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Capturing Nuclear Sequence-Specific DNA-Binding Proteins by Using Simian Virus 40-Derived Minichromosomes

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We have used recombinant simian virus 40 (SV40) minichromosomes to retrieve sequence-specific DNA-binding proteins derived from the cell nucleus of COS-7 cells. We showed that the transcription factors AP-1 and Sp1 are stably bound to the SV40 DNA late in viral infection. Under similar conditions, minichromosomes carrying the rat insulin (rINS1) enhancer, which is under negative regulation in COS-7 cells, bound two proteins which mapped to distinct regions of the rINS1 enhancer. The SV40 P element competed for one of these proteins which bound to the region from −198 to −230. This factor may be related to AP-1. The other factor selectively bound a regulatory element in the region from −92 to −124 of the insulin enhancer. These proteins may play a role in regulating the rINS1 enhancer function.

Eucaryotic gene transcription is controlled by *cis*-acting regulatory elements previously defined as enhancers and promoters (3, 5, 19, 32, 33, 35, 44, 50). The function of these elements is presumably mediated via interaction with nuclear *trans*-acting proteins (11, 40, 42). Until now, most of the information concerning the interactions of such factors with functionally important DNA sequences has been obtained from in vitro binding studies involving extracted cellular proteins (38, 41, 43, 56). In some cases, however, discrepancies have been found in the patterns of in vitro binding versus those of in vivo binding (10, 14, 43). We have developed a system which allows retrieval and analysis of factors binding to specific DNA sequences in the cell nucleus. The system is based on simian virus 40 (SV40) minichromosomes which replicate to a high copy number within the cell nucleus and can be isolated with proteins selectively bound to the DNA (2, 26).

To optimize the amount of viral DNA per cell and hence to increase the yield of bound factor(s), the SV40 recombinant vector was packaged in viral particles prior to infection of COS-7 cells (18, 39). In this way, more than 90% of the cells exposed to the virus contained the exogenously introduced DNA (data not shown). Minichromosomes were extracted from the cell nucleus at 40 h after infection, which is the peak time of viral-DNA replication (48). At this stage of infection, the cells contained an average of 10^4 extrachromosomal viral-DNA molecules per cell (data not shown).

AP-1 binding to SV40 enhancer late in viral infection. Plasmid pLSVPC (Fig. 1A), containing the SV40 late gene, was used to carry the SV40 enhancer (22, 54, 57) and early promoter (4, 20, 36, 48, 49) into COS-7 cells. These elements regulate both the early and late SV40 genes (6–8, 15, 17, 21, 31, 49). The pLSVPC vector replicates and is packaged into viral particles in these cells because the required T antigen is present (18, 39).

pLSVPC DNA (10 μg) was digested with *Bam*HI to remove the pBR322 sequence, religated, and transfected into ~2 × 10^6 COS-7 cells by using the calcium-phosphate precipitation technique (55). Virus obtained from these transfected cells was propagated through five cycles of infection in COS-7 cells. These viral stocks were then used to infect 4 × 10^7 to 5 × 10^7 cells (at 20 PFU per cell). At 24 h after infection, [^{3}H]thymidine was added to the medium to a final concentration of 25 μCi/ml to label the nascent DNA. After 15 h, during the period of active viral replication, the cells were transferred to 4°C and washed once with phosphate-buffered saline and once with hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.8], 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg of leupeptin per ml, 100 U of aprotinin per ml). The cells were then scraped from the plates in the same hypotonic buffer and broken in a Dounce homogenizer (pestle type B). Nuclei were pelleted at 2,000 rpm in an HB-4 rotor for 10 min and were suspended in 800 to 1,000 μl of HEPES hypotonic buffer. Minichromosomes were allowed to leak out of the nuclei for 3 h at 4°C with occasional agitation (23). Centrifugation at 10,000 × *g* for 45 s pelleted the nuclei. The supernatant was treated with 10 μg of RNase per ml for 1 h at 4°C and then loaded onto a 15 to 45% linear sucrose gradient in 50 mM NaCl–10 mM Tris (pH 8)–0.1 mM EDTA–0.1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–2-μg/ml leupeptin–10-U/ml aprotinin. After centrifugation at 40,000 rpm for 2 h at 4°C in the SW41 rotor, 0.4-ml fractions were collected, from which 50-μl samples were taken for the determination of ^{3}H counts per minute. Two ^{3}H peaks, A and B, were resolved with each virus at 40 h postinfection (Fig. 1B). At 50 h postinfection, peak A predominated, while peak B diminished (Fib. 1B). Thus, peak A represented minichromosomes packaged as viral particles, while peak B contained replicating free minichromosomes (12, 23). No similar peaks were found in noninfected cells (Fig. 1B).

