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A Nuclear Single-Stranded-DNA Binding Factor Interacts with the Long Terminal Repeats of the 1731 *Drosophila* Retrotransposon

FRÉDÉRIQUE FOURCADE-PERONNET,* SOPHIE CODANI-SIMONART, AND MARTIN BEST-BELPOMME

Groupe de Génétique Moléculaire et Cellulaire, URA Centre National de la Recherche Scientifique 1135, Université Pierre et Marie Curie, 7, Quai St.-Bernard, 75005 Paris, France

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Using gel mobility assays, we have detected two proteins that bind in the U3 region of the 1731 retrotransposon long terminal repeats (between positions −110 and −73) in nuclear extracts from *Drosophila melanogaster* cultured cells. The first one binds double-stranded DNA, whereas the other binds the mRNA-like strand in a sequence-specific manner. We report here the characterization of the latter protein, named NssBF for nuclear single-stranded-DNA binding factor. Gel filtration shows an apparent molecular mass of 95 kDa for NssBF. The points of contact between NssBF and its single-stranded DNA target were determined. This protein binds neither the complementary strand nor the corresponding RNA sequence. A possible role of NssBF in transcription is discussed.

Retrotransposons have been shown to exist in many eukaryotic species. They display the overall organization of the provirus form of vertebrate retroviruses with long terminal repeats (LTRs) flanking the protein-coding region (reviewed in references 7, 20, and 21). They are widely represented in the *Drosophila melanogaster* genome, in which an important proportion of spontaneous mutations is known to be associated with their insertion (11). Transpositions of the yeast Ty retrotransposon and the mouse intracisternal A particles have been shown to require an RNA intermediate (1, 17), and other evidence suggests that this requirement is a general rule among retrotransposons (7). Thus, it appears that the rate of retrotransposition may be correlated with the rate of transcription. Identification of factors that modulate expression of retrotransposons by interacting with their regulatory sequences will therefore provide a better understanding of the control of retrotransposition events.

The *D. melanogaster* 1731 retrotransposon was isolated during a search for genes modulated by 20-hydroxyecdysone (28), the steroid hormone of insects. There are about 10 to 15 copies of 1731 elements per haploid genome in flies and 20 to 25 copies per haploid genome in cultured cells. At least some of these copies are transcriptionally active. The nucleotide sequence of 1731 was determined and shows the existence of 336-bp LTRs, which flank typical *gag* and *pol* genes (12). By functional dissection of the LTR, three separate regions were defined (33): a promoter region, required for transcription, containing a TATA box located about 50 bp upstream from the transcriptional start site (+1); an activator region which greatly enhances transcriptional efficiency (positions −168 to −111); and a silencer which reduces transcriptional efficiency (positions +90 to +168). The activator region consists of two juxtaposed nearly perfect repeats containing a CAAT motif (positions −157 to −152 and −129 to −124) and differing by 2 bp, which results in an octanucleotide perfectly homologous to the simian virus 40 enhancer core in the second repetition (positions −124 to −117) (Fig. 1). Moreover, the LTR sequence corresponding to positions −168 to −73 is required for the expression of 1731 in *Drosophila* cells (32a). As shown in Fig. 1, this DNA segment includes several putative targets for DNA binding factors (and could therefore bind some proteins implicated in the regulation of 1731 expression). We thus have attempted to detect a DNA binding protein(s) that interacts with this part of the LTR. In the present work, we focus on the existence of a nuclear protein that specifically binds in vitro to the coding strand, i.e., the mRNA-like strand, of the 1731 LTR between positions −99 and −74, without any detectable binding on the complementary strand. As far as we know, this is the first example of a nuclear single-stranded-DNA binding factor which specifically interacts with a *Drosophila* retrotransposon LTR.

MATERIALS AND METHODS

Cells and cell culture. The *D. melanogaster* cell clone 14-XI-23 derived from the original Kc line (8) was used throughout these experiments. Cells were grown in suspension culture at 23°C in D22 medium supplemented with 5% fetal calf serum.

Extraction and chromatography buffer. The buffer used is buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES, pH 7.6], 10% glycerol [vol/vol], 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and KCl as indicated (e.g., 0.1 M D means buffer D plus 0.1 M KCl).

