A 32-kDa Proteolytic Fragment of Transcription Factor Stat3 Is Capable of Specific DNA Binding*

(Received for publication, April 15, 1997, and in revised form, June 16, 1997)

Birgit Dreier‡§, Stefan Fritz‡, Gudrun Volkert‡, Bent Brachvogel‡, Friedrich Lottspeich‡, and Georg H. Fey‡¶
From the ‡Chair of Genetics, University of Erlangen-Nürnberg, D-91058 Erlangen and the ¶Max Planck Institute for Biochemistry, D-82152 Martinsried, Federal Republic of Germany

Fractions of characteristic size retaining the ability of sequence-specific DNA binding were generated by partial proteolysis of transcription factor Stat3 with trypsin, chymotrypsin, or Staphylococcus V8 proteinase. The molecular masses of the smallest DNA-binding fragments were 75, 48, and 32 kDa after digestion with V8 proteinase, chymotrypsin, and trypsin, respectively. The fragments contained major parts of the domain controlling the sequence specificity of DNA binding (amino acids 406–514), the SH3 and SH2 domains, and the phosphorylated tyrosine residue Tyr-705, but not the C-terminal 20 amino acids. The N terminus of the 32-kDa tryptic fragment (ANCDASLIV) matched the sequence of amino acids 424–432 deduced from cDNA. The fragments were observed after proteolytic treatment of preformed complexes between DNA and native factors eluted from rat liver nuclei or recombinant, tyrosine-phosphorylated rat Stat3 from insect cells. It was possible to elute all three minimal fragments from their complexes with DNA and to obtain specific re-binding. The minimal fragments eluted from complexes with DNA still contained the phosphorylated Tyr-705 and the SH2 domain suggesting that they were probably bound to DNA as dimers. The DNA-binding domain of Stat3 identified by these experiments overlapped the domain previously identified by genetic experiments as the domain controlling the sequence specificity of DNA binding. The DNA-binding domain defined here by partial proteolysis probably represents an autonomously folding portion of Stat3.

Stat3 is a transcription factor mediating the effects of a variety of cytokines including interleukin 6 (IL6) and IL6-related cytokines on the transcriptional induction of their target genes (1–6). In particular, Stat3 is the factor primarily responsible for the cytokine-mediated induction of class II acute phase genes during an acute phase response of the liver (1, 7–15). In the absence of cytokine signals, Stat3 is present as a functionally latent monomer in the cytoplasm. In response to cytokine signals Stat3 is phosphorylated at a single tyrosine (Tyr-705) by receptor-associated JAK/TYK kinases. This process, referred to as the “activation” of Stat3, leads to the dimerization of monomers via their phosphoryrosines and SH2 domains in an antiparallel orientation and to the translocation of dimers to the nucleus (2–5, 16, 34). An analogous process leads to the formation of heterodimers, for example between Stat1 and Stat3 (4). Stat3 dimers bind at specific response elements in the control regions of their target genes, including the class II acute phase gene α2-macroglobulin (α2M), and mediate their transcriptional induction (1, 2, 8, 9, 11, 12, 15, 17). Heterodimers often occupy the same palindromic response elements as homodimers, because the members of the Stat factor family bind to similar DNA target sequences. These binding sites generally have a palindromic symmetry allowing the factors to bind in an antiparallel orientation. Binding sites deviating from perfect palindromic symmetry have been observed in a number of genes such as the rat α2M gene, and a set of rules have been derived specifying sequences that are preferred binding sites for various members of the Stat factor family. These rules describe the binding specificity as a function of the distances between the half-sites of the palindrome, of the spacer sequences between the half-sites, and the sequences of the half-sites (18). Stat3 dimers are capable of cooperative binding to dimers of other Stat factors. The cooperative interaction between dimers bound at tandem sites is mediated by the N-terminal domains of the Stat factors (19–21). The transcriptional transactivator domain is located in the C-terminal 40 amino acids of Stat3. Naturally occurring C-terminally truncated molecules lack the transactivator function. These truncated proteins still carry the DNA-binding domain and compete with the intact molecules for the same DNA-binding sites. Thus, they act as dominant negative inhibitors of the full-length factors (2–5, 22).

