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At Least Two Nuclear Proteins Bind Specifically to the Rous Sarcoma Virus Long Terminal Repeat Enhancer

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We used the sensitive gel electrophoresis DNA-binding assay and DNase I footprinting to detect at least two protein factors (EFI and EFII) that bound specifically to the Rous sarcoma virus (RSV) enhancer in vitro. These factors were differentially extracted from quail cell nuclei, recognized different nucleotide sequences in the U3 region of the RSV long terminal repeat, and appeared to bind preferentially to opposite DNA strands as monitored by the DNase I protection assay. The EFI- and EFII-protected regions within U3 corresponded closely to sequences previously demonstrated by deletion mutagenesis to be required for enhancer activity, strongly suggesting a functional significance for these proteins. Only weak homologies between other enhancer consensus sequence motifs and the EFI and EFII recognition sites were observed, and other viral enhancers from simian virus 40 and Moloney murine sarcoma virus did not compete effectively with the RSV enhancer for binding either factor.

Enhancers are a class of *cis*-acting regulatory DNA sequences that can activate transcription from both homologous and heterologous eucaryotic promoters in a relatively orientation-, position-, and distance-independent manner. Enhancers have been identified in many viral genomes (for reviews, see references 23, 43, 55) and near or within a variety of cellular genes (1, 13, 22, 24, 26, 41, 44, 60 64), where they often potentiate transcription in a cell type-specific, and sometimes inducible, fashion. Different enhancer elements bear no extensive sequence homologies, although several short, degenerate consensus sequences occur in certain subsets of enhancers (27, 38, 68, 71, and references therein). Despite the lack of sequence homology, enhancers do seem to share an organizational consensus. Extensive site-specific mutagenesis and deletion analyses have established that the simian virus 40 (SV40) and polyomavirus enhancers (28, 29, 71), as well as several other well-characterized enhancers (4, 14, 32, 46, 67), are mosaics of multiple different and often redundant sequence motifs. Each relevant sequence motif appears to be contained within a separable functional domain that possesses little enhancer activity unless combined with another domain. The functional domains of an enhancer, although nonhomologous, can compensate for each other in satisfying this multiplicity requirement (29, 30, 32, 63, 65, 71).

The mechanism(s) by which this modular arrangement of multiple short sequence motifs synergistically leads to the long-range activation of transcription from a promotor is not known for any enhancer. However, evidence from both functional (36, 50, 52, 54, 69) and structural (6, 16) studies has been rapidly accumulating that the action of viral and cellular enhancers involves their interaction with specific protein factors. Recently, the interaction of such *trans*-acting factors in nuclear extracts with specific domains of the SV40 (70), polyomavirus (3, 18, 45), and insulin gene (40) enhancers has been directly demonstrated in vitro by DNase I footprinting, exonuclease III protection, or retardation of DNA electrophoretic mobility assays. The identity of these protein factors is not yet known, nor has their role in conferring enhancer activity been evaluated. Nonetheless, the purification and characterization of *trans*-acting factors for a variety of enhancers will be critical for establishing the mechanism(s) by which modular arrays of *cis*-acting sequences can interact with protein factors and cooperatively act to enhance transcription.

It is to this end that we have endeavored to identify proteins that may bind in a sequence-specific manner to the enhancer of an avian retrovirus, Rous sarcoma virus (RSV) (8, 32, 33). The RSV enhancer is a pivotal component of the strong transcriptional control region located within the long terminal repeat (LTR) sequences flanking either end of the proviral DNA (9). In conjunction with promotor sequences, the RSV enhancer provides for very high levels of expression of the viral genome; up to 10 to 20% of total polyadenylated RNA can be virus specific in infected avian cells (62 and references therein). As a consequence of its incorporation into this strong transcriptional unit, efficient expression of the viral oncogene *src* is assured. However, even among RNA tumor viruses that lack their own transforming gene, the retroviral enhancer may play a significant role in oncogenic strategies (7, 39, 42, 47, 61, 66).

The activity of the RSV enhancer is not confined to avian cells (25). In fact, its existence was first confirmed by its ability to augment transcription of the herpes simplex virus (HSV) thymidine kinase gene after stable transfection of linked constructs into mouse L cells (33). Subsequent analysis by deletion mutagenesis has demonstrated that the RSV enhancer is also composed of multiple functional domains (32). Two domains reside within the LTR, while a third may lie immediately upstream of the LTR, in sequences not conserved among different strains of the virus. Alternatively, it has been suggested that the RSV enhancer lies entirely within the LTR (8).

In any case, we have detected two protein factors which bind specifically to the Schmidt-Ruppin A (SR-A) RSV LTR. They can be distinguished by the significantly different ionic strengths required for their extraction from avian cell nuclei and by the different functional domains of the RSV enhancer

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to which they bind independently in vitro. We used DNase I footprinting to map the nucleotide sequences recognized by each protein factor and found that EFI and EFII asymmetrically protected 25- to 30-nucleotide regions on alternate DNA strands of the RSV LTR enhancer. The protected regions reside within that portion of U3 shown previously to be required for RSV enhancer activity. The EFI- and EFII-protected nucleotides had only weak homologies with several consensus sequence motifs known to be essential for the activity of other enhancers, and both protein factors appeared to bind specifically to only the RSV LTR enhancer.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extracts were prepared from QT-6 cells (a line of chemically transformed but uninfected quail fibroblasts [37]) maintained in medium 199 supplemented with 10% tryptose phosphate broth, 0.14% sodium bicarbonate, 5% fetal calf serum, and 1% chicken serum. Cells were harvested from monolayer cultures by brief incubation at 37°C in phosphate-buffered saline containing 1 mM EDTA and collected by centrifugation. All subsequent steps were performed at 4°C. The cell pellet was washed once in phosphate-buffered saline and then suspended in 10 volumes of a solution containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 8, 0.5 M sucrose, 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per liter, 0.1 μM pepstatin, and 10 mM benzamidine (buffer A). After vigorous vortexing, nuclei were collected by centrifugation at 1,000 × *g* for 5 min, suspended in 10 volumes of buffer A, and collected again by centrifugation. The nuclear pellet was then suspended by vortexing in 1 ml of buffer B (10 mM HEPES, pH 8, 25% glycerol, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 7 mM β-mercaptoethanol, and protease inhibitors) per 10^8 cells. The mixture was placed on ice for 15 to 30 min and vortexed every 5 min. After centrifugation at 1,000 × *g* for 5 min, the supernatant was saved for dialysis as described below, and the nuclear pellet was suspended in buffer B (1 ml/10^8 cells) except that the NaCl concentration was 300 mM. After incubation on ice as described above, the mixture was collected at 1,000 × *g* for 10 min and the supernatant was removed for dialysis. The nuclear pellet was suspended in buffer B (1 ml/10^8 cells) containing 530 mM NaCl. After incubation on ice as described above, the mixture was collected at 4,000 × *g* for 10 min. The supernatants from this and previous extractions were dialyzed overnight against 10 mM HEPES (pH 8)–7 mM β-mercaptoethanol–50 mM NaCl–50% glycerol.

In some cases extracts were additionally fractionated by $(NH_4)_2SO_4$ precipitation. The glycerol concentration of the extract was lowered to less than 5% by addition of 10 mM HEPES (pH 8)–7 mM β-mercaptoethanol–50 mM NaCl before slowly adding 472 mg of solid $(NH_4)_2SO_4$ per ml. The mixture was then supplemented with 1 μl of 1 N NaOH per g of $(NH_4)_2SO_4$ added and maintained on ice for 1 h. The precipitate was collected by centrifugation at 13,200 × *g* for 30 min and dissolved in 10 mM HEPES (pH 8)–7 mM β-mercaptoethanol–50 mM NaCl before dialysis as described above. Extracts were stored at −14°C, and final protein concentrations ranged from 0.5 to 4.0 mg/ml as determined by the method of Schaffner and Weissman (51), with bovine serum albumin as the standard.

Preparation of radiolabeled DNAs. The plasmid pRSV-LTR was derived from pPvuIIDG (58) by digestion with *Pvu*I, religation, and transformation into the *Escherichia coli* host DH-1. Transformants were screened for a plasmid containing a single copy of the 330-base-pair (bp) LTR (pRSV-LTR).

