Identification of a Novel Repressive Element in the Proximal lck Promoter*

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The T-cell-specific protooncogene lck, a src-related tyrosine kinase, is under the control of two promoters that give rise to transcripts differing only in their 5'-untranslated regions. The distal promoter is transcriptionally active in both peripheral and thymic T-cells, whereas expression of the proximal promoter is highest in developing thymocytes. The proximal promoter has also been shown to be selectively activated in a number of colon carcinoma cell lines. Approximately 570 base pairs of proximal promoter sequence is required for expression in both T-cells and colon carcinoma cell lines. Protein binding studies were initiated with an oligonucleotide homologous to a region that, when deleted, causes an increase in promoter activity in transgenic animals. Two proteins with approximate molecular masses of 35 and 75 kDa were found to bind to this region as determined by UV cross-linking studies. Absence of specific protein binding is correlated with a high level of proximal promoter expression. Competitive gel retardation analysis identified a 9-base pair binding site within the proximal lck promoter that is necessary for repression of transcription in cells that contain specific binding activity. Mutants of this binding site do not repress transcription. Repression does not occur in a cell line that expresses lck and lacks this activity. These data support the hypothesis that activation of lck transcription in colon carcinoma is due, at least in part, to the loss of a transcriptional repressor.

In T-cells, expression of lck, a src-related tyrosine kinase, is driven by two promoters located approximately 35 kilobases apart that give rise to transcripts differing only in their 5'-untranslated regions (1). Transgenes containing lck proximal promoter sequences are actively transcribed in thymocytes of transgenic animals, whereas in similar studies the distal promoter can support transcription in both thymocytes and peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells (2, 3). Therefore, these two apparently independently acting promoters may lead to both the peripheral T-cells.
Cloning and Site-directed Mutagenesis—All subclones used in this study were derived from a 1.9-kilobase BamHI-EcoRI upstream Ick fragment, provided by R. Benarous (22). A 650-bp PstI-BamHI fragment was subcloned into Bluescript KS+ by standard methods. Site-directed mutagenesis was performed as described (23). Both wild type and mutant fragments were subcloned into pCAT Basic (Promega) by standard methods (20).

Concatamerization of both the binding site and a corresponding mutant was performed by donning double-stranded oligonucleotides with flanking BglII and BamHI sites into a pCAT vector constructed by H.L. Grimes. By utilizing an EcoRI site within the pCAT sequence and sequential BamHI/EcoRI and BglII/EcoRI ligations, the binding site was duplicated and doned into a TKCAT vector, also provided by H. L. Grimes. All plasmids were sequenced using Sequenase in a procedure described by the manufacturer (U.S. Biochemical Corp.).

Transfections—All cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum with the exception of HeLa, which was maintained in Dulbecco’s minimal essential/F12, 5% fetal bovine serum. All cells were transfected using Lipofectin (Life Technologies, Inc.) by procedures described by the manufacturer. Cells were cotransfected with 0.25 μg of a Rous sarcoma virus-luciferase vector kindly provided by E. Lai. Each plasmid used for transfection was titrated to insure that expression was a linear function of DNA concentration and that no competition between the promoters occurred. Cells were lysed after 24 h by using 75 μl of 1 × reporter lysis buffer (Promega) containing 0.2 M phenylmethylsulfonyl fluoride. A portion of the lysate was assayed for luciferase activity using a Berthold Lumat luminometer. CAT assays were performed as described (24) with minor modifications. Briefly, 50 μl of lysate was incubated with a solution containing 25 μCi of [3H]acetyl-coenzyme A, 5 μg acetyl-coenzyme A, and 0.8 μg chloramphenicol for 1 h at 37 °C. This mixture was extracted with 1 ml of ethyl acetate, dried by lyophilization, resuspended in 30 μl of ethyl acetate, spotted onto TLC plates (Whatman), and chromatographed in 95% chloroform, 5% methanol. All CAT assays were quantitated using a Fuji phosphor-imaging system.

Propriety initiation of transcription of the TKCAT templates was determined by RNase protection analysis (12) of total RNA isolated from transfected cells. The Riboprobe was comprised of sequences between –109 and +52 of the thymidine kinase (TK) promoter.