Apart from their SH2 domains Stat factors have no sequence homology with other known transcription factors, and therefore their DNA-binding domains, transcriptional transactivator domains, and nuclear localization domains are of unknown types. This situation presents a rare opportunity to analyze a novel type of DNA-binding domain. The position of the DNA-binding domain within Stat3 is not known with precision, but it cannot reside in the C-terminal 40 amino acids, because these are dispensable for DNA binding. Attempts to confine this domain by construction of mutants with progressive deletions extending inward from the N and C termini have met with limited success. As soon as deletions from the C terminus extended beyond Tyr-705, efficient DNA binding was abolished, because dimerization apparently is a prerequisite for high affinity DNA binding. Similarly, mutants in which Tyr-705 was replaced by a different amino acid have been constructed. The mutant factors had lost the ability to dimerize and to bind DNA (4). It is not formally excluded that Stat factor
monomers may be able to attach to their specific DNA-binding sequences with very low affinity. However, if this binding mode existed, it must have gone undetected by the methods used to date. Deletions from the N terminus have also been of limited use, because the corresponding mutant factors were not efficiently phosphorylated. The N terminus of Stat3 factors is important for docking to the receptor-JAK/TYK kinase complex, and this in turn is essential for the phosphorylation of Stat3 factors (4). Thus, the genetic approach to map the DNA-binding domain of Stat3 factors has been of limited use.

A region controlling the specificity of DNA binding has been identified by domain-exchange experiments, so-called “domain swap” experiments, between Stats 1 and 3 or Stats 1 and 6 (18, 23). The region located between amino acids 406 and 514 in Stat3 has been mapped by this approach. However, it was not clarified whether this region contained the actual DNA-contact domain of Stat3 or whether it represented only a domain controlling the specificity of DNA binding without entering into direct contact with DNA.

To overcome these difficulties and to identify the DNA-contact domain of Stat3, we have relied on an approach including the initial tyrosine phosphorylation of the intact molecule and the subsequent removal by partial proteolysis of all parts that were no longer required for DNA binding once tyrosine phosphorylation had been achieved. This approach has allowed us to define a fragment of approximately one-third the size of Stat3 that was still capable of sequence-specific DNA binding. Here we describe the delineation and immunological characterization of this fragment.

EXPERIMENTAL PROCEDURES

Gel Mobility Shift Experiments—Nuclear extracts from rat livers were prepared and electrophoretic mobility shift assays (EMSA) were performed as described previously (17, 24–26). The radiolabeled, double-stranded oligonucleotide TB2 used in most DNA-binding reactions carries two copies of the core site of the IL6 response element of the rat αM gene (27, 28) and is a binding site of intermediate strength for Stat3 (12, 17). Competition EMSA experiments were performed using the nonradiolabeled, double-stranded oligonucleotides TB1, mTB1, and CA1 in a 100-fold (TB1, mTB1) or a 50-fold molar excess (CA1), respectively. TB1 is a 24-base pair oligonucleotide containing one copy of the core site of the IL6 response element of the αM gene (27, 28). mTB1 is a mutant of TB1, in which the critical hexanucleotide CT-GGGA was replaced by a permutation with identical base composition, and CA1 is a 49-base pair oligonucleotide carrying the bipartite IL6 response element of the rat αM gene in its authentic configuration (27, 28). JR1 is a palindromic variant of the TB1 core site (TTCGCGGAA) and a strong binding site for Stat3.2 Proteolytic clip-shift assays were performed as described previously for the digestion of Stat3 with trypsin and chymotrypsin (29, 30). Digestion with Staphylococcus V8 proteinase (Sigma) was performed for 10 min at 37 °C in CP buffer (31, 32). For the verification of the specificity of DNA complexes with the radiolabeled double-stranded oligonucleotide, gel retardation assays were performed with nuclear proteins from rat livers excised 6 h after induction of an actoside-induced E. coli protease (from Drs. S. McKnight and U. Schindler), respectively, and subsequent digestion of DNA-bound Stat3 was performed for 10 min at 37 °C with V8 proteinase in 500 μl of CP buffer supplemented with 100 μM KCl. Trypsin (14 units), chymotrypsin (8 units), and V8 proteinase (375 units) were used to treat 250 μg of crude insect cell containing recombinant rat Stat3. After completion of the reaction the beads were separated magnetically and washed twice with 200 μl CP buffer supplemented with 100 μM KCl. DNA-bound proteins were eluted with 200 μl of CP buffer supplemented with 600 mM KCl and 2 μg of bovine serum albumin. For EMSA assays, the eluted protein was used directly. For Western blot analysis eluted proteins were precipitated with trichloroacetic acid and resuspended in SDS sample buffer (32).

