STAT5a and STAT5b are two highly related transcription factors that control essential cellular functions. Several STAT5 targets are known, although it is likely that many remain uncharacterized. To identify a more complete set of STAT5-regulated genes, we used a modification of the chromatin immunoprecipitation procedure, which does not presuppose any information regarding these targets. Employing Ba/f3 cells in which STAT5 is activated by interleukin-3, we have identified novel STAT5 binding sites that may be regulatory regions for nearby genes. These sites are typically found far from transcription start sites, and most do not contain CpG islands, indicating that they are not in traditional promoter regions. Nonetheless, when the expression of genes near these STAT5 binding sites was examined, all were expressed in Ba/f3 cells, and most were modulated by interleukin-3. Furthermore, genes identified by this strategy show unique expression patterns in acute leukemias, tumors characterized by activated STAT5. Whereas both STAT5 isoforms bind to all promoters tested, STAT5a and STAT5b bound with different kinetics, suggesting that at least some of the differences between the functions of these two proteins are mediated by their DNA binding activity. Therefore, this method of transcription factor target identification represents an effective strategy to isolate transcription factor targets in an unbiased fashion, and it has revealed many novel STAT5-dependent regulatory regions outside of traditional promoters.

STAT1 proteins are a family of transcription factors that regulate genes important for growth, differentiation, and apoptosis (1, 2). Upon cytokine or growth factor stimulation, STATs become phosphorylated and translocate to the nucleus, where they bind to promoters of their target genes (3). In addition to essential functions in normal cells, STATs have been implicated in the pathogenesis of numerous solid and hematopoietic malignancies (4, 5).

STAT5 refers to two highly related proteins, STAT5a and STAT5b, which are encoded by separate genes. These proteins are more than 90% identical at the amino acid level (6). Despite their similarities, some functional differences exist between these two proteins. For example, there may be differences in the DNA binding affinities of STAT5a and STAT5b (7, 8), although this has not been uniformly seen (9). Mice lacking STAT5a or STAT5b show phenotypic differences in both mammary development (10, 11) and hematopoiesis (12–14). These studies indicate that there are differences between STAT5a and STAT5b that may result from either their expression patterns or differential target selection. Clarifying this point is an essential step in understanding the biology of STAT5.

Whereas there are genes known to be directly regulated by STAT5 (15–17), most STAT5 targets are unknown. The location of STAT5-dependent promoters has been shown to be close to the transcription start site, such as the STAT5 binding sites in the CIS and cyclin D1 genes (18, 19). However, the STAT5 binding site in the bcl-x promoter is found in the first intron (15, 20), and thus, it is likely that the STAT5 binding sites for other genes may be located in introns as well. To identify STAT5 target genes in an unbiased fashion, we utilized the technique of chromatin immunoprecipitation (ChIP), which is a method to identify DNA regions that are bound by a transcription factor in vivo. We combined ligation-mediated PCR with subtraction steps to isolate pure STAT5 targets. We present data showing that this strategy is efficient at identifying STAT5 targets in an unbiased fashion, and we show that the STAT5 binding sites are often in introns. We also show that STAT5a and STAT5b bind to all targets tested, although each isoform binds to any given promoter with different kinetics compared with the other isoform. This demonstrates that STAT5a and STAT5b have distinct differences in their promoter binding behaviors that may underlie their functional differences.

MATERIALS AND METHODS

Cell Lines—Ba/f3 cells were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and were maintained in RPMI supplemented with 10% fetal calf serum and 10% WEHI-3B conditioned media. WEHI-3B cells were obtained from ATCC (Manassas, VA).

Antibodies and Cytokines—Antibodies recognizing STAT5a (sc-1081) and total STAT5 (sc-835) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-STAT5b (71-2500 and 13-5300) was obtained from Zymed Laboratories Inc. (South San Francisco, CA). Phosphospecific anti-STAT5 (935–IL) was obtained from Cell Signaling (Beverly, MA). Recombinant murine IL-3 was obtained from R&D Systems (Minneapolis, MN). Normal rabbit IgG was obtained from Zymed Laboratories Inc. (South San Francisco, CA).

