Specific nuclear proteins interact with the Rous sarcoma virus internal enhancer and share a common element with the enhancer located in the long terminal repeat of the virus

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ABSTRACT

We have documented that the Rous sarcoma virus (RSV) internal enhancer functions in the nontransformed Baby Hamster Kidney (BHK) cell line. The sequences within this region were assayed for their ability to bind to specific factors present in BHK nuclear extracts using the gel retardation assay and DNase I footprinting. At least two sequences within the internal enhancer which can specifically bind nuclear factors in vitro have been identified. These regions are located between nucleotides 813-850 and 856-877. These sites map within the overall region of the internal enhancer which has been shown to be essential for enhancer activity and within the specific region which can function as an orientation independent enhancer. Using the DNase I footprinting and binding data to design an oligonucleotide, we have demonstrated that an oligonucleotide extending from nucleotides 804-877 will substitute efficiently as an enhancer. We also demonstrate that the SV40 enhancer does not compete for the factors which bind to the RSV internal enhancer, whereas an oligonucleotide to the binding site for EII in the LTR can compete for factor binding to the internal enhancer.

INTRODUCTION

The long terminal repeats (LTRs) of retroviruses have long been considered to contain all the cis-acting elements required for retroviral gene transcription (1,2,3,4,5). However, several recent lines of evidence suggest that other areas within the actual coding region of the viral genome may play some regulatory role (6,7,8,9). A region of the gag gene between nucleotides 630 and 1149 of the Rous sarcoma virus (RSV) has been found to be required in cis for the efficient transformation of NIH-3T3 cells (9). This same region (in RSV, RAV-0, and PSV) has also been demonstrated to possess an enhancer activity (6). This enhancer functions with some orientation dependence and can also augment the activity of the previously identified enhancer located within the LTR.

The internal enhancer region, as well as the LTR enhancer, has also been found to contain a prominent DNase I hypersensitive site within the provirus in mouse cells (10), chicken cells (11) and hamster SR3/1a cells (L. Karnitz, unpublished observation). Although the functional significance of this
observation is unclear, such structural evidence has been found to strongly correlate with regions required for gene regulation. DNase I hypersensitivity is most likely the result of specific nonhistone protein-DNA interactions (12,13,14,15). Many specific proteins which interact with enhancers or other transcription regulatory sequences have recently been documented (16,17,18,19,20,21). Consequently, we have begun to identify cellular proteins which specifically interact with the internal enhancer of RSV. Using extracts from BHK cells (the parental line of the RSV-transformed SR3/la cells (22)), we have identified protein factor(s) which specifically interact with the regions between 813-850 and 856-877. Finally, the binding data were used to construct an oligonucleotide extending from nucleotides 804-877. This oligonucleotide was demonstrated to possess about 60% of the enhancer activity of the entire fragment.

Sequence comparisons of identified enhancers have not revealed more than short sequence homologies (23,24,25 and references therein). One such homology is the enhancer core sequence GTGCTTG. This sequence was previously noted to be within the internal enhancer (6), although we have not identified any specific binding to this sequence. Furthermore, binding of our factors is not competed by the SV40 enhancer sequences, although there is competition for some factor(s) between the LTR enhancer and the internal enhancer.

MATERIALS AND METHODS

Nuclear extracts from BHK cells were prepared basically as described by Sealy and Chalkley (16). In summary, BHK cells were grown as a monolayer in DMEM with 10% fetal bovine serum. The cells were grown to confluence and harvested with phosphate-buffered saline (PBS) which contained 0.2% EDTA. All procedures were then carried out at 4°C, basically as previously described (16). The cells were washed twice with cold PBS and then nuclei were prepared by suspending the cells in buffer A. The nuclei were then pelleted at 1000xg and washed twice with the same buffer lacking Triton X-100. Nuclear proteins were then extracted from the nuclei with buffer B which contained 0.52M NaCl for 15 minutes and then centrifuged at 1000xg for 30 minutes to pellet the nuclear remains. The supernatant was then incubated with 1/5 volume of Bio-gel A15M which had been equilibrated with the same buffer to adsorb any contaminating histones (R. Chalkley unpublished observations). The Bio-gel A15M was then pelleted at 20,000xg for 15 minutes and the supernatant was collected and slowly adjusted to 70% ammonium sulfate (Bethesda Research Laboratories) and the protein pellet was collected at 100,000xg for 30 minutes. The pellet was then redissolved in 10 mM Hepes, pH 8.0, 300 mM NaCl, 5 mM 2-
mercaptoethanol, 50% glycerol and dialyzed overnight against the same buffer. Protein was quantitated according to the method of Bradford (26).