Cloning, Expression, and Purification of Recombinant Stat3 Fragments and Generation of Polyclonal Antiserum—Protein-coding fragments of rat Stat3 cDNA were inserted into the bacterial expression vector pET15b (Novagen) generating fusion polypeptides with an N-terminal hexahistidine (6xHis)-tag. Construction was verified by sequence analysis, and the constructs were tested for expression in Escherichia coli BL21 (Novagen) after induction with 1 μM isopropylthiogalactoside. Recombinant proteins were purified under denaturing conditions using a nickel nitrilotriacetic acid column (Quia- gen) following the instructions of the manufacturer and dialyzed against phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4). Rabbits and mice were separately immunized with approximately 500 and 50 μg, respectively, of the purified recombinant Stat3 fragments. Immunization, booster injections, collection, and processing of the sera were performed following standard procedures (31) as a commercial service by Charles River Laboratories, Kissing, Germany. The fragments used were EX18 (amino acids 1–159 of the rat Stat3 cDNA sequence; Ref. 17), RD7 (amino acids 231–334), P11.1/10 (amino acids 405–555), and SD4 (amino acids 604–770). The anti-SD4 serum was absorbed with E. coli proteins. To this effect, an insoluble acetone precipitate from E. coli BL21 cells was incubated overnight at 4 °C with the serum. The precipitate was then removed by centrifugation, and the supernatant represented the absorbed serum.

Western Blot Experiments—Western blot experiments were performed after electrophoresis of protein extracts in SDS containing 17.5% polyacrylamide gels (31, 32). For the verification of the specificity of polyclonal rabbit sera 20 μg of crude extract from isopropylthiogalactoside-induced E. coli BL21 bacteria expressing Stat3 fragments were used. The minimal DNA-binding domains of Stat3, the eluted fraction representing 50 μg of input crude extract from insect cells was loaded on the gel. The proteins were transferred to a BA-85 nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked with 3% bovine serum albumin in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.5) and probed with the anti-Stat3 antibodies generated in this study (mouse anti-RDT, mouse anti-PII.1/10, and rabbit anti-SD4) or with the commercial antibodies rabbit anti-C20 (Santa Cruz), mouse anti-SS-N (Translaboratories), and the anti-phosphotyrosine antibody 4G10 (Upstate). Goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma) and goat anti-mouse IgG coupled to horseradish peroxidase (Dianova) were used as second anti- bodies. Signals were detected using the enhanced chemiluminescence system (Amersham Corp.).