Cell Stimulations—Ba/f3 cells were washed twice and resuspended in RPMI containing 10% fetal calf serum. For Western blotting, immunoprecipitations, and ChIP analysis, cells were starved for at least 4 h. For reverse transcription-PCR (RT-PCR) analysis, cells were placed in 1 pg/ml IL-3 overnight, followed by starvation overnight. Cells were

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stimulated with 10 ng/ml IL-3 for 15 min for Western blots and immunoprecipitations and for 30, 90, and 150 min for ChIP. Stimulations for RT-PCR were performed for 2, 6, and 24 h.

RT-PCR—RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). RT-PCR was performed using a Superscript One-Step RT-PCR kit from Invitrogen. Reverse transcription was performed at 50 °C for 30 min followed by cycling 35 times of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Primer sequences are provided in the Supplemental Materials.

Immunoprecipitation and Western Blotting—2 × 10^7 cells were starved of IL-3 for 4 h. Half of the cells were left untreated, and half of the cells were stimulated with IL-3. Immunoprecipitations and Western blotting were performed as described (4).

Chromatin Immunoprecipitation—For each condition, 1.5 × 10^7 cells were used. Cross-linking was performed with 1% formaldehyde for 10 min at room temperature, followed by a quenching of formaldehyde using 0.125 M glycine. Cells were washed with PBS. Nuclei were isolated in 400 μl of cell lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% Nonidet P-40) containing phosphatase and protease inhibitors (sodium orthovanadate, phenylmethylsulfonyl fluoride, pepstatin, aprotinin, and leupeptin) and incubating for 10 min on ice. Nuclei were centrifuged at 2500 rpm in a refrigerated microcentrifuge and were lysed in 100 μl of nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors in ice for 10 min. Two drops of dilution buffer (20 mM Tris, pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.01% SDS) was added, and the chromatin was sheared by sonication to an average size of 500–1000 base pairs. Debris was removed by centrifugation, the supernatant was split into three aliquots, and 700 μl of IP dilution buffer were added to each aliquot. 2.5 μg of antibody were added, and the tubes were rocked overnight at 4 °C. 10 μg of packed packed protein A/G beads (Santa Cruz Biotechnology) were added, and the samples were rocked for several hours at 4 °C. The beads were then washed with IP wash I (20 mM Tris, pH 8.1, 1 mM EDTA, 0.5% SDS) 5 times for 10 min each at 4 °C, followed by one wash with IP wash II (10 mM Tris, pH 8.1, 1 mM EDTA, 0.25 μM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) for 10 min at 4 °C. Three washes with cold TE were performed. After the last wash, 200 μl of wash buffer 9–10× (25 mM Tris, 25 mM NaCl, 1 mM EDTA, 0.1% SDS) for 10 min at room temperature, 16 μl of 5 M NaCl were added, and the cross-links were reversed at 65 °C overnight. The DNA was purified using a Qiagen PCR purification kit from Invitrogen. Reverse transcription was performed at 4 °C. The beads were then washed with IP dilution buffer (20 mM Tris, pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.01% SDS) three times for 10 min each at 4 °C, followed by one wash with IP wash II (10 mM Tris, pH 8.1, 1 mM EDTA, 0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) for 10 min at 4 °C. Three washes with cold TE were performed. After the last wash, 200 μl of wash buffer 9–10× (25 mM Tris, 25 mM NaCl, 1 mM EDTA, 0.1% SDS) was added, and the beads were washed with IP wash III (20 mM Tris, pH 8.1, 1 mM EDTA, 0.25% Triton X-100). This solution was incubated at 95 °C for 10 min and then phenol/chloroform extraction, 10 μl of streptavidin solution (2 μg/μl) final concentration dissolved in TE with 0.15 mM NaCl were added and incubated at room temperature for 5 min. After phenol/chloroform extraction, 10 μl of streptavidin solution was added to the supernatant. After a 5-min incubation, three phenol/chloroform extractions were performed, and the DNA was precipitated with 0.2 M NaCl and 10 μg of glycogen. The precipitated DNA was resuspended in 20 μl of distilled H2O. Two additional washes were performed, and the subtracted DNA was ligated using the TA Cloning Kit (Invitrogen).