To obtain probe DNA for gel electrophoretic mobility binding assays, pRSV-LTR was digested with *Eco*RI and *Mlu*I prior to end-labeling with [α-^{32}P]dATP and *E. coli* DNA polymerase I (New England Biolabs) in a buffer containing 70 mM Tris, pH 7.4, 10 mM magnesium acetate, 70 mM NaCl, and 100 μg of autoclaved gelatin per ml at 4°C. A 600 μM concentration of each of the four nonradiolabeled deoxynucleotides was added before terminating the reaction to generate blunt-ended DNA molecules. A 286-bp DNA fragment containing the RSV enhancer (referred to elsewhere as the 280-bp fragment) was then purified by polyacrylamide gel electrophoresis and subsequent electroelution of DNA from the gel fragment.

DNase I footprinting of plus-strand DNA was performed with the 280-bp *Eco*RI-*Mlu*I DNA fragment end-labeled at the *Eco*RI site as described above. For footprinting of the EFI complex on minus-strand DNA, *Eco*RI-digested pRSV-LTR was treated with bacterial alkaline phosphatase prior to labeling with polynucleotide kinase (International Biotechnologies, Inc.) and [γ-^{32}P]ATP as recommended by the manufacturer. After digestion with *Mlu*I, the 280-bp fragment was purified by electrophoresis as described above. To obtain EFII, III footprints on minus-strand DNA, *Eco*RI- and *Mlu*I-digested pRSV-LTR was end-labeled at the *Mlu*I site with [α-^{32}P]dCTP, [α-^{32}P]dGTP, and DNA polymerase I prior to purification of the 280-bp fragment by acrylamide gel electrophoresis.

Preparation of competitor DNAs. A 276-bp DNA fragment from pBR322 was prepared by treating pBR322 with *Bam*HI and *Sal*I. The digestion products were separated on a preparative polyacrylamide gel, and the 276-bp fragment was recovered by electroelution. The RSV LTR promoter-containing fragment was obtained by digesting pRSV-LTR with *Eco*RI and *Sac*I followed by isolation of the 314-bp fragment by acrylamide gel electrophoresis. The SV40 enhancer-containing fragment was prepared by digesting SV40 form I DNA with *Pvu*II and *Bgl*I, followed by isolation of a 278-bp fragment containing the SV40 enhancer and also including the 21-bp repeats and TATA box consensus sequence. SV40 DNA was obtained by infection of CV-1 cells with SV40 strain WT800 followed by purification of form I DNA by standard procedures. A fragment containing the Moloney murine sarcoma virus (MSV) enhancer was excised from pBR-MSVLTR by digesting with *Cla*I and *Xba*I followed by isolation of a 438-bp fragment containing the enhancer. pBR-MSVLTR is a plasmid constructed in this laboratory containing a segment of Harvey MSV DNA (15) from *Cla*I (nucleotide 2380) to *Bam*HI (nucleotide 3330) cloned at the *Cla*I site of pBR322. This MSV DNA segment was obtained from pMV-5/srcA, a plasmid supplied by P. Luciw, Chiron Corp.

Gel electrophoretic mobility shift assay. Samples of nuclear extracts were mixed with 600 to 950 ng of poly(dI) · poly(dC) (Pharmacia) for 5 min prior to the addition of approximately 0.25 ng of ^{32}P-labeled 280-bp RSV enhancer-containing fragment. Poly(dI) · poly(dC) was dissolved in 10 mM Tris (pH 7.5)–1 mM EDTA–100 mM NaCl and treated as described by the manufacturer except that it was also briefly sonicated (microtip, setting 3, 30 s, Branson Heat Systems sonicator) before use. Incubations were performed at room tempera-

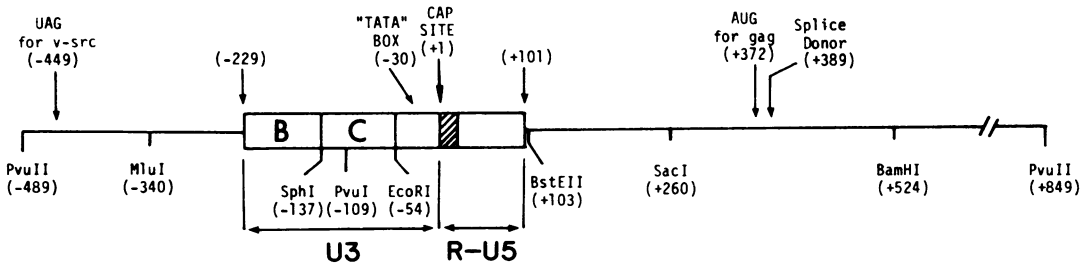

FIG. 1. RSV DNA encompassing the LTR. Diagram of viral DNA in pRSV-LTR. A 1,338-bp insert of SR-A RSV DNA cloned in the *Pvu*II site of pBR322 and containing the 330-bp LTR plus flanking sequences is shown. Numbering is with reference to the initiation site for RSV RNA at the beginning of R. R (+1 to +21) is a 21-bp sequence found at both ends of RSV RNA. U3 (229 bp) and U5 (80 bp) are sequences unique to the 3′ and 5′ ends, respectively, of viral RNA. Functional domains B and C of the RSV enhancer as defined by Laimins et al. (32) are indicated. Remaining data are from Swanstrom et al. (58, 59) and Czernilofsky et al. (10). The number assigned to each restriction site refers to the 5′ base at the position of cleavage within the recognition sequence.

ture for 30 min in a final volume of 20 μl containing 7.5 mM HEPES, pH 8, 2.5 mM Tris, pH 7.5, 0.8 mM EDTA, 5 mM β-mercaptoethanol, 62.5 mM NaCl, 10% glycerol, and, when applicable, 3.3 mM $MgCl_2$. Samples were layered onto 4% polyacrylamide gels (30:1 acrylamide-bisacrylamide weight ratio) containing 25 mM Tris, 190 mM glycine, and 1 mM EDTA, pH 8.5. Gels were electrophoresed in the same Tris-glycine buffer at room temperature for 2 h at 10 V/cm. They were then transferred to Whatman 3MM paper, dried, and autoradiographed.

DNase I footprinting. Samples of nuclear extracts (or bovine serum albumin) were incubated with poly(dI) · poly(dC) and ^{32}P-labeled 280-bp DNA with eight times as much material as described above in an 80-μl final volume for 30 min at room temperature. If necessary, $MgCl_2$ was added to 3.3 mM final concentration, and the samples were treated with DNase I (Worthington, 2,227 U/mg) at final concentrations of between 2 and 6 μg/ml for 1 min at room temperature. Digestions were terminated by adding EDTA to a final concentration of 12 mM and placing the samples on ice. Samples were then loaded onto preparative Tris-glycine-polyacrylamide gels and electrophoresed as described above. Protein-DNA complexes were located by autoradiography of the wet gel at 4°C. Radioactive complexes were excised from the gel, and crushed gel slices were shaken overnight at room temperature in 0.5 M sodium acetate (pH 7)–0.1 mM EDTA–0.1% sodium dodecyl sulfate–20 μg of proteinase K per ml–10 μg of tRNA per ml. Supernatants were collected, filtered through glass wool, and extracted with phenol-chloroform (1:1). After an additional extraction with chloroform-isoamyl alcohol (25:1), the DNA was precipitated twice with ethanol before suspension in 80% (vol/vol) formamide–50 mM Tris-borate–1 mM EDTA, pH 8.3. DNA samples were denatured at 100°C for 3 min and applied to 8% polyacrylamide–7.7 M urea sequencing gels containing 13% formamide. Chemical cleavage of labeled DNAs at purine residues was performed as described (35).