Purification of the Minimal DNA-binding Domains—Five hundred pmol of biotinylated, double-stranded oligonucleotide JR2 (consisting of one JR1 and one TB1 binding site in tandem) was allowed to react with 1 μg of streptavidin coupled to magnetic porous glass beads (Control Pure Glass, CPG Inc., Lincoln Park, NJ) according to the manufacturer’s protocol. Crude extract (250 μg) from Sf21 insect cells coinfected with baculoviral expression constructs for rat Stat3 or murine JAK2 kinase (from Drs. S. McKnight and U. Schindler), respectively, and harvested 50 h postinfection were incubated with the JR2 oligonucleotide coupled to magnetic porous glass beads. Binding was allowed to proceed for 10 min at 25 °C in 500 μl of CP buffer (10 mM Hepes, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 6 mM MgCl2, 1.25 mM CaCl2, 25 μg of salmon sperm DNA) supplemented with 50 mM KCl (17, 24). Subsequent digestion of DNA-bound Stat3 was performed for 10 min at 25 °C with trypsin or chymotrypsin and at 37 °C with V8 proteinase in 500 μl of CP buffer supplemented with 100 mM KCl. Trypsin (14 units), chymotrypsin (8 units), and V8 proteinase (375 units) were used to treat 250 μg of crude insect cell containing recombinant rat Stat3. After completion of the reaction the beads were separated magnetically and washed twice with 200 μl CP buffer supplemented with 100 mM KCl. DNA-bound proteins were eluted with 200 μl of CP buffer supplemented with 600 mM KCl and 2 μg of bovine serum albumin. For EMSA assays, the eluted protein was used directly. For Western blot analysis eluted proteins were precipitated with trichloroacetic acid and resuspended in SDS sample buffer (32).

N-terminal Amino Acid Sequence Analysis—Ten ng of crude extract from Sf21 insect cells coinfected with baculoviral expression constructs for Stat3 and JAK2 and harvested 50 h after infection were incubated with the double-stranded biotinylated JR2 oligonucleotide bound to streptavidin/magnetic porous glass beads. Tryptic digestion of the DNA-bound Stat3 was performed as described above, and the eluted minimal fragment was electrophoretically separated in an SDS-polyacrylamide gel. The relevant fragment was transferred to a Glassybond membrane (Biometa) as described previously (33). N-terminal amino acid sequencing was performed by automated Edman degradation using an Applied Biosystems 492A sequencer.

RESULTS

Proteolytic digestion of rat Stat3 produces defined minimal fragments capable of sequence-specific DNA binding. Nuclear protein extracts from rat livers excited 6 h after induction of an experimental acute phase response were used as the initial source of activated Stat3. Earlier studies had demonstrated that Stat3 was the most abundant activated Stat factor in liver nuclei at this stage of the acute phase response. At this time activated Stat1 was present in negligible concentrations and activated Stat5 was undetectable in the nuclei (17). Protein-DNA complexes with the radiolabeled double-stranded oligo-
nucleotide probe were first assembled in vitro and then treated with increasing concentrations of either trypsin, chymotrypsin, or Staphylococcus V8 proteinase. In all three cases defined complexes were produced that migrated as focused bands in electrophoretic mobility shift experiments (EMSAs) even after digestion with elevated concentrations of the proteinases (Fig. 1). The treated complexes migrated faster than the untreated complex (complex II) indicating a discrete loss of protein mass. DNA binding of the minimal Stat fragments contained in these complexes was still sequence-specific as shown by competition gel mobility shift experiments (Fig. 2). Addition of an excess of nonradioactive oligonucleotides TB1 or CA1, representing efficient Stat factor binding sites (see “Experimental Procedures”) to the DNA-binding reaction prior to addition of the proteinases prevented formation of the faster migrating complexes. Addition of an excess of the mutant oligonucleotide mTB1, an ineffective Stat factor binding site, had no effect. Thus, fragments of Stat3 that remained bound to DNA after proteolytic digestion of preformed complexes remained bound with the same sequence specificity as intact Stat3.

Assembly of Protein-DNA Complexes after Proteolytic Digestion Is Still Sequence-specific—When nuclear protein extracts from acute phase rat livers were first treated with proteinases and assembly of complexes with DNA was attempted subsequently, no more complexes were observed after treatment with V8 proteinase (Fig. 2B). Faster migrating complexes of the same characteristic mobility as in Fig. 2A were also observed after treatment with trypsin and chymotrypsin. These findings suggest the existence of cleavage site(s) for V8 proteinase within the minimum DNA-binding fragment accessible to the proteinase in free Stat3 but not in complexes between Stat3 and its DNA target. By contrast, treatment of free Stat3 with limited amounts of trypsin and chymotrypsin generated fragments capable of sequence-specific DNA binding. The sequence specificity of DNA binding of the proteolytic fragments was established by competition gel mobility shift experiments with appropriate oligonucleotides (Fig. 2B).