**Partial Purification of Extracts**

The 0.3-0.5M NaCl nuclear extracts were diluted to 250mM NaCl, the insoluble material was pelleted at 20,000xg and the supernatant was applied to hydroxyapatite (Biorad) at a concentration of 1 mg protein per ml resin. The specific binding factors were eluted with 30 mM sodium phosphate, 2M NaCl, 5% glycerol, 0.005% NP40 and 5mM β-mercaptoethanol, pH 7.7. The protein peak was then desalted on a Sephadex G-25 column in 10 mM Hepes, pH 8.0, 250mM NaCl, 5% glycerol, 0.01% NP40 and 5mM β-mercaptoethanol. The protein peak was reapplied to hydroxyapatite at 9 mg of protein per ml of resin and washed with 50mM sodium phosphate, pH 7.7, 50% glycerol, 0.01% NP40mM and 5mM β-mercaptoethanol. Specific binding factors were eluted with the same buffer containing 500 mM sodium phosphate, pH 7.7. The protein peak was then desalted on a Sephadex G-25 column containing 10mM Hepes, pH 8.0, 250mM NaCl, 50% glycerol, 0.1 mM EDTA, 5mM dithiothreitol and 0.01% NP40.

**Plasmid constructions.**

The plasmid, pTZ-RSVIEN, was constructed by ligating the XhoI-BclI (nucleotides 622-1143) fragment from pSRA-2 (27), which contained the previously described internal enhancer, into the Sall and BclI sites of pTZ18R (United States Biochemical Corp.). The plasmids used for transfection assays were constructed basically as described by Arrigo et al., except that the enhancer fragment was digested out of the polylinker of pTZ-RSVIEN with EcoRI and HindIII, blunt-ended with the Klenov fragment (PL Biochemicals) and cloned into either the blunt-ended NdeI or BamHI site of pSV1 (28). All plasmids were prepared by alkaline lysis procedure and centrifugation to equilibrium in CsCl and ethidium bromide.

**Preparation of Restriction Fragments.**

Restriction fragments were purified by electrophoresis in 8% acrylamide gels. The running buffer was TBE. The gels were then stained with 0.5 mg/ml ethidium bromide for 10 minutes and the appropriate band was excised and electroeluted in 4.5 mM Tris, 4.5 mM boric acid and 0.13 mM EDTA which contained 0.1% NP40. Fragments were ethanol precipitated before use. Fragments were labelled with either the Klenov fragment or by phosphatasing with bacterial alkaline phosphatase (Bethesda Research Laboratories) and then labeling with γ-32P ATP and T4 polynucleotide kinase (New England Biolabs).

**Oligonucleotide Preparation.**

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer by the Diabetes Core Facility at Vanderbilt University. Oligonucleotides were dissolved in 90% formamide and electrophoresed on a 20%
acrylamide, 7M urea gel, located by ethidium bromide staining and
electroeluted and recovered as described above. The single strands were then
mixed in equimolar amounts at a concentration of 5 mg/ml and heated to 88°C.
This was then allowed to slowly cool to room temperature at an average rate of
about 4°C/hour.

Gel Retardation Assays.

Binding reactions were carried out under the following conditions; 10 mM
Hepes, pH 8.0, 120 mM NaCl, 3 mM MgCl₂, 17% glycerol, 0.01% NP40, 0.2 mg/ml
poly(dI:dC).poly(dI:dC) (Pharmacia). Binding reactions were in 20 μl and
contained 0.2 ng of labelled probe and the specified amount of nuclear
extract. Binding was allowed to proceed for 10-15 minutes at 23°C and then
loaded directly onto a 4% acrylamide gel and analyzed as previously described
(16).

DNAse I Footprinting Reactions.

HindIII-Styl fragments labelled at the StyI site by either polynucleotide
kinase or the Klenow fragment, were incubated with increasing amounts of
partially purified nuclear extracts for 15 minutes at 23°C. DNAse I was then
added to a concentration of 2 μg/ml and allowed to react for 1 minute at 23°C.
The reactions were stopped by adding sarkosyl to 1%, EDTA to 10 mM and
proteinase K to 0.2 mg/ml. The samples were incubated at 65°C for 1 hour,
ethanol precipitated and analyzed on an 8% acrylamide, 7 M urea, 20% formamide
sequencing gel alongside the reaction products of the purine specific cleavage
as previously described (29).