 Screening—Miniprep DNA was isolated using a Qiagen DNA Mini- prep kit. Sequencing was performed at either the Dana Farber Cancer Institute Core Facility or by Seqwright Corp. (Houston, TX). Sequences for these clones are provided in the Supplemental Materials.

RESULTS

STAT5 Activation in Ba/f3 Cells—We chose the IL-3-dependent Ba/f3 cell line as a model system to aid in the identification of STAT5 targets. IL-3 activates STAT5 in these cells, which leads to the increased expression of the prosurvival protein Bel-xl (24, 25). We first confirmed that both STAT5a and STAT5b are present in Ba/f3 cells and that both STAT5 isoforms are phosphorylated in response to IL-3. Immunoprecipitations of lysates from Ba/f3 cells were performed using antibodies specific for STAT5a or STAT5b. No phosphorylated STAT5a or STAT5b was seen in starved cells. However, phosphorylation of both STAT5 isoforms was seen in IL-3-stimulated cells (Fig. 1). Therefore, since IL-3 results in the robust activation of both STAT5a and STAT5b in Ba/f3 cells, we reasoned that these cells would make an attractive model system to identify the targets of both STAT5 proteins.

Both STAT5a and STAT5b Bind to the bel-x Promoter—The STAT5 binding site in the bel-x promoter has been defined by

![Image](https://www.broad.mit.edu/cancer/genecruiser/). Hierarchical clustering was performed with dChip software (23) using 1 − r (Pearson's correlation coefficient) as the distance metric.

**RESULTS**

**STAT5 Activation in Ba/f3 Cells**—We chose the IL-3-dependent Ba/f3 cell line as a model system to aid in the identification of STAT5 targets. IL-3 activates STAT5 in these cells, which leads to the increased expression of the prosurvival protein Bel-xl (24, 25). We first confirmed that both STAT5a and STAT5b are present in Ba/f3 cells and that both STAT5 isoforms are phosphorylated in response to IL-3. Immunoprecipitations of lysates from Ba/f3 cells were performed using antibodies specific for STAT5a or STAT5b. No phosphorylated STAT5a or STAT5b was seen in starved cells. However, phosphorylation of both STAT5 isoforms was seen in IL-3-stimulated cells (Fig. 1). Therefore, since IL-3 results in the robust activation of both STAT5a and STAT5b in Ba/f3 cells, we reasoned that these cells would make an attractive model system to identify the targets of both STAT5 proteins.

**Both STAT5a and STAT5b Bind to the bel-x Promoter**—The STAT5 binding site in the bel-x promoter has been defined by...
electrophoretic mobility shift assay, and it conforms to the TTCN3GAA consensus sequence determined for STAT5 (25). In order to validate our approach to identifying STAT5 targets, we wanted to confirm the in vivo binding of both STAT5 isoforms to the bcl-x promoter. Primers were designed to amplify this region and were used in a ChIP assay. Ba/F3 cells were starved of IL-3 and then left untreated or stimulated with IL-3. After 30 min, chromatin was harvested, and immunoprecipitations were performed using antibodies specific to STAT5a or to STAT5b. In addition, normal rabbit IgG was used as a nonspecific control. Both STAT5a and STAT5b were bound to the bcl-x promoter after IL-3 stimulation (Fig. 2). Often, some basal binding can be detected in starved cells, but this is dramatically increased with IL-3 stimulation. Importantly, the immunoprecipitation is specific for STAT5a and STAT5b, since there was no detection of the bcl-x promoter when a nonspecific antibody was used for immunoprecipitation (Fig. 2).