Generation of Antibodies Specific for Different Epitopes of Stat3—For an immunological identification of the portion of Stat3 contained in the minimal DNA-binding fragments, a set of 7 overlapping fragments, each approximately 100 to 200 amino acids in length, were expressed in E. coli (Fig. 3). Together these fragments covered the complete Stat3 sequence. Fragments EX18, RD7, PI1.1/10, and SD4 were purified and used to immunize rabbits and mice. The N-terminal fragment EX18 was not immunogenic in our experiments. Therefore, the two commercial antibodies S3-N and C20, directed against N- and C-terminal portions of Stat3, respectively, were included in this study. The specificity of the antisera was verified in Western blot experiments with bacterial extracts expressing the fragments used for immunization (Fig. 4). All antibodies specifically reacted only with fragments carrying the epitopes used
transactivator domain. DNA binding; SH2 domain for cooperative binding between dimers of Stat factors at tandem sites; DNA, domain controlling the specificity of DNA binding; SH2, src homology domain 2; Y, tyrosine 705; TA, transcriptional transactivator domain.

|       | EX18 | RB3 | PIL2 |
|-------|------|-----|------|
| EcoRI/XbaI |      |     |      |
| PsI/PstI |      |     |      |
| RD7   |      |     |      |
| PsI/Ddel |      |     |      |
| PII.1/10 |      |     |      |
| SD4   |      |     |      |
| Smal/DraI |      |     |      |


![DNA binding fragment of Stat3](image)

**FIG. 3.** Fragments of Stat3 used for the generation of polyclonal antisera and epitopes recognized by various anti-Stat3 antibodies. Fragments of Stat3 cDNA were cloned into the expression vector pET15b as fusions with a 6xHis-tag. The fragments were expressed in E. coli BL21 and purified on a nickel nitrilotriacetic acid column. Rabbits and mice were immunized with fragments EX18, RD7, PII.1/10, and SD4, respectively. Epitopes recognized by the two commercial antibodies S3-N and C20 are also shown. Shaded N-terminal region, domain for cooperative binding between dimers of Stat factors at tandem sites; DNA, domain controlling the specificity of DNA binding; SH2, src homology domain 2; Y, tyrosine 705; TA, transcriptional transactivator domain.

![Western blot analysis](image)

**FIG. 4.** Specificity of anti-Stat3 antibodies. A, 20 μg of crude extract (for EX18: 1 μg of purified fragment) from E. coli cells expressing Stat3 fragments were loaded for each track of a 17.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue. ST, molecular weight standard; –, extract from E. coli cells not expressing any recombinant Stat3 fragment; B-F, Western blot analysis with the antibodies S3-N (B), RD7 (C), PII.1/10 (D), SD4 (E), and C20 (F).

to generate them and were therefore suitable to map the minimal DNA-binding fragments of Stat3.

**Purification of the Minimal DNA-binding Fragments of Recombinant Rat Stat3 Expressed in Insect Cells**—Further structural characterization of the minimal DNA-binding fragments required the availability of Stat3 in semi-preparative quantities in the 0.1–1 nmol range, with 1 nmol equalling approximately 90 μg. To this effect recombinant rat Stat3 and murine JAK2 kinase were produced from baculoviral expression constructs in insect cells (see “Experimental Procedures”). Double infection of insect cells with both of these constructs produced correctly tyrosine-phosphorylated, specifically DNA-binding rat Stat3 in the required quantities. For purification of the minimal DNA-binding fragments on the semi-preparative scale, proteolytic digestions were performed on Stat3-DNA complexes. For this purpose the biotinylated double-stranded synthetic oligonucleotide JR2 carrying an efficient Stat3 binding-site was used as the DNA target (see “Experimental Procedures”). Tyrosine-phosphorylated Stat3 contained in insect cell extracts was allowed to bind to this target and enriched with streptavidin-coated magnetic beads (see “Experimental Procedures”). The beads were then collected, washed, and treated with the proteinase of choice. After completion of the reaction, the beads were again collected and washed, and the minimal DNA-binding fragments were eluted. The recovered fragments were then used for further analysis in EMSA experiments or for electrophoresis in SDS-polyacrylamide gels and Western blots.