Preparation of nuclear extracts. *Drosophila* cell nuclei were isolated and extracted at 4°C as described by Dignam et al. (6). A 250-ml volume of a cell suspension (6×10^9 cells) was centrifuged (Beckman JS13 rotor; 5,000 rpm, 5 min, 4°C). The pellets were suspended in 5 volumes of phosphate-buffered saline at 4°C, centrifuged as described above, and then resuspended in a solution containing 10 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol. After being allowed to stand for 10 min at 4°C, cells were collected as described above and suspended in 2 volumes of the same buffer. They were disrupted by 20 strokes in a Dounce homogenizer (B-type pestle) and centrifuged (Beckman JA21 rotor; 2,500 rpm, 10 min). The cytoplasmic supernatant was decanted, and the nuclear pellet was centrifuged again (Beckman JA21 rotor; 14,500 rpm, 20 min). The pellet was resuspended in 3 ml of 20 mM HEPES (pH 7.6)–25% (vol/vol) glycerol–0.42 M NaCl–1.5

* Corresponding author.

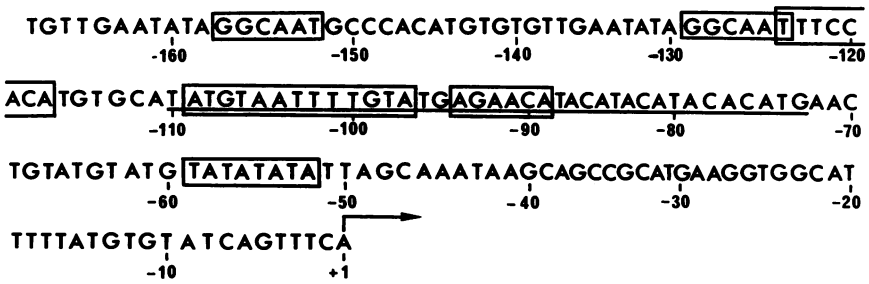

FIG. 1. Nucleotide sequence of the 1731 5′ LTR U3 region (12, 28). Numbers refer to the nucleotide position relative to the transcription initiation site (indicated by the arrow). The location of the CAAT boxes (positions −157 to −152 and −129 to −124), the octanucleotide homologous to the simian virus 40 enhancer core (positions −124 to −117), and the sequences similar to the heat shock element (positions −109 to −97) of heat shock genes and to the hexanucleotide involved in the binding of the glucocorticoid receptor on glucocorticoid-responsive elements (positions −94 to −89) are framed. The sequence of the Bc oligodeoxynucleotide used in the gel retardation experiments is underlined.

mM $MgCl_2$–0.2 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–0.5 mM dithiothreitol. After 10 strokes in a Dounce homogenizer (B-type pestle), the suspension was gently stirred and then centrifuged (Beckman JA21 rotor; 14,500 rpm, 30 min). The supernatant was dialyzed against 50 volumes of 0.1 M D and centrifuged again (Beckman JA21 rotor; 14,500 rpm, 20 min). The nuclear extract was aliquoted and stored at −70°C. Protein concentrations were estimated with a Bio-Rad assay kit.

Heparin-agarose chromatography. A 4-ml volume of heparin-agarose (0.4 to 0.5 mg of heparin per ml of gel; Pierce Chemical Company) was poured into an Econo-Pac disposable chromatography column (Bio-Rad). The column was equilibrated with 0.1 M D buffer at 4°C. Then the nuclear extracts were applied to the column. The column was washed with 6 volumes of 0.1 M D and eluted with 1 column volume of 0.2, 0.3, 0.4, 0.5, and 0.6 M D successively. Each fraction was then dialyzed against 0.1 M D.

DNA-protein binding assays. Two complementary oligodeoxynucleotides corresponding to positions −110 to −73 of the 1731 LTR promoter (coding strand 5′-TATGTAATTT TGTATGAGAACATACATACATACACATGca-3′, designated Bc, and noncoding strand 5′-tatgCATGTGTATGTAT GTATGTTCTCATACAAAATTACA-3′, designated Bnc) were purchased from Pharmacia (lowercase letters indicate *Nde*I ends). These oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase. The double-stranded B oligodeoxynucleotide was obtained by annealing the labeled Bnc oligonucleotide with an equimolar amount of unlabeled Bc. The oligoribonucleotide corresponding to Bc (5′-UAUGUAAUUUUGUAUGAGAACAUACAUACAU ACACAUGCA-3′, named rBc) was purchased from Genset and labeled as described above.