Transfections.

BHK cells were transfected using the calcium phosphate coprecipitation
procedure of Graham and Van der Eb (30). The day before transfection, BHK
cells were seeded onto 75 cm² tissue culture flasks such that the flasks were
60-70% confluent at the time of transfection. Calcium phosphate-DNA
precipitates (1.5 ml containing 30 μg of DNA per flask) were prepared using
5.0 μg of internal enhancer containing CAT plasmid or an equal molar amount
(4.43 μg) of enhancerless pSV1-CAT and salmon sperm DNA. The suspension was
added to the cell monolayer, incubated for 30 minutes at 23°C, and then
incubated for 4 hours at 37°C after the addition of 15 ml of medium. The cell
monolayers were then treated for 90 seconds with medium containing 20% DMSO as
described by Stow and Wilkie (31) followed by a 42 hour incubation at 37°C in
fresh medium.

CAT Assays

Preparation of cell extracts and CAT assays were performed according to
Gorman et al (3,28). Twenty μl of extract and a 30 minute incubation at 37°C
were used per assay. The autoradiogram of the TLC plate was used as a template
to cut out the regions containing acetylated and unmodified chloramphenicol and quantitated by scintillation counting. Extracts of cells transfected with salmon sperm DNA alone were assayed to determine the background level of CAT activity and was subtracted for calculating the amount of chloramphenicol modified.

RESULTS

The Rous Sarcoma Virus Internal Enhancer also Functions in Hamster Cells.

BHK cells were used as a model system for analysis and identification of proteins which bind to the recently identified internal enhancer of the RSV. This cell line was chosen since we have had an ongoing interest in the chromatin structure of actively transcribed genes and the integrated RSV provirus in the transformed cell line, SR3/1a is an ideal candidate for such a study (22).

We have determined, in agreement with previous observations, that the sequence between XhoI and BclI (nucleotide 622-1149) of the Schmidt-Ruppin A strain, which functions as an enhancer in CV-1 cells (9), chick embryo fibroblasts and NIH 3T3 cells (6), also functions as an enhancer in hamster

<table>
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<tr>
<th>Plasmid</th>
<th>CAT Activity relative to:</th>
<th>pSV1-CAT</th>
<th>p5'(+)CAT</th>
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<td>1.0 (12)</td>
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<tr>
<td>p5'(-)-CAT</td>
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<td>10.5 (7)</td>
<td>-</td>
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<td>p3'(-)-CAT</td>
<td></td>
<td>7.1 (7)</td>
<td>-</td>
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<tr>
<td>pOLIGO5'+CAT</td>
<td></td>
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<td>0.58 (6)</td>
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Measurement of Chloramphenicol Acetyl Transferase Activity in Transfected BHK Cell Extracts. The transfection of BHK cells and the quantitation of CAT activity were performed as described in Materials and Methods. The number of transfections performed is indicated in parentheses. BHK cells were transfected with pSV1-CAT or derivatives containing an insert of the Schmidt-Ruppin A gag gene fragment (nucleotides 623-1142). The inserts are p5', gag fragment inserted upstream at the NdeI site of pSV1-Cat; p3', gag fragment inserted downstream of the CAT gene at the BamHI site. The orientation is given as (+) or (-), where + indicates the same orientation of the fragment with respect to CAT gene transcription as the fragment would have in the viral genome with respect to transcription. p5'(+)CAT, p5'(-)CAT and p3'(-)CAT were normalized to the activity of pSV1-CAT and the fold enhancement is given in the table. pOLIGO5'(+)-CAT was normalized to the enhancer activity of p5'(+)CAT and is the fold enhancement in the table.
Figure 1. Assay of Binding to Various Restriction Fragments within the Internal Enhancer. (A) Each labelled fragment was incubated with the indicated amounts of crude nuclear extract for 15 min. The binding reactions were then electrophoresed on a 4% TGE gel and the dried gel was exposed to film at -70°C. Part B show the schematic map of the internal enhancer. Nucleotide numbers are shown for the Schmidt-Ruppin A strain of the virus and nucleotide numbers on the 3' end of the sequence shown are only approximate. GTGGTTTG shows the position of the enhancer core sequence within the internal enhancer. Restriction sites are X, XhoI; N, NarI; Ha, HhaI; P, PvuII; St, StyI; Sc, ScaI; B, BamHI; E, EcoRI; H, HindIII. The HindIII and EcoRI sites are derived from the polylinkers of pTZ18R. The figure is a composite of separate gels and therefore fragments of different sizes appear to have the same mobility.