RESULTS

Detection of proteins that bind to the RSV LTR enhancer. A 1,338-bp molecular clone of RSV DNA encompassing the viral LTR is diagrammed in Fig. 1. This *Pvu*II DNA fragment was originally obtained from a permuted, linear, full-length clone of unintegrated SR-A RSV DNA containing two copies of the LTR (12). One copy of the LTR was subsequently deleted by standard cloning techniques, so that the remaining LTR in pRSV-LTR is flanked upstream by viral DNA found at the 3′ end of the virus and downstream by sequences normally found 3′ of U5 at the 5′ end of the viral genome. Transcription of the viral genome is initiated within the LTR at the R sequence (59 and references therein). An element required for accurate initiation among a wide variety of eucaryotic promotors, the TATA box, is located within U3 sequences at −30 (58). Transcription of RSV LTR DNA in a transcriptional extract (23) in vitro demonstrates that sequences 3′ of the *Eco*RI site (position –54) are sufficient to direct accurate initiation of this promotor in vitro (B. Delorbe, personal communication).

In contrast, sequences 5′ of the *Eco*RI site (–54) to the beginning of U3 (–229) have been demonstrated by deletion mutagenesis studies (9, 32, 33) and enhancer trap studies (66) to constitute the minimal DNA segment exhibiting enhancer activity. More specifically, the work of Luciw et al. (33) and Cullen et al. (8, 9) has indicated that the 3′ boundary of the RSV enhancer may lie at about –136 to –143, while the 5′ boundary of the enhancer, at least in the SR-A strain of RSV, may lie slightly outside U3 to approximately 11 bp upstream. However, Cullen et al. (8, 9) propose that the RSV enhancer lies entirely within the LTR, and Laimins et al. (32) observed a requirement for sequences immediately upstream of the LTR only if sequences 3′ to –139 have been deleted. Significantly, the results of Laimins et al. (32) were obtained with the Prague strain of RSV, in which the sequences immediately upstream of U3 differ from those found in the analogous position of the SR-A strain that were implicated by Luciw et al. (33) in enhancer function.

Due to the uncertainties involved in defining the exact boundaries of the RSV LTR enhancer, a DNA fragment from pRSV-LTR certain to encompass the extremities of this element (but no other known transcriptional control sequences) was chosen for studies aimed at detecting proteins which bind specifically to the RSV LTR enhancer. Thus, an approximately 280-bp *Mlu*I-*Eco*RI DNA fragment extending from positions −54 to −340 was isolated, end-labeled, and mixed with various extracts obtained from QT-6 cell nuclei by sequential washing with 0.1, 0.3, and 0.53 M NaCl. The mixtures were then analyzed on low-ionic-strength polyacrylamide gels. DNA fragments bound to protein migrate more slowly through such gels than unbound DNA fragments (17, 20), and in the presence of an agent to minimize nonspecific protein-DNA interactions, such as poly(dI) · poly(dC), discrete mobility shifts arising from specific protein-DNA interactions can be observed (3, 5, 45, 56, 57).

We tested each of the three nuclear extracts (or these extracts after further ammonium sulfate fractionation) for their ability to generate discrete electrophoretic mobility

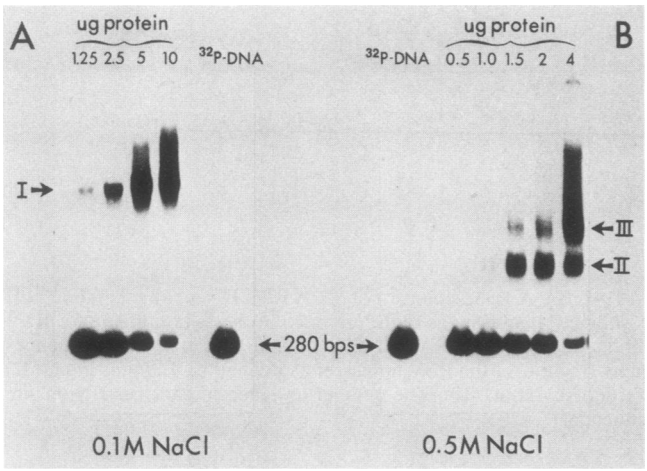

FIG. 2. Identification of proteins which bind to RSV enhancer-containing DNA. (A) End-labeled 280-bp *Mlu*I-*Eco*RI DNA fragment from pRSV-LTR was mixed in the presence of 1 μg of poly(dI) · poly(dC) with increasing amounts of protein extracted from QT-6 cell nuclei by 100 mM NaCl as described in the text. After analysis of the samples on a 4% Tris–glycine–polyacrylamide gel, the gel was dried and exposed to X-ray film. (B) Same as panel A except that proteins were extracted from quail cell nuclei between 0.3 and 0.53 M NaCl and then further fractionated by $(NH_4)_2SO_4$ precipitation. Complexes I, II, and III are indicated.

shifts with ^{32}P-labeled 280-bp DNA in the presence of various amounts of poly(dI) · poly(dC) and in the presence and absence of divalent cations such as Mg^{2+} and Zn^{2+} (Fig. 2). A constant amount of radiolabeled 280-bp enhancer-containing DNA fragment was mixed with increasing amounts of QT-6 nuclear protein obtained by 0.1 M NaCl extraction (Fig. 2A). In the absence of extract, the 280-bp DNA migrated as a single band, as expected. In the presence of extract, another distinct band of slower mobility (band I) was observed which increased proportionally as additional extract was added to the DNA.

A different set of additional bands was observed when the 280-bp RSV enhancer DNA fragment was mixed with increasing amounts of QT-6 cell protein extracted from nuclei between 0.3 and 0.53 M NaCl. A single discrete band of retarded mobility (band II) was observed when a small amount of this nuclear extract was mixed with the 280-bp DNA (Fig. 2B). Band II increased in proportion as more extract was added to the DNA, and another discrete band exhibiting an even greater reduction in mobility (band III) was observed. Bands II and III were not readily detected unless the QT-6 0.3 to 0.53 M NaCl extract was further fractionated by precipitation with 70% $(NH_4)_2SO_4$ (hereafter referred to as the 0.5 M NaCl extract). Moreover, the formation of bands II and III was highly dependent on the addition of $MgCl_2$ (2.5 mM) to the 0.5 M NaCl extract-DNA incubation (Fig. 2B). In contrast, band I (Fig. 2A) formed equally well in the presence and absence of divalent cations, although incubations with the 0.1 M NaCl extract were generally performed in the absence of $MgCl_2$ to minimize nuclease activity. Finally, proteinase K treatment of the 0.1 and 0.5 M NaCl extracts abolished the formation of band I or II and III, respectively (data not shown), demonstrating that these complexes represented protein-DNA interactions.

The gel assay failed to detect the formation of any distinct complexes of reduced electrophoretic mobility when proteins extracted from QT-6 nuclei between 0.1 and 0.3 M NaCl were mixed with the 280-bp enhancer DNA before or after $(NH_4)_2SO_4$ fractionation of the extract and under any specific incubation conditions we tested. We have not examined extracts obtained from nuclei by treatment with NaCl concentrations above 0.53 M due to the eventual complicating presence of some core histone proteins in such extracts. However, at least two different protein factors capable of binding DNA containing the RSV LTR enhancer appeared to be present in QT-6 cell nuclei. These factors differ in the affinity with which they are associated with QT-6 nuclei and in the electrophoretic mobility of the complexes they form with RSV enhancer-containing DNA.

At least two protein factors bind specifically to the RSV LTR enhancer. To determine whether complexes I, II, and III represent specific protein-DNA interactions, a series of competitions with various unlabeled DNA fragments were performed. In these experiments, increasing amounts of the unlabeled DNAs were mixed with either the 0.1 or 0.5 M NaCl extracts immediately prior to the addition of a constant amount of radiolabeled 280-bp enhancer DNA. Figure 3A presents the results of three competition experiments performed with the 0.1 M NaCl extract. When the 280-bp *Mlu*I-*Eco*RI DNA fragment containing the RSV enhancer was used as a competitor, formation of complex I rapidly disappeared as the concentration of competitor DNA increased. However, when a similar-sized DNA fragment obtained from pBR322 (*Bam*HI-*Sal*I, 276 bp) was included in the incubation, a 50- to 100-fold excess of pBR322 DNA did not affect the formation of complex I. Apparently, the protein(s) combining with radiolabeled 280-bp DNA to form complex I was recognizing sequences specifically found in the enhancer-containing DNA fragment. This conclusion was substantiated by a third experiment in which a 314-bp *Eco*RI-*Sac*I restriction fragment from pRSV-LTR was used as the competitor. This DNA fragment (Fig. 1) contained the "promotor" elements of the viral LTR, including a consensus binding site for the TATA box transcription factor (11) and the start site of transcription. The fragment extends 3′ 160 nucleotides into the 5′ untranslated region of the viral transcription unit. The presence of up to 50- to 100-fold excess of this 314-bp DNA fragment in the incubation did not disrupt the formation of complex I (Fig. 3A).