For the gel retardation assays, 1.5 μg of proteins was preincubated in a final volume of 22 μl for 10 min at room temperature with 30 mM HEPES (pH 7.6), 100 mM KCl, 0.06 mM EDTA, 1 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, 0.7 mM dithiothreitol, 7% glycerol, 3.5% Ficoll 400 (Pharmacia), and 5 μg of sonicated herring sperm DNA as the carrier (heat denatured in the case of single-strand probes). One microliter of end-labeled single- or double-stranded oligonucleotide (0.04 pmol, 10^4 cpm) was then added. After an incubation of 20 min at room temperature, DNA-protein complexes were analyzed by electrophoresis in nondenaturing 4% polyacrylamide gels (acrylamide-bisacrylamide, 29:1) in 0.04 M Tris-acetate–2 mM EDTA buffer at 4°C. For the competitive assays, preincubation was performed with an increasing molar excess of the competitor.

In the case of the oligoribonucleotide probe or competitor, each protein sample was treated with 1 U of RNase block (Stratagene) for 1 h at room temperature before the binding reaction. Yeast RNA (2.5 μg; Boehringer) was added in the preincubation mixture.

After autoradiography, the bands corresponding to bound and free DNAs were cut and counted in scintillation liquid.

Determination of the native size of NssBF by gel filtration. Gel filtration was carried out on a Sephadex G-100 column (8-ml bed volume and 11-cm height) equilibrated with 0.1 M D buffer. The 0.5 M KCl–heparin-agarose fraction containing NssBF (a nuclear single-stranded-DNA binding protein) was concentrated 10-fold by centrifugal filtration with an Ultrafree-MC 10,000 NMWL filter unit (Millipore). A 100-μl volume of the concentrated fraction (150 μg of proteins) was loaded onto the column and eluted with 0.1 M D buffer at 8.4 ml/h. Samples of each fraction were assayed for DNA binding by using the Bc probe.

Missing-contact probing of DNA-protein interactions. Specific contacts between protein and DNA were analyzed by the method of Brunelle and Schleif (3). The Bc oligodeoxynucleotide was end labeled with [γ-^{32}P]ATP, and base modifications were performed. The binding reaction was set up as described above, but a 100-fold molar excess of unlabeled Bc was added just before loading onto the gel. The bound and free DNAs were then separated on a 1% low-melting-point agarose gel, extracted, and subjected to piperidine cleavage. The resulting fragments were analyzed on a 20% sequencing gel.

RESULTS

Identification of a protein that interacts with the single-stranded oligodeoxynucleotide Bc. Assuming that the sequence from positions −168 to −73 located upstream of the TATA box was necessary for 1731 expression, we used several oligodeoxynucleotides covering this region in gel retardation assays to detect the interactions of cellular factors. Nuclear extracts were prepared from *D. melanogaster* 14-XI-23 cells and partially purified by heparin-agarose chromatography. Each fraction of the column was then individually assayed for DNA binding activity. The synthetic oligonucleotide covering positions −110 to −73 (named B for the double-stranded form, Bc for the coding or mRNA-like strand, and Bnc for the noncoding strand) holds our