cells. Using a construct similar to that described by Arrigo et al. (see materials and methods), we have demonstrated (table 1) comparable enhancement of CAT (E. coli chloramphenicol acetyl transferase) gene expression in transient expression assays. However we have not observed the same orientation dependence in the BHK cell line which was displayed in other cell lines (6,9). We find that transcription is enhanced about 8 fold independent of orientation with the entire XhoI-BclI fragment. Based on the footprinting and oligonucleotide binding data, an oligonucleotide which extends from nucleotides 804–877, was tested for enhancer function. This oligonucleotide was inserted in the + orientation at the NdeI site of pSV1-CAT and as can be seen in table 1 functions about 60% as efficiently as does the entire fragment.

Distinct Regions of the Internal Enhancer Bind Nuclear Proteins.

Fragments covering the entirety of the subcloned internal enhancer were
assayed by a gel retardation assay (32,33). The binding conditions are described in the Materials and Methods section. We used nuclear extracts containing proteins which were extracted from the nuclei between 0.05 to 0.52 M NaCl and precipitated in 70% saturated ammonium sulfate. Various overlapping and partially deleted restriction fragments covering the 520 basepair (bp) enhancer were prepared. As indicated in figure 1A, each labelled fragment was incubated with increasing concentrations (0,2,4,8 ug of protein) of a nuclear extract. The diagram in figure 1b, shows the location of each restriction enzyme site used to generate the various fragments. The EcoRI site and the HindIII site are the sites in the polylinker of the vector pTZ18R which are near the original BclI and XhoI sites respectively. The results for each binding assay are shown in figure 1A. Very little binding is evident for the HindIII to NarI restriction fragment, even at the highest protein concentration. On the other hand, binding results for the restriction fragment, NarI-PvuII, indicate that a fraction of the labelled DNA fragment is retarded into a well resolved, three band pattern. The next restriction fragment in the enhancer, PvuII-Styl, showed that an even greater proportion of the labelled restriction fragment is retarded by increasing amounts of added extract. The pattern of binding seen for this fragment resembles that which was seen for NarI-PvuII. The remaining sequence of the enhancer, Styl-EcoRI, was then assayed for binding activity. This fragment also showed a significant ability to bind factors within the nuclear extract, although the overall appearance of the retarded bands is characteristically different than the other fragments. All of the binding activities were destroyed by addition of SDS to 1%, indicating that they were not due to covalent modification (unpublished data).

In an attempt to map the regions of binding on a finer scale, many different labelled fragments were tested. A second large region which did not show specific binding was identified and is present in the restriction fragment, HhaI-Scal (nucleotides 868-1068). This 200 basepair (bp) fragment located approximately in the middle of the enhancer region and which contains the core enhancer sequence GTGTTTG (34), shows a small amount of material which is retarded into a set of bands (figure 1A). However, the binding appeared to be nonspecific, since these bands could not be competed by any specific or nonspecific competitor. These results demonstrate that most of the binding is occurring around the PvuII site and to the 3' side of the Scal site. Since PvuII-Styl binds very well (see figure 1) and since HhaI-Scal does not show much binding activity, it was concluded that a binding site or a significant part of a site, must be located within the 16 basepairs between PvuII and HhaI or it might be directly over the HhaI restriction site. Also,
Figure 2. Binding Sites are Sequence Specific. Labelled restriction fragments from Figure 1 were incubated with the crude nuclear extracts in the presence of increasing amounts of competitor fragments and analyzed using the gel retardation assay. The labelled fragment which was used is shown and the competitor and the molar excess used are also shown. The competitors used are N-P, NarI-PvuII; P-S, PvuII-StyI; S-E, StyI-EcoRI from the internal enhancer and pBR, the BamHI-SalI fragment of pBR322.

this figure indicates that there is no binding which might be located near the StyI site which was being destroyed by using this as a fragment boundary.

Binding Activities are Sequence Specific.