Similar results were obtained for competition experiments with the 0.5 M NaCl extract. Neither a 100-fold excess of the 276-bp pBR322 DNA fragment nor the 314-bp LTR promotor fragment competed with radiolabeled enhancer DNA for binding the proteins involved in forming complexes II and III. Only DNA containing the RSV enhancer eliminated complex formation with radiolabeled DNA. Thus, we have identified at least two proteins extractable from QT-6 nuclei at different ionic strengths which bind specifically to sequences in a 280-bp DNA fragment containing the RSV enhancer.

Protein complexes involving different domains of the RSV LTR enhancer. Although we have demonstrated that at least two different quail cell proteins can bind specifically to the 280-bp *Mlu*I-*Eco*RI DNA fragment, we cannot yet definitively conclude that these proteins bind to the RSV enhancer due to the presence of at least some DNA sequences outside the 5′ boundary of the enhancer in the 280-bp fragment. Therefore, we next attempted to more specifically localize binding domains for the proteins within the 280-bp DNA fragment. The recent work of Laimins et al. (32) has divided the RSV LTR enhancer (Prague strain) into three separate functional domains: A, B, and C. The A domain lies immediately 5′ to the LTR, possibly extending as far as 65 bp upstream. (Different sequences occupy an analogous posi-

tion in the DNA of the SR-A strain used in this work, although domain A sequences are found upstream of the *src* gene.) The B and C domains are sufficient for enhancer function, although A will substitute for C, as will a duplication or triplication of B alone. To localize the binding domains of the proteins in complexes I, II, and III and also to establish whether these proteins recognized the same or different functional domains of the RSV LTR enhancer, another set of competition experiments was performed.

We dissected the 280-bp LTR enhancer DNA fragment into C and B+A (plus additional upstream sequences) domains by appropriate restriction enzyme digests followed by isolation of an approximately 200-bp *Mlu*I-*Sph*I (A+B domain) fragment and an approximately 80-bp *Sph*I-*Eco*RI (C domain) fragment. The 80- and 200-bp subfragments were then assayed for their ability to compete with the entire enhancer for combining with proteins in the 0.1 or 0.5 M NaCl extracts to form complexes I or II and III, respectively. The protein(s) present in the 0.1 M NaCl extract responsible for forming complex I recognized sequences present in the C domain of the RSV LTR enhancer, although the 80-bp fragment competed somewhat less efficiently than the entire enhancer for protein binding (Fig. 4A). In any case, the B domain and sequences upstream have no effect on complex I formation.

In contrast, the proteins in the 0.5 M NaCl extract which bound specifically to the 280-bp enhancer-containing DNA

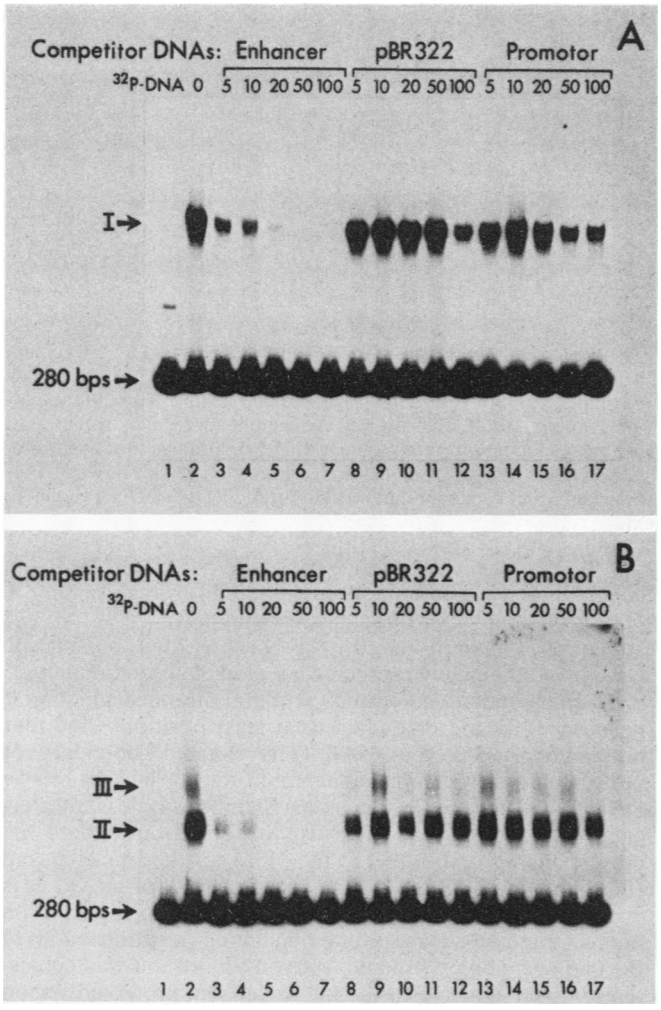

FIG. 3. Complexes I, II, and III form specifically with RSV enhancer-containing DNA. (A) End-labeled 280-bp DNA fragment containing the RSV enhancer (lane 1) was mixed with 1 μg of poly(dI) · poly(dC) and 2.5 μg of protein extracted from QT-6 cell nuclei by 0.1 M NaCl in the absence (lane 2) or presence (lanes 3 to 17) of 5- to 100-fold molar excess of nonradiolabeled DNA fragments as indicated. Enhancer, 280-bp *Mlu*I-*Eco*RI fragment from pRSV-LTR; pBR322, 276-bp *Bam*HI-*Sal*I fragment; promotor, 314-bp *Eco*RI-*Sac*I fragment from pRSV-LTR. After analysis of samples on a 4% Tris–glycine–polyacrylamide gel, the gel was dried and exposed to X-ray film. (B) Same as panel A except that 1.5 μg of 0.5 M NaCl nuclear extract protein was used.