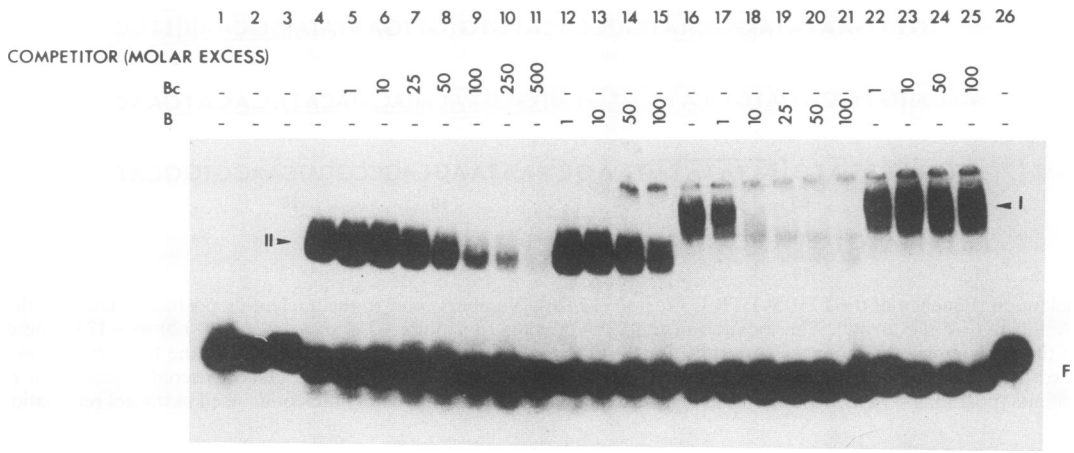

FIG. 2. Binding of factors from the 0.5 M KCl fraction to single-stranded Bc and double-stranded B oligodeoxynucleotides. Autoradiogram of a gel retardation assay examining the 0.5 M KCl–heparin-agarose fraction. Proteins (1.5 μg) were preincubated with 5 μg of herring sperm DNA and competitor DNA as indicated. The labeled oligonucleotide was then added to these extracts and the mixtures were further incubated as described in Materials and Methods. The labeled probes were Bc (lanes 4 to 15), B (lanes 16 to 25), and Bnc (lane 26). Lanes 1, 2, and 3 correspond to the same amount of Bc, Bnc, and B probes incubated without protein extracts, respectively. Complexes I and II (NssBF) and the free DNA (F) are indicated.

attention because the 0.5 M KCl fraction exhibits two distinct binding activities for it, one of them specific to only the Bc strand. No binding activity was observed with the complementary strand Bnc. We then focused on this 0.5 M KCl fraction (Fig. 2). The complexes formed with B and Bc were named complexes I and II, respectively. These complexes are eliminated by treatment with proteinase K, which shows that proteins are involved (data not shown). When comparing the migrations of these two complexes, we observed a lower level of mobility for the B-protein complex (complex I). Since the difference in mobility between the complexes is far greater than that between the free B and Bc probes, it appears that the proteins implicated in each complex are different. Moreover, the proteins implicated in complexes I and II are specific to their own targets, as complex II is undetectable with the B probe and complex I is undetectable with the Bc probe. It is noteworthy that to avoid any ambiguity between single-stranded Bc and double-stranded B binding activities, we labeled the B probe by first labeling the single-stranded oligodeoxynucleotide Bnc (in which no binding activity is detectable) and then annealing the radioactive Bnc with equimolar amounts of single-stranded unlabeled Bc. As indicated by the self-competition experiments (Fig. 2, lanes 5 to 11 and 17 to 21) with the double-stranded B probe, a fivefold molar excess of unlabeled B reduces complex I by 50%, and with the single-stranded Bc probe, a 25-fold molar excess of unlabeled Bc reduces complex II by 50%. By taking into account the isotopic dilution which occurs in the self-competitions, the affinity of protein II for its single-stranded Bc target can be estimated to be about 10^9 M^{-1}, whereas its binding to the complementary strand Bnc is undetectable (Fig. 2, lane 26). This protein was named NssBF for nuclear single-stranded-DNA binding factor. The binding affinity of NssBF for Bc can also be inferred from the concentration of salt needed to inhibit formation of the complex. We therefore incubated the 0.5 M KCl fraction with the Bc probe in the presence of increasing concentrations of KCl. Complex II remains stable at 0.4 M KCl, and concentrations greater than 0.8 M KCl are required to fully dissociate the DNA-protein complex, which corroborates the high level of affinity of NssBF for binding to oligodeoxynucleotide Bc (data not shown). The fact that NssBF does not bind Bnc constitutes an internal negative control, demonstrating that NssBF does not bind all single-stranded DNAs but selectively binds the single-stranded oligodeoxynucleotide Bc. Moreover, NssBF fails to bind other single-stranded oligodeoxynucleotides covering positions −141 to −96 of the LTR (data not shown). Taken together, these results show that NssBF binds single-stranded DNA with sequence specificity. For studying the possible interactions between the factors implicated in complex I and NssBF, reciprocal competitions were also performed. The 0.5 M KCl fraction was incubated with increasing amounts of unlabeled B or Bc before addition of the labeled Bc or B probe, respectively (Fig. 2, lanes 12 to 15 and 22 to 25). With the double-stranded B probe, no competition with as much as a 100-fold molar excess of unlabeled Bc is observed, showing that factor I exhibits a strict double-strand specificity. With the single-stranded Bc probe, a 75-fold molar excess of unlabeled B reduces complex II by 50%, suggesting that the protein which is implicated in complex I might modify the affinity of NssBF for Bc (see Discussion).