Each of the fragments capable of binding protein (as documented in figure 1) was then tested to determine if the binding activities were specific. Figure 2 demonstrates that the three fragments (NarI-PvuII, PvuII-StyI, and StyI-EcoRI) which were able to bind factors in the nuclear extracts also show specific competition. The labelled NarI-PvuII fragment was incubated with a nuclear extract along with a 0, 10, 50 or 100-fold molar excess of either the unlabelled NarI-PvuII fragment or an equivalent molar excess of a pBR322 BamHI-SalI 275 bp fragment. A significant fraction of the binding is removed by a 10 fold molar excess of the specific competitor. In contrast, no competition is seen even at a fifty-fold molar excess with the pBR322 fragment. Restriction digested total vector DNA also shows no ability to compete for binding (data not shown), indicating that the lack of competition is not confined to this particular fragment of pBR322. As can also be seen in figure 2, binding to PvuII-StyI is specifically and efficiently competed by the corresponding unlabelled fragment and is not competed by the pBR322 275 bp fragment.
Figure 3. Specific Binding is Competed by Oligonucleotides. Oligonucleotides were prepared as described in the Materials and Methods section and also are depicted schematically in figure 4B. The indicated labelled fragments were incubated with the crude nuclear extracts in the presence of increasing molar excesses of the indicated duplex oligonucleotide.

On the other hand, binding to the Styl-EcoRI fragment which encompasses the 3' portion of the enhancer and which generates a more complex pattern than that seen for the 5' fragment behaves somewhat differently. Not all of the bands in this pattern are specifically competed, and indeed only the lower set of bands in this pattern show specificity of binding in the presence of an excess of the Styl-EcoRI fragment.

**Competition for Binding by Specific Duplex Oligonucleotides.**

Since the sequences just 3' of the PvuII sites appeared to be absolutely essential for the binding activity of the PvuII-Styl fragment, we have further probed for specific DNA-protein interactions by competition with specific oligonucleotides. Four oligonucleotides were constructed. The first encompasses the sequence from the PvuII site and extends downstream 30 bp (OpuvII3') from nucleotides 851-881, the second extends 15 bp to either side of the PvuII site from nucleotides 826-856 (OpuvII). The third starts 30 bp to
the 5' side of PvuII and extends to the PvuII site and includes nucleotides 821-850 (OpvuII5'). Another 40 bp oligonucleotide (Oconl) was also synthesized as a control competitor. It consisted of nucleotides 779-820, which did not appear to contain any binding domains. The annealed duplex oligonucleotides were used as competitors for binding to the labelled Narl-PvuII fragment (figure 3 upper left panel) and to the PvuII-Styl fragment (figure 3 lower left panel). As can be seen in figure 3, OpvuII3' functions as a very efficient competitor for binding to both labelled fragments and the other oligonucleotides less efficiently. Oconl which overlaps into the labelled Narl-PvuII fragment does not compete at all. Both OpvuII and OpvuII5' show competition, though much less efficiently, demonstrating that neither of these oligonucleotides contains a complete binding site. These data also demonstrate that some binding factor must be common to the binding sites both 3' and 5' of the PvuII site. Since the previous results indicated that all the binding seen within the region between HindIII and Styl was located on two adjacent fragments (Narl-PvuII and PvuII-Styl), binding to the fragment HindIII-Styl was tested. Results of such an analysis are shown in figure 3 (upper right panel) and demonstrate that the same basic binding pattern is seen for this fragment as was seen for the Narl-PvuII and the PvuII-Styl fragments. Further all of the binding to this fragment is removed by competition with OpvuII3'. We conclude that all binding which occurs between HindIII-Styl must share at least a common binding aspect, since one duplex oligonucleotide (OpvuII3') can compete for binding at all sites.

Duplex Oligonucleotides which Specifically Compete for Binding Can also Bind Nuclear Factors.

Figure 4 shows the binding of nuclear extracts to the labelled oligonucleotides. The results are consistent with the competition data. Oconl shows no binding, OpvuII and OpvuII5' show a minor amount of binding and OpvuII3' shows significant binding in a pattern very similar to that seen for either the Styl-PvuII or Narl-PvuII fragments, except that the protein-DNA complexes are retarded to a much greater degree. These data suggest that the competitions with the oligonucleotides were the result of specific protein-DNA interactions.

Competition for Binding Using other Retroviral Fragments and the SV40 Enhancer.