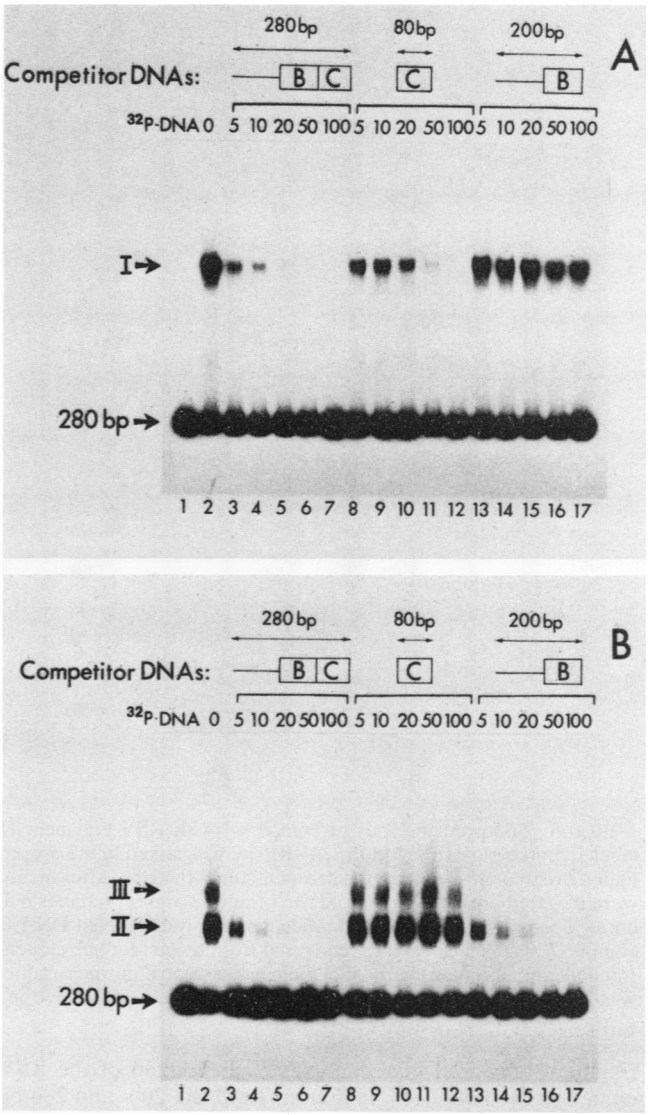

FIG. 4. Proteins in complexes I and II-III recognize different domains of the RSV enhancer. (A) End-labeled 280-bp *Mlu*I-*Eco*RI DNA fragment (lane 1) containing the RSV enhancer was mixed with 1 μg of poly(dI) · poly(dC) and 2.5 μg of protein extracted from QT-6 nuclei by 0.1 M NaCl in the absence (lane 2) or presence (lanes 3 to 17) of 5- to 100-fold molar excess of nonradiolabeled DNA fragments from pRSV-LTR containing the entire RSV enhancer (*Mlu*I-*Eco*RI, 280 bp), the C domain (*Sph*I-*Eco*RI, 80 bp), or the B domain plus additional upstream sequences (*Mlu*I-*Sph*I, 200 bp) as indicated. After electrophoresis of samples on a 4% Tris–glycine–polyacrylamide gel, the gel was dried and exposed to X-ray film. (B) Same as panel A except that 1.5 μg of 0.5 M NaCl nuclear extract proteins was used.

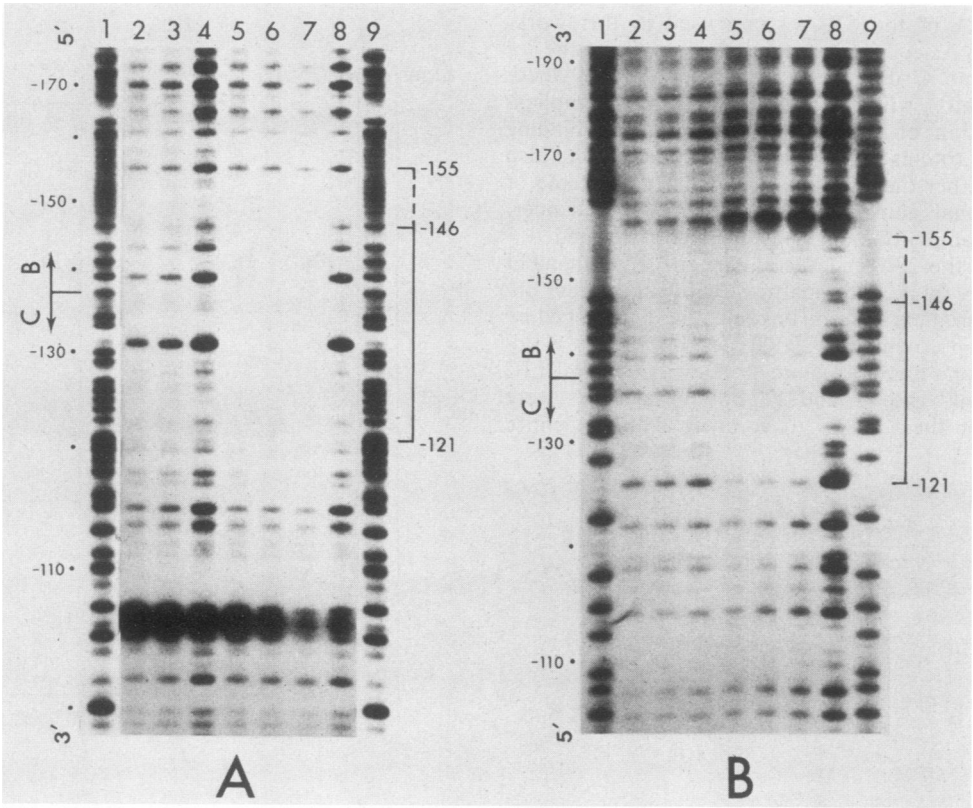

FIG. 5. Footprint analysis of complex I with RSV enhancer DNA. (A) Approximately 2.5 ng of a 280-bp *Eco*RI-*Mlu*I DNA fragment labeled on the plus-strand at the *Eco*RI site was mixed in the presence of 8 μg of poly(dI) · poly(dC) with 40 μg of 0.1 M NaCl protein extract (lanes 2 to 7) or 40 μg of bovine serum albumin (lane 8). After incubation at room temperature, $MgCl_2$ was added to 3.25 mM, and the samples were digested with 4 (lanes 2 and 5), 5 (lanes 3 and 6), 6 (lanes 4 and 7), or 2 (lane 8) μg of DNase I per ml. Samples were electrophoresed on a 4% Tris–glycine–polyacrylamide gel, and radiolabeled DNA fragments corresponding to the free-migrating band (lanes 2 to 4 and 8) or complex I (lanes 5 to 7) were recovered from the gel for subsequent analysis on an 8% polyacrylamide–urea sequencing gel as shown. Lanes 1 and 9 contain the products of chemical cleavage at purine residues. (B) Same as panel A except that the 280-bp *Eco*RI-*Mlu*I DNA fragment was labeled on the minus-strand at the *Eco*RI site as described in the text.

fragment appeared to recognize the B domain of the RSV enhancer or sequences upstream. Both the 200- and 280-bp DNA fragments competed equally well with radiolabeled DNA for binding proteins involved in forming complexes II and III. The 80-bp C domain had no effect on the formation of either complex. Thus, the proteins we had previously distinguished by extractability from QT-6 nuclei and by the ability to specifically form complexes of different electrophoretic mobilities with 280-bp RSV enhancer-containing DNA apparently recognized different domains of that 280-bp DNA as well.

Identification of nucleotide sequences protected in RSV enhancer-protein complexes. DNase I footprinting of protein-DNA complexes (19) is a widely used technique for precisely delineating the nucleotide sequences protected from cleavage by specific protein binding. It can be coupled with the gel electrophoretic assay described above to identify the exact nucleotides protected in specific protein-DNA complexes under conditions in which unprotected DNA may be in excess. We subjected complexes I, II, and III to DNase I footprinting analyses (Fig. 5 and 6). End-labeled 280-bp DNA containing the RSV enhancer was incubated with either the 0.1 or 0.5 M NaCl extract and treated briefly with DNase I prior to analysis on low-ionic-strength polyacrylamide gels. Nuclease treatment had no significant effect on the intensity or mobility of the complexes detected. Complexes I, II, and III were excised from the gels and radiolabeled DNA was subsequently recovered for analysis on 8% polyacrylamide–7.7 M urea sequencing gels.

The protein(s) in the 0.1 M NaCl extract comprising complex I completely protected a region of approximately 25 to 35 nucleotides on the plus (coding) strand beginning at position −121 and extending to at least position −146, but not beyond −155 (Fig. 5A). (The exact 5′ boundary of protection is not clearly demarcated due to lack of strong DNase I cutting within this area.) Significantly less interference with DNase I cleavage was observed throughout the corresponding region (−122 to −155) on the minus-strand (Fig. 5B, lanes 5 to 7). We consistently observed less protection in several attempts to footprint complex I on minus-strand DNA. Also, one nucleotide (position −158) at the end of a polypyrimidine tract on this strand was consistently hypersensitive to the nuclease. The protected region on the coding strand included the *Sph*I site at position −137 and thus overlapped the boundary between functional domains B and C of the RSV enhancer as described by Laimins et al. (32). The decreased ability of the 80-bp C domain fragment to act as a competitor in comparison to the 280-bp fragment containing the entire enhancer (Fig. 4A) may reflect the fact that the last 10 to 19 nucleotides protected by the protein(s) in complex I were missing from the 80-bp fragment.