The Minimal Stat3 DNA-binding Fragments Are Capable of Sequence-specific Re-binding after Elution from DNA—Full-length Stat3 and its minimal DNA-binding fragments generated by treatment with the three proteinases were analyzed for their ability to re-bind DNA in EMSA experiments (Fig. 5). Re-binding was assayed using the radiolabeled synthetic oligonucleotide JR1. Stat3 from insect cell extracts and eluted Stat3 formed a complex III of standard mobility, consisting of one Stat3 dimer (Fig. 5, tracks 2 and 3). The eluted and re-bound minimal tryptic and chymotryptic fragments generated complexes migrating with the same increased mobilities as those originally detected in Fig. 1 (Fig. 5, tracks 2 and 3). The eluted and re-bound V8 proteolytic fragment also generated a complex of indistinguishable mobility as the minimal minimal complex shown in Fig. 1. Re-binding of the eluted V8 minimal fragment was sequence-specific as shown by competition gel shift experiments with appropriate competitors (Fig. 5B).

**Composition of the Minimal DNA-binding Fragments; Epitope Analysis**—To identify the portions of Stat3 contained in the minimal DNA-binding fragments, Western blot experiments were performed with the eluted fragments (Fig. 6). The antibodies PII.1/10, SD4, and the anti-phosphotyrosine antibody reacted with full-length Stat3 and the minimal DNA-binding fragments generated by all three proteinases. These fragments had approximate molecular masses of 32, 48, and 75 kDa for the tryptic, chymotryptic, and V8 fragments, respectively (Fig. 6A). The C20 antibody reacted only with full-length Stat3 (Fig. 6B). The RD7 antibody reacted with full-length

4 S. Fritz, unpublished data.
and were tyrosine-phosphorylated. They did not include the DNA were capable of sequence-specific re-binding. 4) Minimal DNA-binding fragments of recombinant Stat3 eluted from tryptic digestion of both free and DNA-bound Stat3 re- 

kDa, respectively. 2) The minimal fragments generated by proteolytic digestion of both free and DNA-bound Stat3 retained the ability of sequence-specific DNA binding. 3) Minimal DNA-binding fragments of recombinant Stat3 eluted from DNA were capable of sequence-specific re-binding. 4) Minimal DNA-binding fragments of Stat3 contained the SH2 domains and were tyrosine-phosphorylated. They did not include the extreme N- and C-terminal portions of Stat3. 5) The minimal DNA-binding fragments also contained the region previously described by other authors as the region controlling the sequence specificity of DNA binding (amino acids 406–514), but the fragment generated by tryptic digestion did not contain amino acids 406–423.

**DISCUSSION**

The main new results and conclusions drawn from this study were as follows. 1) Digestion of rat Stat3 with trypsin, chymotrypsin, and *Staphylococcus* V8 proteinase generated minimal DNA-binding fragments of characteristic sizes (32, 48, and 75 kDa, respectively). 2) The minimal fragments generated by proteolytic digestion of both free and DNA-bound Stat3 retained the ability of sequence-specific DNA binding. 3) Minimal DNA-binding fragments of recombinant Stat3 eluted from DNA were capable of sequence-specific re-binding. 4) Minimal DNA-binding fragments of Stat3 contained the SH2 domains and were tyrosine-phosphorylated. They did not include the

**FIG. 5.** Recombinant Stat3 and its proteolytic DNA-binding fragments eluted from DNA are capable of re-binding to DNA. Recombinant Stat3 expressed in Sf21 insect cells was tested for DNA binding activity in EMSA experiments using the JR1 oligonucleotide. The equivalent of 5 μg of crude extract was loaded on each lane. A, DNA re-binding of the minimal fragments; lane 1, no protein; lane 2, crude extract; lane 3, Stat3 re-bound after elution; lane 4, re-bound tryptic fragment; lane 5, re-bound chymotryptic fragment; III, complex III consisting of a single dimer of Stat3 bound at a palindromic site. B, gel mobility shift competition for binding of the minimal V8 fragment (V8) to oligonucleotide TB2; I, no competitor; 2, high molar excess of competitor TB1; 3, mTB1; 4, CA1. Target and competitor oligonucleotides as described under “Experimental Procedures.”