The SV40 enhancer was tested as a competitor for the RSV internal enhancer binding proteins. The SV40 fragment used for the competitor assays extended from PvuII-BglII (nucleotides 5235-270) of the SV40 strain WT800 (4). The fragment located between the LTR and the internal enhancer region was also included in the competition as a control. This fragment extended from BstEII-
Figure 4. Nuclear Proteins Also Bind to the Oligonucleotides. The oligonucleotides which were used as competitors for figure 3 were labelled and the duplex form gel purified. These were then incubated with the indicated increasing amounts of the crude nuclear extracts and analyzed using the gel retardation assay.

BamHI (nucleotides 103-524). Figure 5A demonstrates the results of these competitions. The SV40 fragment cannot effectively compete for the binding sites which are located between HindIII-StyI. The other internal fragment which was tested (BstEII-BamHI), showed a small amount of competition. The exact nature of this competition is unknown at this time, although little sequence homology is evident between these regions.

We have used fragments from the plasmid, pRSV-LTR (16), in competitions for factor(s) which bind to the internal enhancer HindIII-StyI fragment (figure 5B). A fragment which includes the LTR and a portion of the 3' LTR flanking sequence between src and the 3' LTR was obtained by digesting pRSV-LTR with PvuII and BstEII (nucleotides -489 to +103 in RSV Schmidt-Ruppin A) and recovering the 594 bp fragment. This fragment competes very effectively for binding to the internal enhancer. Using other fragments from this region as competitors, the competing zone was found to lie between MluI and EcoRI.
Figure 5. Competition for Nuclear Proteins Binding to the Internal Enhancer with the SV40 Enhancer and Other Retroviral Fragments. (A) labelled HindIII-Styl was incubated with the crude nuclear extract from BHK cells and analyzed by the gel retardation assay. Competitions were performed using increasing molar excesses of the indicated fragments; RSV gag, BamHI-BamHI of RSV Schmidt-Ruppin A; SV40, PvuII-BglII (nucleotides 5235-270) of SV40; pBR, BamHI-SalI fragment of pBR322. (B) The same type of analysis as for (A) was performed, except that an LTR containing fragment (RSV LTR, nucleotides -489 to +103) was used as a competitor as indicated. The oligonucleotides EFI and EFII were also used in the indicated molar excesses as competitors.

(data not shown). Since previously reported protein-DNA interactions had been documented within the LTR (16) and these had been footprinted, we used oligonucleotides of these sites for competition assays. The sequence of the oligonucleotide for EFI is

\[
\text{GAGAAAAAGCACCGTCATGCCGATTGGTGGAAGCTCTTTTTCGTGGCACGTACGGCTAACCACCTTC}
\]

\[
\text{CTCTTTTGTGCCACGTACCGCTAACCACCTTC}
\]
Figure 6. Binding of Partially Purified Factor to Enhancer Containing Fragment. A labelled HindIII-Styl fragment was incubated with increasing amounts of partially purified protein fraction with no competitor present (lanes 2-6), with 20 ng of specific 0pvuII3' competitor present (lanes 7-11) or 20 ng of nonspecific Ras/NFI competitor present (lanes 12-16). Each set of reactions labelled NC (no competitor), 3'P (3'P is the oligonucleotide 0pvuII3') or NF I (NF I is the oligonucleotide Ras/NFI) were performed with increasing amounts of partially purified factors, with lanes 2,7,12 and 3,8,13 and 4,9,14 and 5,10,15 and 6,11,16 showing the shift pattern with 0.4, 0.9, 1.4, 1.8 and 2.3 ug of protein respectively. Lane 1 is no protein added to the binding reaction and lane 17, labelled, C, is the shift pattern seen for this fragment with a crude extract.

and the sequence of the oligonucleotide for EFII is

GTAGCTTATTGCAGAAATTGCTGCTGCTG
CATCAGATTACCCGAGACATCAGAACGTTCT

The results in figure 5, demonstrate that an oligonucleotide for EFII competes with approximately the same efficiency as does 0pvuII3', whereas the binding activity is not at all affected by an oligonucleotide of EFI.

Partially Purified Protein Fractions Have the Same Binding Pattern.