The proteins in the 0.5 M NaCl extract which had been shown to bind specifically to the B domain of the LTR

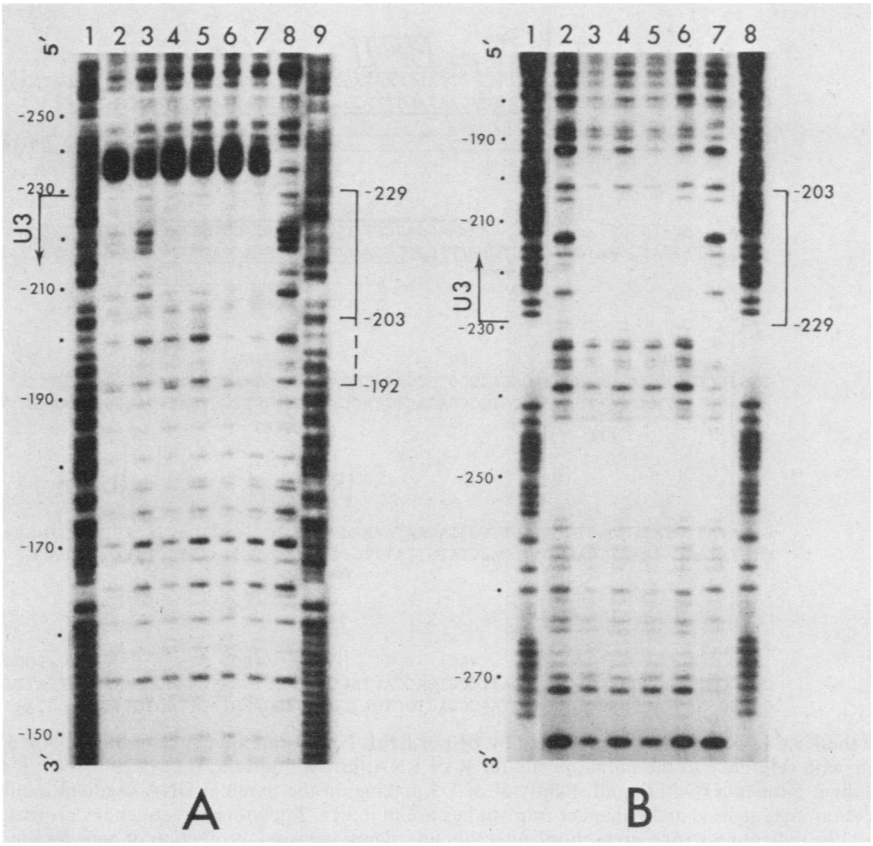

FIG. 6. Footprint analysis of complexes II and III with RSV enhancer DNA. (A) Approximately 2.5 ng of a 280-bp *Eco*RI-*Mlu*I DNA fragment labeled on the plus-strand at the *Eco*RI site was mixed in the presence of 5 μg of poly(dI) · poly(dC) with 16 μg of 0.5 M NaCl protein extract (lanes 2 to 7) or 40 μg of bovine serum albumin (lane 8). After incubation at room temperature the samples were digested with 2 (lane 8), 3 (lanes 2, 4, and 6) or 5 (lanes 3, 5, and 7) μg of DNase I per ml. Samples were electrophoresed on a 4% Tris–glycine–polyacrylamide gel, and the radiolabeled DNA fragments corresponding to the free-migrating band (lanes 2, 3, and 8), complex II (lanes 4 and 5), or complex III (lanes 6 and 7) were recovered for subsequent analysis on an 8% polyacrylamide–urea sequencing gel as shown. Lanes 1 and 9 represent chemical cleavage at purine residues. (B) Same as panel A except a 280-bp *Mlu*I-*Eco*RI DNA fragment labeled on the minus-strand at the *Mlu*I site was used. DNA in lanes 2 through 6 was mixed with 0.5 NaCl extract protein, and DNA in lane 7 was mixed with bovine serum albumin. Samples were digested with 2 μg (lane 7), 3 μg (lanes 3 and 5), or 5 μg (lanes 2, 4, and 6) of DNase I per ml. After electrophoresis on a polyacrylamide gel, radiolabeled DNA fragments corresponding to the free-migrating band (lanes 2 and 8), complex II (lanes 3 and 4), or complex III (lanes 5 and 6) were recovered for subsequent analysis as above. Lanes 1 and 8 represent chemical cleavage at purine residues.

enhancer or to sequences upstream in fact protected a region within the B domain at the 5′ extremity of U3. However, in this instance strong protection was observed on the minus-strand DNA (Fig. 6B, lanes 3 to 6) from nucleotides −229 to −203. The corresponding 27 nucleotide stretch on plus-strand DNA was only marginally protected from nuclease activity (Fig. 6A, lanes 4 to 7). In fact, among several different footprinting experiments with plus-strand DNA over a wider range of DNase I concentrations than that shown in Fig. 6B, we consistently detected only minor protection of nucleotides from approximately −220 to −208 with the protein(s) forming complex II. This region of limited protection was extended to approximately −192 with proteins comprising complex III. In contrast, we could discern no differences in footprinting between complexes II and III on minus-strand DNA (compare Fig. 6A, lanes 3 to 5 and 4 to 6). Since the regions protected by proteins in complexes II and III primarily or completely overlapped, we suggest that complex III may comprise complex II plus other proteins or multiple copies of the same protein involved in additional protein-protein, rather than extensive additional protein-DNA, interactions.

In any case, we have identified at least two protein factors differentially extracted from QT-6 cell nuclei that specifically bind to and protect different 25- to 30-nucleotide regions of the RSV LTR. These proteins apparently interact preferentially with opposite strands of the DNA, as monitored by susceptibility to DNase I attack. The two protected regions lie within that segment of U3 known to be required for enhancer activity. Hence, we will refer to the protein(s) present in the 0.1 M NaCl extract which bound to nucleotide sequence −121 to −146/−155 as enhancer factor I (EFI), and those proteins present in the 0.5 M NaCl extract which bound to nucleotide −229 to −203/−192 as enhancer factor II (EFII).

EFI and EFII specific for the RSV LTR. The nucleotide sequences protected by EFI and EFII are shown in Fig. 7. Some limited similarities with other known enhancer elements are exhibited by these sequences. For example, the seven-nucleotide sequence (−129)-GTGGAAG-(−123) in the EFI-protected region was homologous to the core enhancer consensus sequence GTGGAAAG (68) except for an A which is not present in the RSV sequence. We also observed limited homology to a region essential for SV40 enhancer function known as the *Sph* motif (71), with the consensus sequence of alternating purine/pyrimidines,

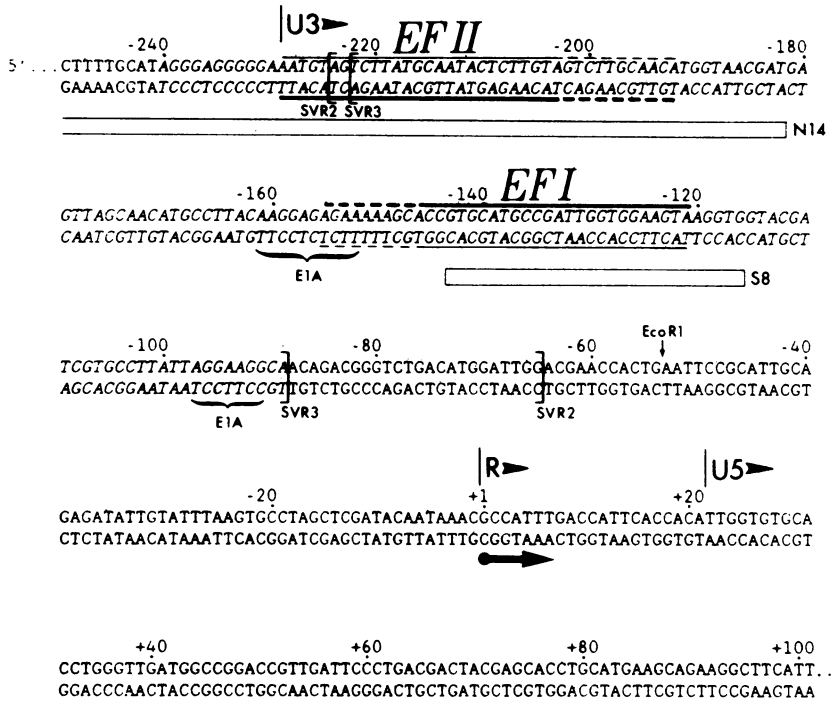

FIG. 7. Sequence of the RSV LTR and regions protected by EFI or EFII. Nucleotide sequence of the SR-A RSV LTR is from Swanstrom et al. (58). Numbering is with reference to the initiation site for RSV RNA (arrow), and the U3 (−229 to −1), R (+1 to +21), and U5 (+22 to +101) regions are shown. Sequences within and slightly 5′ of U3 making up the minimal DNA segment exhibiting enhancer activity as defined by previous deletion mutagenesis and enhancer trap studies are in italics. Locations of sequences protected by EFI or EFII binding are shown, with a heavy line indicating strong protection and a thin line signifying weak protection of appropriate strands. Also indicated by open boxes are sequences missing from two different RSV DNA deletion mutants constructed by Luciw et al. (33) that lack enhancing activity (pAV-3/TKΔN14 [N14] and pAV-3/TKΔS8 [S8]). Sequences in U3 captured by two SV40-RSV recombinant viruses, SVR2 and SVR3, in enhancer trap experiments (66) are bracketed, and sequences AGGAGAGAAA at −160 to −151 and AGGAGGG at −97 to −91 which resemble the E1A enhancer consensus sequence repeats (27, 32) are indicated.