**FIG. 6.** The minimal DNA-binding fragments include the SH2 domain and are tyrosine-phosphorylated. Two hundred fifty μg of crude extract from Stat3-expressing Sf21 insect cells were incubated with oligonucleotide JR2 coupled to magnetic beads and digested with the proteinases as described before. As a negative control, JR2/magnetic beads were incubated with proteinases in the absence of added crude extract. The purified proteins were precipitated with trichloroacetic acid, resuspended in SDS sample buffer, and electrophoresed in a 17.5% SDS-polyacrylamide gel. Proteins were blotted on a nitrocellulose membrane and incubated with the anti-Stat3 antibodies shown in the left margin. Detection was done with the enhanced chemiluminescence system (Amersham Corp.). A, anti-PII.1/10 (1:2500), anti-phosphotyrosine (1:2000), anti-SD4 (1:10000); B, anti-S3-N (1:10000), anti-C20 (1:5000), anti-RD7 (1:2000). Arrows pointing to S3, full-length Stat3; T, tryptic fragment; C, chymotryptic fragment; V8, V8 fragment. + and − symbols in top row, reactions with or without added Stat3.
Hence, in this respect Stat3 is similar to many other eukaryotic DNA-binding proteins that contain functionally autonomous DNA-binding domains. The DNA-binding function of Stat3 thus is not generated by the combined contributions of several discontinuous sub-domains dispersed over the entire molecule.

Interestingly, the minimal DNA-binding domain contains a cleavage site for V8 proteinase that is protected in DNA-bound Stat3 but accessible in free Stat3. This finding suggests that Stat3 either undergoes a previously not recognized conformational change upon DNA binding or that the bound DNA constitutes a sterical hindrance for the accessibility of the cleavage site by V8 proteinase.

The competition gel mobility shift experiments (Fig. 2) document sequence specificity of DNA binding for the minimal tryptic and chymotryptic fragments (Fig. 6). These minimal fragments contained both the SH2 domain and phosphorylated Tyr-705. It is therefore reasonable to assume that they were bound as dimers, because this is the generally accepted DNA-binding mode of intact Stat factors. However, no direct evidence (such as protein-DNA cross-linking data or competition studies with a phosphotyrosine peptide, Ref. 18) was provided here for a dimeric organization of the DNA-bound minimal fragments. It is only the simplest interpretation of our data to assume that partial proteolysis of a DNA-bound dimer removed terminal portions of the protein not involved in DNA binding without changing the organization of the DNA-bound core of the dimer. At present, it is not clear whether dimerization is an absolute prerequisite for the sequence specificity of Stat factor binding to DNA or only a mechanism enhancing the affinity of binding. All available evidence points to dimerization as a requirement for high affinity binding detectable with the currently available methods. However, weak sequence-specific binding of Stat factor monomers below the detection threshold of current methods has not been formally excluded.

The immunological mapping of the portion of Stat3 contained in the minimal DNA-binding fragments produced results compatible with the mapping by partial amino acid sequence analysis. Our results are also consistent with previous conclusions of other authors (18, 23) who reported that the domain controlling the sequence specificity of DNA binding is approximately located between amino acids 400 and 500. Our results suggest that this specificity-controlling region may in fact be identical with the DNA-contact domain of Stat3. However, our data do not allow us to locate the C-terminal boundary of the minimal DNA-contact domain with precision. This is due to the fact that the minimal binding fragments defined here carry not only the DNA-contact domain but also all sequences required for dimerization. We cannot decide from our data whether the minimal DNA-contact domain ends in the vicinity of amino acid 500 or extends beyond this region and includes sequences between amino acids 500 and 706. This question could probably be solved by employing an artificial mechanism of antiparallel dimerization operating independently of the SH2 domain and phosphorylation of Tyr-705.