To demonstrate that the purified material was binding in the same manner as did the crude extract, a labelled fragment extending from HindIII-Styl was incubated with 1.6 µg of protein from a crude extract and 2 µg poly(dI:dC).poly(dI:dC)(figure 6, lane 17). The same labelled DNA was also incubated with 0.4, 0.9, 1.4, 1.8, 2.3 ug of partially purified protein and 500 ng poly(dI:dC).poly(dI:dC), respectively (lanes 2-6). Competitions under the same binding conditions with 20 ng of both specific (0pvuII3', lanes 7-11) and nonspecific (Ras/NFI, lanes 12-16) oligonucleotides were performed. The oligonucleotide, Ras/NFI, which contains a strong NFI binding site, was
Figure 7. DNase I Footprinting of Internal Enhancer Binding Proteins. Labelled DNA was incubated with increasing amounts of partially purified nuclear extract and DNase I was added for 1 minute at 23°C. The DNase I digestion products were then electrophoresed on a sequencing gel alongside the purine specific cleavage reaction (denoted by A/G). The coding strand (figure 7A) was incubated with 0, 0.9, 1.4, 1.8, 2.2, 2.8 μg of partially purified binding factor in each set shown (A1, A2 and A3). Set A1 (NC) is the protection pattern seen in the absence of competitor, set A2 (3′P) is the protection pattern seen in the presence of 10 ng of the oligonucleotide, OpvuII3′, and set A3 (NFI) is the protection pattern seen in the presence of 10 ng of the oligonucleotide, Ras/NFI. 7B demonstrates the same type of reactions for the noncoding strand with each set of reactions (B1, B2, B3 and B4) representing incubation with 0, 0.9, 1.8, 2.8 μg of partially purified factor. Competitions are the same as for 7A except 5′P is a competition using 10 ng of the oligonucleotide OpvuII5′.

used as a control and was the same as that described by Jones et al. (35). As increasing amounts of factor are added to the reaction, an initial complex, followed by the formation of more highly retarded complexes is evident. These highly retarded complexes (presumably reflecting multiple binding sites) are specific as demonstrated by the competitions, indicating that multiple protein complexes can form on this fragment of DNA.
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Figure 8. Schematic Diagram of the Binding Sites. The underlined portions of sequence represent those regions which were protected from DNAse I digestion. The position of each oligonucleotide which was used is also indicated.

DNAse I Footprinting of the Binding Sites.

DNAse I footprinting experiments were performed with partially purified factor under binding conditions similar to those used in the last section (figure 7). HindIII-StyI DNA fragments labelled at the StyI site with either polynucleotide kinase or with the Klenow fragment were incubated with increasing amounts of protein in the presence of 50 ng poly(dI:dC).poly(dI:dC) under standard binding conditions for 15 minutes. Reactions were treated with DNAseI, deproteinized and electrophoresed alongside the purine specific reaction products (29) as described in the Materials and Methods.

As can be seen in figure 8, on the coding strand of the DNA, incubation with increasing amounts of partially purified factors (0, 0.9, 1.4, 1.8, 2.2 and 2.8 ug protein, (left to right in set A1, A2 and A3)), generates one footprint denoted by the brackets. The footprint coincides with the oligonucleotide OpvuII3′ and only a very faint footprint is visible on the site 5′ to the PvuII site. In the lanes which follow, footprinting reactions were exactly the same, except 10 ng of specific oligonucleotide competitor was included and as can be seen the footprint is specifically competed for by OpvuII3′ (set A2) but not with Ras/NFI (set A3), demonstrating the specificity of binding to this site.

On the coding strand, two footprints are visible when increasing amounts of partially purified factors (0, 0.9, 1.8, 2.8 ug protein, (left to right in sets B1,B2,B3 and B4)) are used. The footprints on this strand are again denoted by the brackets. One footprint corresponds to the 5′ site (nucleotides 813-850) and the other corresponds to the 3′ binding site (nucleotides 856-877). In the following lanes footprints were performed exactly as for set B1, except that 10 ng of oligonucleotide competitors were included. Both of the footprinting sites are specifically competed by OpvuII3′ (set B2) and OpvuII5′ (set B3) but not with the Ras/NFI oligonucleotide (set B4). We have also seen traces of footprints between nucleotides 780-800, but these are probably the
result of another less abundant or weaker binding factor which has been removed during the purification procedure and is being pursued at this time (Steven Faber, unpublished results). Figure 8 summarizes the binding data and each binding site as determined by the DNase I footprints of the noncoding strand. The appearance of more than one footprint is consistent with the gel retardation studies with partially purified factors which also indicated that more than one factor could bind to a given DNA fragment encompassing both footprint sites.