ChIP-based Gene Identification Strategy—Chromatin immunoprecipitation provides information about the DNA binding activity of a transcription factor in vivo (26). ChIP is performed by formaldehyde cross-linking of protein to DNA and immunoprecipitating these complexes with a specific antibody. Since this method can isolate all of the targets that are bound by the transcription factor at the time of cross-linking, we reasoned that these targets could be identified by a ligation-mediated PCR technique. In this approach, the ChIP product is ligated to linkers and amplified using the linker sequence for priming. At this point, the DNA can be isolated and sequenced. To also identify any major differences in the targets of STAT5a and STAT5b, we added a subtraction step, which is based on the technique to identify differential expression of mRNA species in different tissues (21). There are two advantages to this approach. First, any major differences in targets between STAT5a and STAT5b will be identified, and second, any DNA still bound nonspecifically after the immunoprecipitations will be removed during the subtraction, resulting in a high proportion of STAT5-specific targets.

ChIP-based Identification of STAT5 Targets—To identify STAT5 targets, we performed the subtractive ChIP cloning strategy using product from STAT5a and STAT5b chromatin immunoprecipitations. From the resulting clones, we sequenced 32 potential targets isolated from the STAT5a-enriched pool (designated A1–A32) and 39 potential targets isolated from the STAT5b-enriched pool (designated B1–B39). We analyzed the location of these sequences in the genome using the UCSC Genome Bioinformatics browser. Of the 32 STAT5a targets, three gave no matches, and 11 identified unique targets. The others represented multiple clones. 17 of the STAT5b targets were unique targets. We therefore analyzed a total of 28 separate STAT5 binding sites. All of these were unique; none of the STAT5a targets were also isolated from the STAT5b pool, and no targets from one experiment were isolated in another experiment.

An analysis of the clones revealed that most are close to genes and thus could be regulatory regions. Of the 28 clones, we analyzed 25 that contained full-length sequence and found that 80% contained a STAT consensus sequence, as defined by TTCTN3GAA, whereas 20% contained no consensus sequence. Since CpG islands are present in ~60% of genes at their 5′-end (27), others have combined ChIP with microarrays made from CpG enriched DNA to identify E2F targets (28–30). Therefore, we wanted to determine whether or not the STAT5 binding sites that we have identified contained CpG islands. We determined that 36% contained CpG islands, whereas 64% did not. Furthermore, those with CpG islands were most often found near the 5′-end of a gene, as expected (27). Therefore, the STAT5 binding sites that we have identified are most often found near genes, most contain a STAT consensus site, and most do not contain CpG islands.

Genes Located near STAT5 Binding Sites—Since STAT5 functions to directly regulate the expression of its target genes, a key question is to identify where these newly identified STAT5 binding sites lie in relation to known genes. Only 17% of these binding sites were within 1 kb of the transcription start site. Interestingly, the majority (54%) of the STAT5 binding sites were within introns. Excluding ESTs, in which the structure of the full-length mRNAs is not well defined, 23% of STAT5 binding sites were located in the first intron (Fig. 3).

Genes located near the STAT5 binding sites are listed in Table I. Two binding sites gave no matches using the UCSC Genome Bioinformatics browser, one contained repetitive DNA, and the other 25 binding sites were near genes. These included 19 known and 12 unknown genes. Most of the STAT5 binding sites were near a single gene, but six were near more than one gene. For example, A34 was found in the first intron of 14-3-3-γ, and this entire gene was found in the first intron of the ZP3 gene. An important observation is that none of the genes identified represent known STAT5 targets, and they encode genes of various functions, including cytoplasmic signal transduction, DNA binding, calcium homeostasis, vesicle cycling, cell adhesion, and housekeeping functions.

