protein. Since we have shown that the promoter is probably capable of adopting an H-DNA conformation (27), it is also formally possible that SpY may be involved in stabilizing or promoting such a structure by virtue of its single-stranded binding characteristics, which may then influence promoter strength and transcription initiation.

In conclusion, we report here the first detailed description of the identity of SpY is currently unknown; however, over the last several years a number of factors with both double-stranded and single-stranded binding characteristics have been described, including testis-specific TTF-D (38), THZif-1 (39), estrogen-responsive element-binding factor (40), and a factor(s) binding to the lipoprotein lipase promoter (41). In addition, a growing list of factors that bind to pyrimidine single-strands has been characterized including chk-YB-1b (42), hnRNAP K (43, 44), and PTB (45). None of these factors appear to have SpY-like characteristics, although Bayarsaihan et al. (46) have cloned a single-stranded DNA binding factor thought to be involved in the regulation of the chick a2(I) collagen gene promoter. The binding characteristics of the factor are very similar to SpY with the target sequence containing two copies of the CTTCC motif. We have cloned the human homologue of this factor and are currently testing both its binding and antigenic similarity to SpY.

This current study demonstrates the importance of four Pu:Py tracts (TC1, TC2, TC3, and the shorter tract overlapping the GA2 site) in the regulation of c-Src transcription. Such sequences can form triple-helical structures with appropriate single-stranded oligonucleotides through Hoogsteen base pairing (47). Considerable excitement has been generated over the possibility of an antigenic therapy based on such agents targeted to essential Pu:Py genes such as c-myc (48), c-fos (49), bel-2 (50), and c-Ki-ras (51). We have previously described the characterization of a series of purine based TFOs designed to bind the essential tracts described here (22). In this report we have demonstrated that these same TFOs are able to significantly inhibit the activity of the promoter when annealed to reporter plasmids. The inhibitory mechanism of these TFOs could involve the interference of transcription initiation processes, for example, the binding of transcriptional regulators such as Sp1 and SpY required for proper initiation complex formation, as well as the inhibition of RNA polymerase elongation (52).

In conclusion, we report here the first detailed description of
a c-Src promoter, which will form the framework for future work aimed at elucidating c-Src differential gene expression. In addition, the characterization of the SPy factor will be important, not only for c-Src biology, but for other genes controlled by similar means. The strong dependence of c-Src promoter activity on the described Pu-Py tracts and our demonstration of TFO-mediated inhibition suggests the possibility of a therapeutic approach to specifically target c-Src in cancer of the colon and breast where c-Src overexpression or activation has been documented. We are currently assessing a series of phosphorothioate-modified TFOs for their ability to down-regulate c-Src gene expression in c-Src-overexpressing cell lines.

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