DISCUSSION

We have demonstrated that the Schmidt-Ruppin A RSV internal enhancer is active in hamster cells and that factors within hamster cell nuclear extracts specifically bind to multiple sites within the RSV internal enhancer. This work was done in hamster cells because the RSV provirus in SR31/a cells is an example of vigorously transcribed chromatin and as such is the object of study in this laboratory. The identified binding sites map within the region of DNA which has been determined to be important in enhancer function (6) and an oligonucleotide which contains the majority of two of the binding sites functions efficiently as an enhancer element. Such results indicate that there is a close correlation between binding of the nuclear factors and in vivo function. The binding sites have been named as follows: the site located between NaeI and PvuII is the internal enhancer sequence 1 (IES1), the site directly 3' of the PvuII site (nucleotides 856-877) is internal enhancer sequence (IES2) and the site located between Styl and EcoRI is the internal enhancer sequence 3 (IES3). IES3 has been demonstrated to be within the region which is required for orientation dependence of the enhancer in chick embryo fibroblasts, NIH 3T3 cells (6) and CV1 cells (9), although we do not see an orientation dependent effect with this enhancer in BHK cells. The function of this region and the role of the specific binding, if any, in BHK cells is currently being investigated. We have therefore concentrated our attention upon the other binding sites.

Two of these sites (IES1 and IES2) have been further defined by gel mobility shift studies using specific oligonucleotides as competitors and DNase I footprinting. The region protected from DNase I digestion for IES1 extends from nucleotides 813-850 and a portion of its binding site is bisected by the junction between oligonucleotides Oconl and OpvuII5'. This result might explain why neither of those oligonucleotides was a very effective competitor. The region protected from DNase I digestion for IES2 extends from nucleotides 856-877 and coincides with the oligonucleotide OpvuII3', which was the most
efficient oligonucleotide competitor. Interestingly, OpvuII3' is an excellent competitor for binding not only of restriction fragments containing IES2, but equally well for restriction fragments containing IES1.

Both IES1 and IES2 show similar gel retardation patterns when present on separate restriction fragments and binding to all three sites, when present in the restriction fragment HindIII-StyI, is competed for by OpvuII3'. Thus we anticipated that there might be a sequence element in common between these binding sites. Sequence analysis of the regions of the IES1 and IES2 footprints reveals varying degrees of sequence homology. These sequence homologies are summarized below (with '.' indicating the lack of a nucleotide at that position).

IES2 (coding) TGCAATTGCGC
IES2 (noncoding) CGCAATTGCAG
IES1 (coding) ATC.ATTGCGG
IES1 (noncoding) CGCAATGATAG

Analysis of these sequences reveals that each of these homologies within IES2 comprises one half of a perfect inverted repeat based on the sequence: GCAA. The homologous sites identified by footprint analysis in IES1 do not contain pure inverted repeats and also do not appear to bind as tightly as observed for the site in IES2. In such sequences, only a portion of the inverted repeat is evident. Indeed the EFII binding site within the LTR contains the sequence TGCAAT, which may be sufficient to account for its ability to compete. Such small sequence homologies between enhancers have previously been reported (6) and may explain the ability of EFII to compete for binding. Based on the potency of OpvuII3' as a competitor and its almost exact correspondence with IES2, we surmise that the inverted repeat sequence shown for IES2 may approximate the binding site(s) for the protein factors to the internal enhancer.

We have found that in all the binding studies to the various restriction fragments (including binding to the oligonucleotide OpvuII3') we observe at least two specific complexes. This could be a consequence of three possible modes of interaction between the DNA fragment and the protein factors. (a) Two different proteins could bind to two independent sites on the fragment, (b) two different proteins could bind to different sites except that the binding of one protein would exclude the other and (c) there might be only one DNA binding protein and a second protein might bind to the DNA-protein complex itself. Recent work with more highly purified proteins has indicated that the binding activity can be resolved into two components each of which leads to the formation of a specific complex, which would exclude the third possibility. Discrimination between the first two possibilities should be
possible after further purification of the separate factors. As purification proceeds we will also be able to determine the relationship of the factors which recognize IES1 and IES2 to those which recognize EFII in the LTR.

Finally, the organization of this enhancer element resembles that of other enhancer elements, in that multiple binding sites reported here resemble the multiple block organization of other enhancers (25,36,37). If IES1 and IES2 are indeed repeated sequence motifs, the enhancer may resemble mutated enhancers which have been produced by duplicating any one of the blocks (25,36,37 and reference therein). We are currently determining the minimum sequence requirements for this enhancer and if a repetition of one of these blocks will generate a fully active enhancer.

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