RESULTS

Gel Retardation Analysis of Protein Binding to –520 to –460 of the Proximal Ick Promoter—Deletion analysis of the proximal Ick promoter has allowed the identification of a region that, when deleted, causes a decrease in transcription of both reporter genes and of Ick in transient assays and in transgenic animals (2, 17). To investigate the potential importance of this region to the expression of Ick in colon carcinoma, gel retardation experiments were performed with an end-labeled 60-bp oligonucleotide representing sequences from –520 to –460 of the proximal promoter and nuclear extracts isolated from a variety of cell lines. The results of one of these experiments are shown in Fig. 1. In panel A nuclear extracts from HT29, a well differentiated primary colon carcinoma cell line that does not express Ick, were analyzed. A number of complexes are observed in the lane marked 0 (no competitor). To assess the specificity of the complexes, competition analysis was performed using the unlabeled –520 to –460 oligonucleotide in 10, 100, and 1000-fold molar excess (lanes 3–5) as well as a 1000-
fold molar excess of a nonspecific oligonucleotide (lane N). 1000-fold molar excess of the specific oligonucleotide reduces the level of two of the complexes (arrows). The upper complex is occasionally resolved into two closely migrating complexes that show the same competition pattern (see below). In panel B, the experiment was repeated with nuclear extracts isolated from COLO205, an undifferentiated human colon carcinoma cell line that expresses high levels of Ick. As shown in the left side of panel B, COLO205 nuclear extracts do not specifically shift this oligonucleotide. COLO205 extracts do specifically shift an oligonucleotide containing a consensus AP1 site as shown in the right portion of panel B. A summary of the results of the gel retardation experiments performed in several cell lines is shown in Table I. Protein binding is tabulated as a function of Ick expression, as determined by RNase protection analysis (24, 31) and quantitated by phosphor imaging. The two cell lines that express high levels of Ick (CEM, a human T-cell leukemia cell line, which expresses high levels of Ick from both the proximal and distal promoters (24, 31), and COLO205) do not show specific binding to the −520 to −460 oligonucleotide. Three cell lines that do not express Ick (HT29, Hela, a well differentiated cervical carcinoma cell line, and T84, another well differentiated colon carcinoma cell line) show specific binding to the −520 to −460 oligonucleotide. SW620, a human colon carcinoma cell line derived from a lymph node metastasis expresses small amounts of Ick and also specifically binds this oligonucleotide. Thus, in the two cell lines tested that express high levels of Ick, specific protein binding to the −520 to −460 oligonucleotide is not detected.

Mapping of the Protein Binding Sites in HT29 and SW620—A competitive gel retardation analysis was used to map the binding site of this protein(s). A series of wild type and mutant oligonucleotides representing different regions of the −520 to −460 oligonucleotide (Fig. 2A) were added in 1000-fold molar excess as competitors in the gel retardation assay. The first five lanes of Fig. 2B demonstrate the specific binding to the original −520 to −460 oligonucleotide. Neither the most 5′ 30-bp oligonucleotide, nor the middle 30-bp oligonucleotide competed for binding of the protein. However, the most 3′ 30-bp oligonucleotide competed with the original oligonucleotide, localizing the binding site to this region. The 3′ oligonucleotides containing 6 bp changes (Fig. 2A) were then analyzed similarly. As shown in Fig. 2B, oligonucleotides 2 and 3 did not compete, further narrowing the binding region. Oligonucleotides, 4, 5, 6, and 7, with 3 bp changes were used as competitors in this analysis (Fig. 2, A and C). Of these only oligonucleotides 5 and 6 did not compete, thus mapping the binding site to the sequence TTTCATCAG, represented in boldface letters within the original −520 to −460 oligonucleotide in Fig. 2A. Identical results were obtained using nuclear extracts isolated from both HT29 and SW620. As described above, the upper complex shown in Fig. 2B is resolved into two complexes as seen in Fig. 2C; the significance of this is, at this time, unknown. Therefore, by competitive gel retardation the binding site was mapped to positions −474 to −466 of the Ick proximal promoter.