AAGC/TATGCA. The last six nucleotides of this consensus sequence were present in the EFII-protected region (−219)-TATGCA-(−214) and the middle nucleotides were present as an *Sph*I site in the EFI region (−141)-GCATGC-(−136). A longer region of homology with the *Sph* motif was also present in the EFI-protected region from −150 to −139 (AAGCACCGTGCA), except for the inserted CCG. It is difficult to assess the significance, if any, of these very short nucleotide homologies. Thus, we wished to test directly whether the protein factors making up complexes I, II, and III with RSV enhancer DNA could also bind to other enhancers.

Accordingly, we performed competition experiments with DNA fragments containing the SV40 enhancer or the enhancer identified in the LTR of Moloney MSV (31). Neither EFI nor EFII appeared to interact with the SV40 or MSV enhancer (Fig. 8). The small decreases in complex formation when a 50- to 100-fold molar excess of SV40 or MSV competitor DNA was present were probably due to nonspecific DNA binding (a similar result was sometimes observed with pBR322 DNA; see Fig. 3), although some weak cross-recognition cannot be excluded. The decrease in complex formation was especially apparent with a 50- to 100-fold molar excess of MSV DNA. However, this DNA fragment was larger (438 bp) than the other competitor DNAs used (approximately 300 bp), and therefore a higher molar concentration of nucleotides would be present to increase nonspecific binding.

We conclude that recognition of enhancer DNA by EFI and EFII is primarily if not entirely limited to the sequences present in the RSV LTR. We have not yet tested other viral enhancers or any known cellular enhancers, such as those found within the heavy- and light-chain immunoglobulin genes or upstream of the insulin gene. However, the lack of homology to sequences protected by EFI and EFII coupled with the strong cell type specificity of many cellular enhancers so far identified would seem to make it unlikely that cross-recognition would occur. Since EFI and EFII are present in uninfected avian cells, we presume that a normal target(s) for these proteins, besides the RSV LTR, exists in the host genome. Further clarification of the nucleotides essential for EFI and EFII recognition may assist in defining the binding sites for these proteins in uninfected cells.

DISCUSSION

Fractionation of RSV enhancer-binding proteins by differential extraction of nuclei. We exploited the differential extraction of quail cell nuclei with buffers of increasing ionic strength to identify at least two protein factors which bind specifically and independently to RSV enhancer DNA. We first detected these factors in nuclear extracts by the gel electrophoresis DNA-binding assay (17, 20), an increasingly popular approach that has already established its usefulness in identifying factors in crude nuclear extracts which bind to other transcriptional control regions, such as the adenovirus major late promotor (5), the immunoglobulin heavy-chain promotor (56), and the polyomavirus enhancer (3, 45). Of the

sequence-specific RSV enhancer-binding proteins we detected, one factor, EFI, was readily dissociated from nuclei by relatively low NaCl concentrations (100 mM or less). A second factor was more strongly associated with quail cell nuclei. EFII was dissociated from nuclei between 0.3 and 0.53 M NaCl, although this nuclear extract required further fractionation by $(NH_4)_2SO_4$ precipitation to readily detect EFII complex formation with enhancer DNA by the electrophoretic mobility shift assay. We suspect that the $(NH_4)_2SO_4$ fractionation step is necessary primarily to remove histone H1 [which is soluble in 70% $(NH_4)_2SO_4$] from this extract. By 0.53 M NaCl, H1 is beginning to be dissociated from chromatin (2). Extraction of even a small proportion of this relatively abundant protein, with its very high affinity for naked DNA, could easily interfere with the formation of a limited number of specific complexes of other proteins with enhancer DNA.

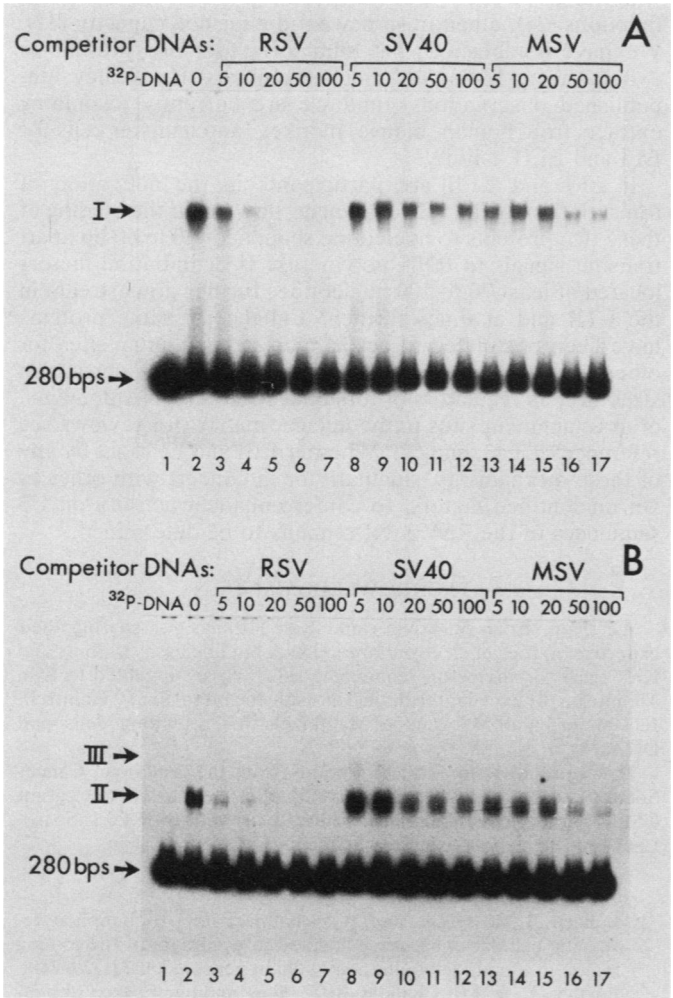

FIG. 8. EFI and EFII bind specifically to the enhancer of RSV. (A) End-labeled 280-bp DNA fragment containing the RSV enhancer (lane 1) was mixed with 1 μg of poly(dI) · poly(dC) and 2.5 μg of protein extracted from QT-6 nuclei by 0.1 M NaCl in the absence (lane 2) or presence (lanes 3 to 17) of 5- to 100-fold molar excess of nonradiolabeled DNA fragments as indicated. RSV, 280-bp *Mlu*I-*Eco*RI fragment from pRSV-LTR; SV40, 278-bp *Pvu*II-*Bgl*I fragment from SV40 form I DNA; MSV, 438-bp *Cla*I-*Xba*I fragment from pBR-MSV LTR (see text). After electrophoresis of samples on a 4% Tris–glycine–polyacrylamide gel, the gel was dried and exposed to X-ray film. (B) Same as panel A except that 1.5 μg of 0.5 M NaCl extract protein was used.