Points of contact between NssBF and the single-stranded oligonucleotide Bc. The contact points between native NssBF and single-stranded oligodeoxynucleotide Bc were identified by the method described by Brunelle and Schleif (3). Figure 3A shows the effect of partial depyrimidation (C+T) and depurination (G+A), i.e., random mutagenization, of Bc on the binding. A comparison of the intensities of bands corresponding to bound and free DNAs shows a significant effect when bases in positions −99 to −94 and −88 to −74 are missing. All other positions show either no effect or very weak effects. These data are summarized in Fig. 3B.

Gel filtration chromatography of NssBF. The fraction from the heparin-agarose chromatography which contained the binding activity for Bc, i.e., the 0.5 M KCl fraction, was concentrated and loaded onto a Sephadex G-100 column equilibrated in 0.1 M D buffer. Each fraction was then assayed for binding activity to Bc. The result, shown in Fig. 4, is a peak of Bc binding activity which elutes with an

FIG. 3. Points of contact of NssBF and Bc. (A) Depurination and depyrimidination interference experiments with the 0.5 M KCl–heparin-agarose fraction. Protein-bound and free DNAs are indicated. A+G and C+T: Maxam and Gilbert sequence of the Bc oligodeoxynucleotide as a control. (B) Summary of the results shown in panel A. Vertical lines represent the importance of the corresponding bases for the binding of NssBF. Horizontal arrows emphasize a palindromic sequence.

apparent molecular mass of 95 kDa. Since factor I failed to be detected in the NssBF fractions with B used as a probe, we can conclude that if this factor favors the binding of NssBF to Bc, its presence is not absolutely required.

NssBF does not bind to RNA. Proteins which bind single-stranded DNA sometimes display a significant affinity for single-stranded RNA. Because of the overall structure of retrotransposons (flanked by LTRs with the characteristic U3-R-U5 organization), the nucleotide sequence corresponding to Bc, located upstream from the transcription start site in the 5′ LTR, is repeated and therefore transcribed in the 3′ LTR. We therefore investigated the possibility that NssBF is actually a sequence-specific RNA binding protein that would also bind DNA in in vitro conditions. An oligoribonucleotide corresponding to Bc, rBc, was synthesized and assayed in gel retardation experiments as described in Materials and Methods. The results (Fig. 5) show no binding activity when rBc was used as a probe. Moreover, a 500-fold molar excess of unlabeled rBc does not reduce the formation of complex II (Bc-NssBF) in the presence of the Bc probe, indicating that rBc cannot act as a competitor to Bc. We thus conclude that NssBF is indeed a DNA-specific binding protein.

DISCUSSION

The present data show that a short segment of the 1731 retrotransposon LTR located at positions −110 to −73 with regard to the transcription initiation site is the target of two different proteic factors. This was investigated by using a synthetic oligonucleotide (B) corresponding to this region in gel retardation experiments. One of these factors, named factor I, binds to the double-stranded oligonucleotide B, whereas the other, named NssBF, binds solely to the mRNA-like strand Bc. No binding to the complementary strand Bnc was detected. Functional analysis of the 1731 LTRs has shown that this part of the LTR plays a role in the regulation of 1731 expression (33). More recently, we have shown that the oligodeoxynucleotide B promotes the expression of the chloramphenicol acetyltransferase reporter gene under the control of the thymidine kinase minimal promoter in *Drosophila* cells (32b). It was therefore of biological interest to attempt to characterize these proteins. This paper focused on the analysis of NssBF.