UV Cross-linking Defines Two Proteins of Different Molecular Weight—To determine the approximate molecular weight of proteins that bind to the oligonucleotide, a −520 to −460 probe was used in UV cross-linking studies. HT29 nuclear extracts were incubated with a labeled probe, exposed to a UV transilluminator, and immediately subjected to SDS-polyacrylamide gel electrophoresis. The results of this analysis are shown in Fig. 3A. Three protein-DNA complexes migrating at 35, 65, and 75 kDa are visible in the lane containing no competitor (lane 0). Upon competition with either 100- or 1000-fold molar excess of

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**Table I**

| Cell line     | Derivation* | Ick expressionb | Protein binding |
|---------------|-------------|----------------|-----------------|
| CEM           | T-cell leukemia | 1191.0         |                |
| SW620         | Colon-lymph node | 59.6           |                |
| HT29          | Colon-primary  | <0.01          |                |
| Hela          | Cervical carcinoma | <0.01          |                |
| T84           | Colon-lung metastasis | <0.01          |                |

* Each cell type and the site of derivation is shown.  

b Ick expression was determined by RNase protection analysis and is expressed in phosphor imaging units/mm².
Transcriptional Repression of the lck Promoter

Fig. 3. Molecular characterization of proteins binding the −520 to −460 oligonucleotide. Panel A shows a 10% SDS-polyacrylamide gel electrophoresis gel indicating the relative migration of the protein-DNA complexes from either HT29 or SW620 nuclear extracts that bind to a doubly labeled probe corresponding to −520 to −460 of the proximal promoter after UV cross-linking. The molecular weight marker is listed at the left. The lane marked 0 is no competitor added; 100 × and 1000 × indicate where either 100- or 1000-fold molar excess of specific competitor was used. Panel B shows the migration patterns of both the upper and lower protein-DNA complexes excised from gel retardations using either HT29 or SW620 nuclear extracts.

The Binding Site Is Required for Transcriptional Repression—To begin to determine the functional significance of the binding site in the lck proximal promoter, a 650-bp PstI-BamHI fragment containing 570 bp of promoter sequence was cloned into a Basic CAT vector and cotransfected with a Rous sarcoma virus-luciferase reporter gene into SW620. HT29, HeLa, and COLO205 cells. Resultant CAT activity for SW620 and HT29 is shown in Fig. 4. These experiments demonstrated that the fragment containing 570 bp of lck promoter sequence was sufficient for expression of CAT activity in SW620 and COLO205 but not in HT29 or HeLa, in which it was only slightly active. Transient assays were performed using the wild type proximal lck promoter and a proximal promoter carrying a mutation in the binding site (GGGCATCAG), which abolished protein binding in gel retardation experiments. The results of a representative experiment are shown in Fig. 4. SW620 expresses high levels of CAT activity from the wild type lck promoter, while in HT29 this promoter is only slightly active. These data suggest that the proximal lck promoter is activated in trans in SW620. The mutation in the lck promoter has no detectable effect on transcription in SW620 and shows a 2-fold increase in transcription over the wild type promoter in HT29 cells.

Since loss of transcriptional repression at this site may not be sufficient to fully activate this promoter in HT29, the effect of the site on a minimal promoter was investigated. An oligonucleotide containing the binding site was cloned in one or two copies just upstream of the herpes simplex virus TK minimal promoter in a CAT-containing expression vector (Fig. 5A) and these constructs were used in transient transfection assays. The results of a representative experiment for HT29 and SW620 are shown in Fig. 5, B and C, respectively. Comparison of CAT activity driven either by the TK promoter alone or with one or two sites just 5′ to the TK promoter reveals the ability of this element to completely abolish transcription from this minimal promoter element in HT29 (Fig. 5A), and identical results were obtained in HeLa (data not shown). These experiments were repeated in SW620, and although complete repression was not documented, this site caused an 8-fold reduction in promoter activity in this cell line. As a control, a mutant binding site, GGGCATCAG, was also cloned upstream of the TK promoter, and the transient assays were repeated. For both HT29 (Fig. 5B) and SW620 (Fig. 5C) a mutant binding site that did not compete for protein binding in gel retardation analysis had no effect on the transcriptional activity of the TK promoter.

Fig. 4. The effect of a site-directed mutation in the mapped binding site on the proximal lck promoter. Comparison in HT29 and SW620 of promoter activity of the wild type and mutant measured by CAT activity, quantitated by phosphor imaging and expressed as relative to the luciferase activity obtained by cotransfection of Rous sarcoma virus-luciferase. Transfections were done in triplicate in three independent experiments. A representative experiment is shown.
in either of these cell lines. To insure that the CAT activity measured was due to accurate transcription initiation in the TK promoter, RNase protection analysis was performed using a probe containing the TK promoter. Proper initiation of transcription of the TKCAT construct was observed (data not shown).