Interestingly two sequence motifs previously identified by other authors (23) as essential for sequence specificity of Stat factor binding to DNA are included in the minimum tryptic fragment reported here. These residues, VTEEL and SLPVVV (432–436 and 458–463 in rat Stat3), are located close to the N terminus of the minimal tryptic fragment (position 424; Fig. 8). This observation still does not allow us to conclude formally that the sequences jointly contained in the region defined here and by the genetic approach (23; amino acids 424–500 approximately) must contain the DNA-contact domain. However, this suggestion is now very plausible.

An alternative way to identify the minimal DNA-contact domain would consist in mapping amino acid residues engaged in direct contact with DNA by protein-DNA cross-linking. Such experiments are currently underway. Finally, it should be possible to delineate the DNA-contact region by x-ray diffraction of protein-DNA crystals. However, such crystals and the crystal structure of free Stat3 have so far not been obtained. One of the reasons why it may have been difficult to generate crystals of free Stat3 probably is that this molecule consists of several autonomously folding domains, such as the DNA-contact domain and the transcriptional transactivator domain. These regions most likely are connected by flexible linkers carrying the cleavage sites for the proteinases employed here and may not assume a sufficiently well defined position in space to allow the formation of crystals. The fact that it was possible to define a minimum DNA-binding fragment of only one-third the size of intact Stat3 suggests that this fragment may represent an
autonomously folding domain. The observation that this mini-
mal tryptic fragment was well defined and formed a single
sharp band in SDS-polyacrylamide gels5 is a further argument
in favor of this view. If this fragment indeed represented an
autonomously folding domain, then it may be possible to crys-
tallize it alone or in a complex with specific DNA sequences and
to obtain x-ray diffraction data. Attempts to crystallize this
region both alone and in complex with DNA are currently
underway.

Acknowledgments—We thank Dr. J. Miller (Lilly) for advice on the
construction of baculoviral expression vectors and Drs. S. McKnight
and U. Schindler (Tularik Inc., San Francisco) for providing the bacu-
loviral expression construct for mouse JAK2. We are grateful to Dr. L.
Miller for providing the insect cells.

REFERENCES
1. Akira, S., Nishio, Y., Inoue, M., Wang, X. J., Wie, S., Matsusaka, T., Yoshida,
K., Sudo, T., Naruto, M., and Kishimoto, T. (1994) Cell 77, 63–71
2. Kishimoto, T., Taga, T., and Akira, S. (1994) Cell 76, 253–262
3. Schindler, C., and Darnell, J. E., Jr. (1995) Annu. Rev. Biochem. 64, 621–651
4. Ihle, J. N. (1996) Cell 84, 331–334
5. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
6. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) Cell 76, 253–262
7. Zhang, D., Sun, M., Samols, D., and Kushner, I. (1996) J. Biol. Chem. 271, 9503–9509
8. Wegenka, U. M., Bushmann, J., Luettiken, C., Heinrich, P. C., and Horn, F. I.
(1993) Mol. Cell. Biol. 13, 276–288
9. Wegenka, U. M., Luettiken, C., Buschmann, J., Yuan, J., Lottspeich, F.,
Mueller-Esterl, W., Schindler, C., Raeb, E., Heinrich, P. C., and Horn, F.
(1994) Mol. Cell. Biol. 14, 2186–2196
10. Ruff-Jamison, S., Zhong, Z., Chen, K., Darnell, J. E., Jr., and Cohen,
S. (1994) J. Biol. Chem. 269, 21933–21935
11. Wang, Y., Morella, K. K., Ripperger, J., Lai, C.-F., Gearing, D. P., Fey, G. H.,
Campos, S. P., and Baumann, H. (1995) Blood 86, 1671–1679

5 B. Dreier, unpublished data.