NssBF-Bc complexes are stable at a high ionic strength (0.8 M KCl), indicating a high level of affinity of NssBF for binding single-stranded DNA. Moreover, Scatchard analysis of the self-competition assays performed with Bc (Fig. 2) leads to an apparent affinity constant of 10^9 M^{-1}. The fact that the binding of NssBF decreases when factor I is trapped by the addition of B suggests that this factor may favor the NssBF binding activity. Nevertheless, this is unlikely since B is fully inhibited by only fivefold molar excess of itself. An alternative explanation is that a low percentage of the double-stranded B competitor is partially opened, allowing the binding of NssBF to the accessible coding strand. Preliminary results indicate that this might be the case.

The high level of affinity of NssBF for its single-stranded target allows us to determine its contact points by random mutagenization of Bc. This nuclear protein binds to positions −99 to −74 with regard to the transcription initiation site and presents two single-strand binding domains separated by the five nucleotides GAACA. Deletion of these nucleotides in Bc suppresses the binding of NssBF (data not shown), suggesting that the distance between the domains has to be maintained. The observation that the contact points partially overlap a palindrome (Fig. 5) should indicate that NssBF binds an internal double-strand conformation, but this is unlikely because the complementary strand is not bound by NssBF. A computer search through the GNOMIC data bank