Confirmation of STAT5 Binding—In order to confirm that the binding sites that we have identified were true STAT5 targets, we designed primers encompassing these binding sites and performed PCR from multiple ChIP products obtained using STAT5a and STAT5b antibodies. We analyzed 12 targets and found that nine are bound by STAT5 (Fig. 4). Often there was some binding of STAT5 to these targets under starvation conditions.
conditions, but binding generally increased with IL-3 stimulation, as was the case with B60 (Fig. 4). Interestingly, A34 showed a strong signal in starved cells that was not greatly increased with IL-3 stimulation. This binding is specific for STAT5, since there is no binding to A34 when a nonspecific IgG is used in the immunoprecipitation (Fig. 4). A20 and A25 showed robust binding to STAT5, although there was variability in the binding of STAT5 to these promoters in response to IL-3 (data not shown). Therefore, they are likely STAT5 binding sites, but the variability in the data does not allow us to conclusively determine the extent to which IL-3 regulates both promoters. Importantly, none of the targets were isolated by a nonspecific IgG antibody, indicating that the binding is specific for STAT5 (Fig. 4). Only one of the potential binding sites showed little to no binding (A12), and thus we conclude that this is probably not a STAT5 target (data not shown). Therefore, we conclusively show that nine of the 12 DNA sequences isolated represent true STAT5 binding sites, with eight being regulated by IL-3, and that all nine are bound by both STAT5a and STAT5b.

STAT5a and STAT5b Bind to Promoters with Distinct Kinetics—Both STAT5a and STAT5b bound all of the targets tested, but the magnitude of binding of each isoform was different between promoters (Fig. 4). Therefore, we sought to ascertain whether or not binding of STAT5a or STAT5b changed over time. We tested the binding of STAT5 proteins to three promoters after 30, 90, and 150 min of IL-3 stimulation. The binding of STAT5a was similar at each time point on the B60 promoter (Fig. 5), whereas STAT5b shows greatest binding at 90 min. The binding of STAT5a to the B47 promoter increases at 30 and 90 min, whereas STAT5b binding is maximal at 30 min and decreases at later time points. STAT5a binding to the bcl-x promoter increases continually through 150 min, whereas STAT5b is bound maximally at 90 min. The kinetics of phosphorylation of both STAT isoforms is similar through 150 min (data not shown), suggesting that any differences of DNA binding are not due to differences in phosphorylation. Thus, STAT5a and STAT5b behave differently on various promoters when both are activated in the same cell.

STAT5 Targets Are Modulated by IL-3 in Ba/f3 Cells—To determine whether the association of STAT5 with a particular binding site has functional consequences, we determined whether any of the genes containing these binding sites were regulated by IL-3 in Ba/f3 cells. We tested five of the newly