Since this sequence acts to repress transcription in cells containing specific binding activity, transfection studies were performed in a cell line lacking this activity. As shown in Fig. 6, the sequence does not suppress the TK promoter in COLO205, a cell line that expresses high levels of the proximal lck transcript. Proper transcript initiation was also observed in this cell line (data not shown). Thus, repression of promoter activity seems to require both the intact binding sequence and the binding activity.

**DISCUSSION**

In this study we have defined a sequence in the human lck proximal promoter located at positions −474 to −466, which acts as a strong repressor of transcription. The approximate molecular masses of proteins that specifically bind this sequence are 35 and 75 kDa. The binding activity was detected in four human tumor cell lines; three of these do not express lck (HT29, T84, and HeLa), and the other expresses nominal amounts (SW620). Specific binding was not detected in two cell lines that express high levels of proximal lck promoter transcripts. This sequence acted to repress transcription of a heterologous promoter in cell lines that contained the binding activity but had no effect on the promoter in a cell line lacking this activity. These data suggest that activation of the proximal lck promoter is at least partly due to a loss of transcriptional repression mediated by the loss of specific protein binding to this 9-bp sequence.

These findings are consistent with results from transgenic mouse experiments, which demonstrated protein binding to an analogous 50-bp region in the murine proximal promoter with nuclear extracts from spleen, a tissue in which this promoter is inactive, and a lack of binding in cells expressing the transgene (2). Comparisons between mouse and human have demonstrated a high degree of sequence conservation in the proximal promoter element, indicating that this promoter may be similarly regulated in both species. This conservation also extends to the binding site mapped to −474 to −466 in this study (7/9-bp). These data indicate that the murine system may have a functionally similar transcriptional repressor.

Our data, which show measurable promoter activity using 570 bp of the human proximal promoter sequence, varies somewhat from previously reported transient assays in which, when using identical sequence, deletion to −512 was necessary to show modest promoter activity (17). In transgenic animals,
however, 584 bp of the murine proximal promoter sequence was sufficient for promoter activity (2). In this report CAT activity was measured rather than primer extension of transient β-globin mRNA, possibly allowing for the detection of lower amounts of promoter activity. The binding site located within the −520 to −460 oligonucleotide was mapped to the sequence TTTCATCAG at positions −474 to −466 by competitive gel retardation assays. A computer search of the Transcription Factor Data Base revealed no strong homology to known transcription factor binding sites. However, we noted some homology to the 3' portion of the composite binding site for NFAT within the interleukin-2 promoter, i.e. TTTCATCAG, the portion thought to bind Fos or Jun (25-27). The proteins detected in this study are unlikely to be identical to those in the T-cell NFAT complex, as neither an oligonucleotide containing an NFAT motif nor one containing an AP-1 motif competes in 1000-fold molar excess in a gel retardation assay (data not shown).

Although the region from −520 to −460 was shown to contain a repressive element (2) and gel retardation experiments correlated a lack of binding to this region with a high level of lck transcription, the portion thought to bind Fos or Jun (25–27). The proteins detected in this study are unlikely to be identical to those in the T-cell NFAT complex, as neither an oligonucleotide containing an NFAT motif nor one containing an AP-1 motif competes in 1000-fold molar excess in a gel retardation assay (data not shown).

To determine the effect of the binding site on a minimal promoter the binding site was cloned upstream of a TK promoter containing CAT vector and used in transient transfection assays. The binding site acted to completely abolish transcriptional activity of this minimal promoter in both HeLa and HT29. It is likely that other factors are required for full activation of this promoter.

This paper describes a novel binding site in the proximal lck promoter that acts as a strong repressor of transcription in cell lines that either do not express proximal lck transcripts (HT29 and HELA) or only express small amounts (SW620). The correlation of repressor activity, protein binding activity, and lck expression suggests that this repressor does regulate lck transcription. Whether expression of the wild type lck transcript in colon carcinoma regulates aspects of the transformed phenotype remains to be established. However, the loss of a transcriptional repressor may have more global regulatory effects in colon cancer. In addition, the proximal lck promoter is most active in developing thymocytes; the transcriptional repressor complex defined in this report may play an important role in the silencing of this promoter in peripheral T-cells. Purification and cloning of the binding proteins will allow further characterization of the function of this repressor.

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