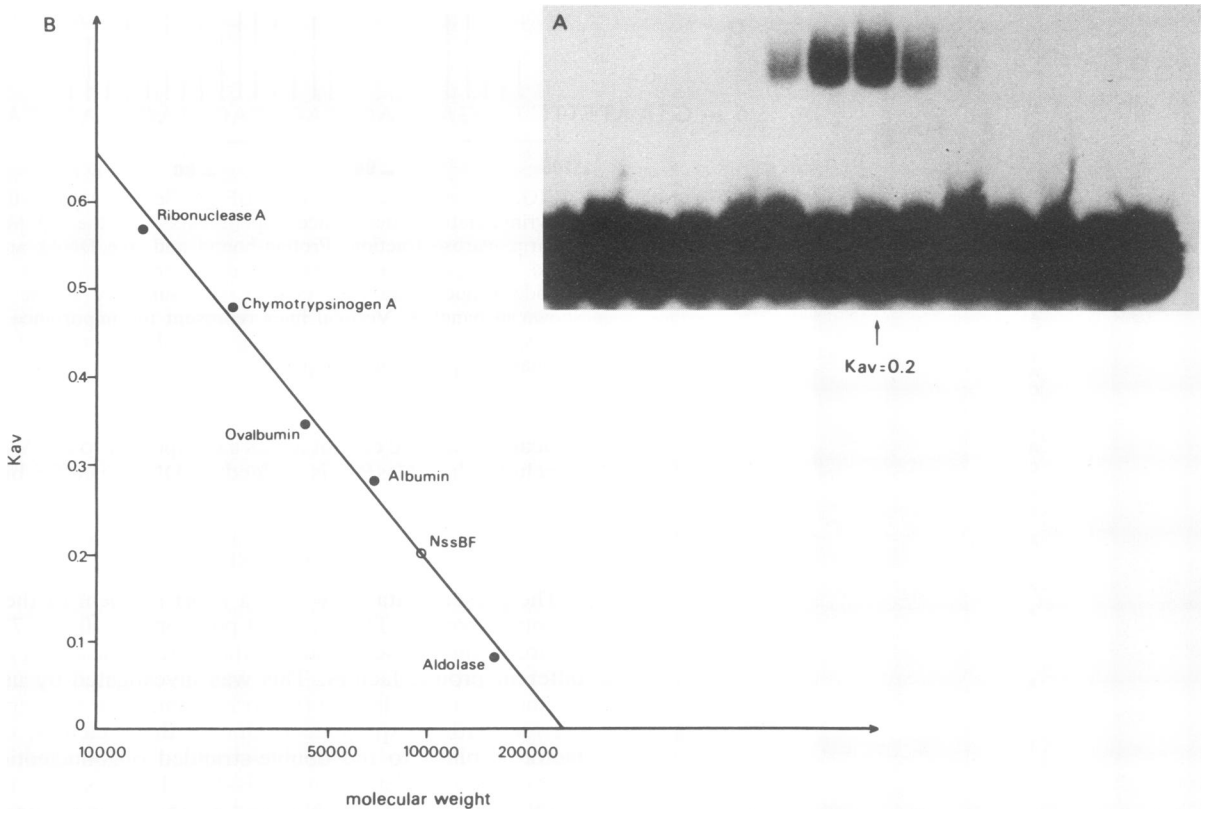

FIG. 4. Determination of the native size of NssBF by gel filtration. The 0.5 M KCl–heparin-agarose fraction was loaded on a Sephadex G-100 column calibrated by standard proteins: aldolase, albumin, ovalbumin, chymotrypsinogen A, and RNase A with molecular weights of 158,000, 67,000, 43,000, 25,000, and 13,700, respectively. (A) Autoradiogram of a gel retardation assay performed with 14 μl of each Sephadex G-100 fraction with Bc used as a probe (see Materials and Methods). (B) Calibration curve (logarithmic scale for molecular weights). Kav = $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for the protein, V_0 is the column void volume, and V_t is the total bed volume.

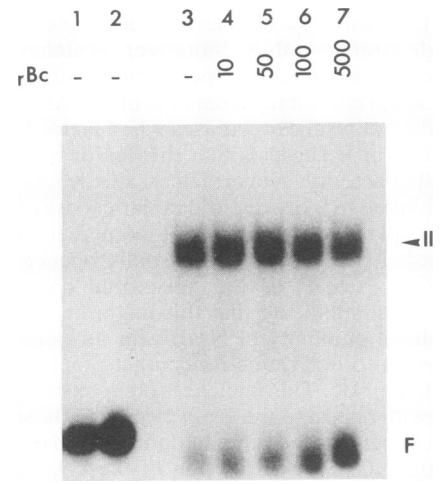

FIG. 5. Interaction of NssBF with RNA. Gel retardation assays were performed with 1.5 μg of protein from the 0.5 M KCl–heparin-agarose fraction with the rBc (lane 2) or the Bc (lanes 3 to 7) probe (see Materials and Methods) and the rBc competitor as indicated. Lane 1 corresponds to the same amount of rBc probe incubated without protein extract. Complex II (NssBF) and free DNA (F) are indicated.

reveals no significant similarities to the NssBF binding site. Gel filtration chromatography shows that NssBF is eluted as a single peak, and this allows us to determine an apparent molecular mass of 95 kDa for NssBF. Moreover, it indicates that NssBF binding does not require the presence of factor I or the presence of other factors such as ATP. Whole-cell extracts of *Drosophila hydei* and *Drosophila virilis*, two species whose genomes do not harbor 1731, show the same single-stranded-DNA binding activity as the extracts from *D. melanogaster* (unpublished results). We can thus conclude that NssBF is not encoded by 1731 itself but is the product of a cellular gene. Lastly, as explained in Results, it was of obvious interest to show that NssBF does not bind to RNA. In both prokaryotic and eukaryotic cells, the existence of single-stranded-DNA binding proteins has been reported. Many of them belong to the helix-destabilizing class of proteins and exhibit the ability to bind to single-stranded DNA without sequence specificity, a property shared by the RecA protein of *Escherichia coli* (5, 29). These proteins are known to be involved in DNA replication, recombination, and repair and/or transcriptional regulation, for example, by stimulating DNA polymerase (2, 4, 16, 19, 23, 24). More recently, there have been some reports on the presence of single-stranded-DNA binding proteins that also exhibit sequence specificity (9, 10, 13, 15, 18, 22, 25, 27, 32). These proteins have been shown to be involved in several functions such as replication (13, 32), transcription (14, 18, 22, 25), or recombination (9).

Interestingly, the NssBF binding site partially overlaps a long purine-pyrimidine alternative stretch (positions −91 to −73 and −71 to −51) (Fig. 1). Such sequences are known to adopt a transient Z-DNA conformation (30) possibly involved in gene transcription (reference 26 and references therein). Moreover, the single-stranded character of the B-Z junction has been reported (reference 31 and references therein). We can thus hypothesize that NssBF binds in vivo to DNA rendered single stranded at a B-Z junction. For these reasons, added to its specific binding to a U3 sequence which is involved in the promoting of 1731 expression, it is proposed that NssBF might play a transactivator role in transcription.

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