### Table I

| STAT5 binding site | Nearest gene | Accession number | Binding site location |
|--------------------|--------------|------------------|----------------------|
| A2 Rab19           | AK017590     | 5' first exon, first intron |
| A6 Secretagogin    | BC016093     | ~10 kb 3'         |
| A7 No matches      |              |                   |
| A12 mKIAA1450      | AK122512     | Fifth intron      |
| A20 EST            | CA786702     | First intron      |
| A21 Unknown clone  | BC026716     | ~15 kb 5'         |
| A22 Repetitive     |              |                   |
| A23 ssa            | AK046469     | First intron      |
| A25 Unknown clone; EST | AK047164; BU708653 | 3' of clone; last intron of EST |
| A29 Early transposon Eth | AB003517 | ~30 kb 3'        |
| A34 14-3-3γ ZIP3 gene | M26410; BC008129 | First intron of both |
| A42 Unknown clone  | AK030034     | Last intron       |
| A43 0–44 protein homologue | AK011889 | 5' and first exon |
| A46 Hypothetical PDZ domain protein | BC028439 | Last intron       |
| A47 Arnt           | AK040475     | First exon, first intron |
| A49 Ksr            | U43585       | First intron      |
| B50 No matches     |              |                   |
| B51 Hypothetical Src homology 3 domain protein | AK029292 | ~5 kb 5' |
| B52 Hexosaminidase A; EST | AK075911; BY055800 | Immediately 3'; first intron |
| B55 S19; Oct2b    | AK013524; X53654 | Second intron; third intron |
| B56 CCL3/MIP1α   | AF065939     | ~10 kb 3'         |
| B57 B7-H3         | AY190318     | Spans third and fourth introns |
| B60 Srp9; B2 transcribed repetitive sequence | BC039648; M31444 | ~15 kb 5'; ~10 kb from transcript |
| B63 EST           | BY122638     | First intron      |
| B65 Unknown clone | AK0352120    | ~8 kb 5'          |
| B68 Nurr2         | AB014889     | ~500 bp 5'        |
| B72 Sialyltransferase | X60000 | Immediately 5'   |
| B78 Tubby-like protein 2; nucleobindin | AK054304; AK045087 | First intron; 5', first exon, first intron |

![Fig. 4. Confirmation of STAT5 binding to target sequences.](http://www.ncbi.nlm.nih.gov)
identified STAT5 target genes for expression. As a control, we examined the known STAT5 target \textit{bcl-x} and found that its expression is increased after IL-3 stimulation (Fig. 4). Ksr, Srp9, and 14-3-3\(\gamma\) were up-regulated by IL-3 stimulation. Srp9, 14-3-3\(\gamma\), and \textit{bcl-x} levels stay high even at 2 h of IL-3 stimulation, whereas the levels of Ksr are maximal at 2 h and then return to base line. The STAT5 binding sites associated with Ksr and Srp9 show an IL-3-dependent increase in STAT5 binding (Fig. 4). However, this is not the case for the STAT5 binding site for 14-3-3\(\gamma\). STAT5 is strongly bound to the 14-3-3\(\gamma\) gene under conditions of IL-3 starvation or stimulation (Fig. 4). Therefore, any increase in 14-3-3\(\gamma\) transcription cannot solely be due to STAT5 binding but may involve other STAT5 functions, such as the binding of coactivators. The aryl hydrocarbon receptor nuclear translocator (Arnt) and “similar to sperm antigen” (ssa) were down-regulated after IL-3 stimulation. Interestingly, the levels of Arnt are repressed even after 24 h of IL-3 stimulation, whereas the levels of ssa begin to recover after 6 h. Therefore, the genes that we have identified as STAT5 targets by ChIP are expressed in Ba\(\sim\)3 cells and show altered expression in response to IL-3. These results also suggest that STAT5 may act as an activator or repressor of transcription.

\textbf{STAT5 Target Genes Show Unique Expression Patterns in Human Leukemias}—To determine whether the genes identified by this method are physiologic targets of STAT5, we considered a system in which STAT5 is known to be activated. Among cancers, STAT5 has been reported to be constitutively activated in acute leukemias (4, 5). To evaluate whether these genes showed unique expression in leukemias, we analyzed the global cancer map, which contains expression microarray data from a panel of tumors. Whereas this data set provides information about the relative expression of genes among these tumors, it does not contain normal control samples, and thus, this method cannot determine whether gene expression levels in the represented tumors are aberrant. The mouse genes identified in our screen that had human homologues represented on the global cancer map were analyzed for expression patterns in the various cancers. These genes showed a distinct pattern of expression in leukemias, with many of these tumors showing notably increased or decreased expression of these putative STAT5 targets (Fig. 7). Therefore, several of the newly identified STAT5 targets are uniquely expressed in a variety of acute leukemias, suggesting that this approach can identify physiologic STAT5 targets.