Although many nonhistone proteins are extracted from nuclei between 0.1 and 0.3 M NaCl (this extract always had the highest final protein concentration of the three), we did not detect any protein factors which bound specifically to the RSV enhancer in this fraction. Perhaps the larger proportion of nuclear proteins in this fraction precluded our detecting any specific complexes with 280-bp RSV enhancer DNA. Further fractionation of this nuclear extract might reveal additional RSV enhancer-binding proteins. Binding activity may also be present in proteins not yet extracted from quail cell nuclei by 0.53 M NaCl. Nonetheless, we found this initial stepwise extraction of nuclei useful in identifying at least two RSV enhancer-binding proteins. We could not detect mobility shifts corresponding to complexes I, II, and III when the electrophoretic assay was performed with nuclear extracts prepared by a single 0.5 M NaCl extraction step, nor have we been able to recombine the individual 0.1 and 0.5 M extracts to generate a combined EFI-EFII-DNA complex with a new and distinctly shifted electrophoretic mobility. Apparently the ratio of nonspecific to specific DNA-binding proteins in such extracts is sufficiently high to overwhelm the ability of a simple duplex copolymer such as poly(dI) · poly(dC) to limit nonspecific interactions without adding sufficient quantities to also detract from sequence-specific protein DNA interactions. Identification of a multiple EFI-EFII-DNA complex will most likely require partial purification of the factors.

Nucleotide sequences protected by EFI and EFII are required for enhancer activity. We used DNase I footprinting in conjunction with the gel electrophoretic mobility binding assay to determine the nucleotide sequences protected by EFI and EFII binding to RSV LTR DNA in vitro. The sequences protected (−146/−155 to −121 for EFI and −229 to −203/−192 for EFII) lie within that region of U3 shown by others to be required for enhancer activity (8, 32, 33, 66). Both protected regions were contained within the segments of U3 acquired by SV40-RSV recombinant viruses (Fig. 7), in which RSV sequences complement an enhancerless defect in the SV40 genome (66). The region of EFI-protected nucleotides spans the boundary between two functional domains, B and C, of the RSV enhancer defined by Laimins et al. (32). It also coincides fairly precisely with a deletion mutant (pAV-3/TKΔS8; deletion coordinates −142 to −116) constructed by Luciw et al. (33) that lacked enhancing activity entirely. Interestingly, a second mutant constructed by Luciw et al. (33) harboring a larger deletion (pAV-11/TKΔE1; deletion coordinates −143 to +25) retained nearly wild-type enhancer activity. Inspection of the RSV LTR sequence in Fig. 7 shows that of those nucleotides removed from the EFI binding site by a −143 to +25 deletion, two-thirds of the 23 nucleotides were regenerated except for an insertion of one additional T: (−143)-GTGCATGCCGATTGGTGGAAGTA-(−121) and (+26)-TGTGCACCTGGGTTGATGGCCGGA(+49), including a series of five consecutive nucleotides (GTGCA) in the center of the protected region. (Only 5 of 23 nucleotides were restored in a −142 to −116 deletion.) It would be useful to confirm whether EFI binding activity correlates with the LTR enhancer activity of these mutants.

The region of LTR DNA protected by EFII binding lies at the beginning of U3. In fact, the 5′ boundary of EFII-protected nucleotides coincided with the 5′ border of U3 (± 1 or 2 nucleotides, due to uncertainty in footprinting). A deletion mutant of the RSV LTR enhancer (pAV-3/TKΔN14)

which lacks the 5'-proximal 48 nucleotides of U3 (to −182) and upstream flanking sequences (to −1338) is devoid of enhancer activity (33), suggesting that the 5' extremity of U3 (or possibly upstream sequences) is important for RSV enhancer function. If EFII is involved in mediating the activity of the RSV enhancer, then the location of its target sequences may explain some of the discrepancy about whether the SR-A RSV enhancer lies entirely within LTR sequences. Sequences immediately upstream of U3 may influence the interaction of EFII with enhancer DNA at the 5' extremity of U3, as was proposed previously (8).

Although the mutagenesis studies previously performed are entirely consistent with a role for EFI and EFII in RSV enhancer function, the question of whether small mutations confined solely to EFI- or EFII-protected nucleotides result in equivalent losses of both enhancer activity and factor binding remains to be addressed. Not only are more detailed mutagenesis studies required to evaluate the relevance of EFI and EFII binding for RSV enhancer activity, but such studies should also aid in defining the exact nucleotide sequences recognized (as opposed to protected) by these factors. We have discerned only limited, short homologies of uncertain significance between the sequences protected by EFI and EFII and several consensus sequence motifs known to be important for the activity of other enhancer elements. Two homologies to a consensus sequence motif repeatedly found in the E1A enhancer (27) appear not to be involved in recognizing either of the factors we have detected by the electrophoretic mobility binding assay (Fig. 7). We noted one redundancy in the nucleotide sequence protected by EFII. The sequence (−227)-GTAGTCTTATGCAA-(−213) is repeated (except for an AT deletion) beginning 8 nucleotides later, (-206)-GTAGTCTTGCAA-(−195). If the first 14-nucleotide segment constitutes all or part of the recognition signal for EFII, then the presence of a nearly identical copy of the sequence immediately downstream may explain the formation of complex III, although we did not observe strong protection of nucleotides past −203 on either strand in complex III footprints.

The footprints obtained for both complex I and complexes II and III exhibit a distinct asymmetry in protection of analogous regions on the two DNA strands. At least as monitored by DNase I activity, EFI prefers to interact with the plus or coding strand, while its neighboring factor approximately 40 to 60 bp upstream preferentially binds to the minus or noncoding strand. The interaction of a DNA-binding protein primarily with one DNA strand has previously been observed for other eucaryotic transcription factors, such as TFIIIB (48) and Sp1 (21). We do not know whether this novel feature of EFI and EFII apparently binding to opposite strands of enhancer DNA in vitro will prove to be of any significance for whatever functional roles these proteins assume in vivo.

Are EFI and EFII involved in RSV LTR enhancer function? Experiments which monitor and characterize the binding of specific protein factors to enhancer DNA in vitro, even to target sequences known to be required for enhancer activity, can provide only circumstantial evidence that these proteins serve a function on which enhancer activity depends in vivo. Direct functional studies are also needed, not only to sustain the conclusions drawn from binding assays, but also to identify protein-protein interactions that may provide additional clues to how enhancers act. Recently, some of the effects of enhancers, including cell type-specific activation, have been reproduced in in vitro transcription extracts (49, 53, 69). It may be possible to supplement partially purified transcription initiation factors and RNA polymerase II with EFI and EFII to determine whether they stimulate transcription of promoters linked to the RSV enhancer. However, this will undoubtedly require further purification and characterization of these enhancer-binding factors.

At present, the specific identities of the proteins making up EFI and EFII are not known. An estimate can be made of the abundance of these enhancer-binding proteins by titrating the amount of radiolabeled 280-bp DNA fragment migrating in either complex I or II and III with increasing quantities of nuclear extract (Fig. 2). From these data we calculated that both EFI and EFII were minimally present in approximately 1,000 active copies per cell (assuming quantitative extraction and recovery, which is unlikely). If any of this low level of EFI or EFII is involved in mediating the activity of the RSV enhancer after infection of quail cells, then the same enhancer-binding factors should also be present in the wide range of eucaryotic cell types in which the enhancer functions (25), albeit in somewhat diminished capacity (32). We have established that similar factors are present in extracts from 12-day-old chicken embyros (L. Sealey, unpublished observations), and we are currently examining extracts from human, mouse, monkey, and hamster cells for EFI and EFII activity.

If EFI and EFII are participants in the activation of transcription by the RSV enhancer, how might the binding of these two proteins to nucleotide sequences 40 to 60 bp apart transmit signals to RNA polymerase II or initiation factors located at least 70 to 100 nucleotides further downstream in the LTR and at times further? Enhancer-binding proteins have been postulated to act as bidirectional entry sites for other transcriptional proteins, as organizers of chromatin structure, as regulators of torsional strain in DNA domains, or as attachment sites to the nuclear matrix (for reviews see references 23, 43, and 55). Whether EFI and EFII act by any of these mechanisms, singularly or in concert with other as yet unidentified factors, to confer enhancer activity on U3 sequences of the RSV LTR remains to be determined.

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