\section*{DISCUSSION}

Chromatin immunoprecipitation has become the gold standard to confirm the binding of transcription factors to DNA \textit{in vivo}. ChIP provides a convenient analysis of the DNA binding activities of transcription factors in living cells. In addition to confirming the binding of a protein to a known DNA sequence, ChIP has been a powerful tool to identify previously unknown targets of transcription factors. Recently, several groups have combined ChIP with microarrays to identify targets of transcription factors in higher eukaryotes. These include microarrays made from CpG island-enriched DNA (28, 31, 33); promoter microarrays, containing DNA near the 5'-end of genes (32, 33); and chromosomal microarrays, containing the nonrepetitive portions of human chromosomes 21 and 22 (34–36). All of these studies have given important insight into the genes controlled by various transcription factors. However, each of these techniques has its limitations. For example, only about 60% of mammalian genes contain CpG islands in their promoters (27), limiting the ability of CpG-based microarrays to identify all of the binding sites of a transcription factor, particularly those not found at the 5'-end of genes. Similarly, promoter microarrays contain DNA near the transcription start site (32, 33) and thus are biased toward the 5'-end of genes. The data presented in this report show that a significant portion of STAT5 binding sites are not near the transcription start site. Chromosomal microarrays have also shown that the binding sites for other transcription factors are often located far from the 5'-end of genes (34–36). However, although it is clear that these chromosomal microarrays give invaluable information, they lack 97–98% of the human genome. Therefore, there is no technology at present to easily identify all of the binding sites of a given transcription factor.

One approach to identifying all targets of a transcription factor is a direct cloning approach, such as that used to identify several E2F targets (29). This study showed the utility of identifying novel transcription factor binding sites using ChIP in eukaryotes, and we modified this technique to include a ligation-mediated PCR step to enable the use of a smaller quantity of input chromatin. It is conceivable that this PCR step may preferentially amplify certain genomic regions. However, since most of the newly identified targets are \textit{bona fide} STAT5 binding sites, this method clearly does not amplify many nonspecific sequences. In addition, it has the advantage of allowing the production of a large quantity of DNA from one chromatin immunoprecipitation. We used this technique to isolate 28 previously unknown targets of STAT5. The majority of the newly identified STAT5 binding sites are located within introns, with most being in the first intron. It is not known why most STAT5 binding sites are located in introns, but there is precedence for this finding in that the STAT5 binding site in the \textit{bcl-x} gene is also located in the first intron (15, 20). We did not identify any previously identified STAT5 targets in our

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Kinetics of promoter binding of STAT5a and STAT5b. ChIP was performed on starved cells as well as cells stimulated for 30, 90, and 150 min with IL-3 using the indicated antibodies. PCR was performed using primers specific for the \textit{bcl-x}, B47, and B60 STAT5 binding sites.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Expression analysis of STAT5 target genes. Cells were starved of IL-3 and then left untreated or stimulated with IL-3 for 2, 6, and 24 h. RNA was harvested, and RT-PCR was performed for the indicated genes. Glyceraldehyde-3-phosphate dehydrogenase (\textit{GAPDH}) was used as a control. The expression of \textit{bcl-x}, a known STAT5 target, is shown as a positive control.}
\end{figure}
is therefore plausible that STAT5 may play a role in the pathogenesis, since constitutive activation of STAT5 is well known to be present and activated in a cell.

STAT5 functions by regulating the expression of genes, and it suggests that STAT5a and STAT5b may have different roles under a given set of conditions (34–36).

We have used this ChIP-based gene identification technique to identify targets of STAT5, regardless of their location in the genome. This has allowed the identification of many STAT5 binding sites in novel target genes, and these regions regulated by STAT5 frequently occur outside of traditional promoter regions.

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FIG. 7. Expression of STAT5 targets in human cancer. Using expression data from 170 tumors, hierarchical clustering was performed on the STAT5 targets that had human homologues represented in the data sets. Relative expression is indicated by color, with bright red representing high expression and bright green representing low expression.
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