Proteins bound to the DNA of both peaks were extracted from the DNA and precipitated by the slow addition of ammonium sulfate to 70% saturation in the presence of 1 mg of bovine serum albumin per ml. The precipitated proteins were collected by centrifugation, dissolved in 100 to 200 μl of 20 mM HEPES (pH 7.8)–20% glycerol–1 mM EDTA–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride (buffer A), dialyzed against buffer A containing 0.1 M KCl, divided into equal portions, and stored at −70°C. Binding to

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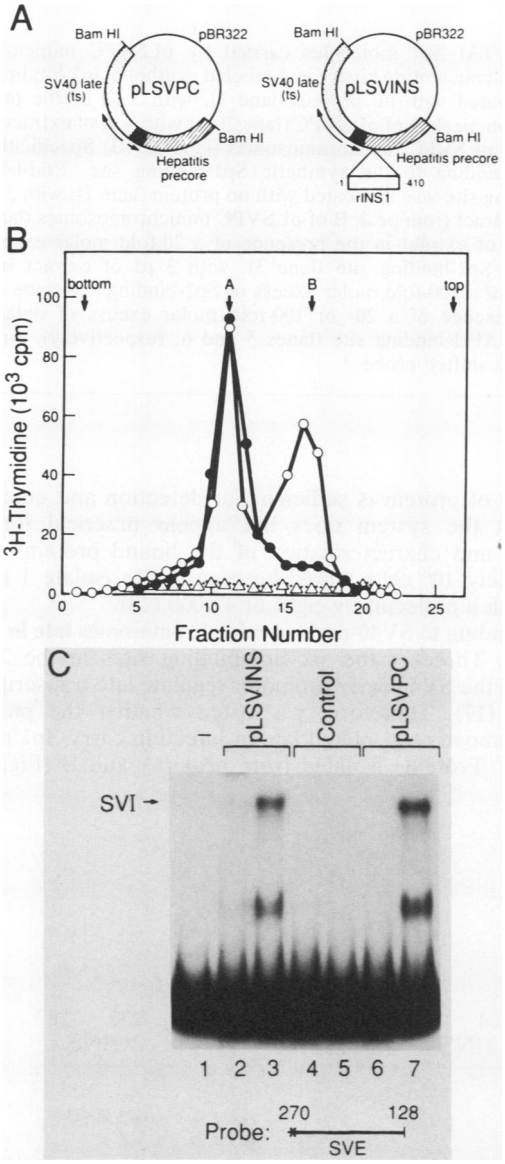

FIG. 1. (A) Structure of pLSVPC and pLSVINS plasmids. Symbols: ——, pBR322 sequences; □, segments of SV40 late genes derived from SV40 777 strain (23); ■, SV40 enhancer, early promoter, and origin of replication; ▨, hepatitis virus sequences encoding the precore protein which lack the hepatitis enhancer (45; the fragment was added to increase the size of the plasmid for more-efficient minichromosome packaging in viral particles); ▭, rINS1 5′-flanking sequences (numbers indicate positions relative to the transcription start site [+1]). ts, Temperature sensitive. (B) Isolation of minichromosomes. Minichromosomes were centrifuged in a 15 to 45% linear sucrose gradient. Samples from the gradient fractions were counted for [^{3}H]DNA. pLSVPC and pLSVINS minichromosomes gave similar profiles. Symbols: ○, cells harvested at 40 h postinfection; ●, cells harvested at 50 h postinfection; △, uninfected cells. (C) Gel retardation assay of sucrose gradient fractions from infected and uninfected cells. Proteins extracted from peaks A and B (panel B) were incubated with an end-labeled SV40 enhancer fragment (SVE) extending from nucleotides 270 (*Pvu*II) to 128 (*Sph*I) (53). Electrophoresis in a low-ionic-strength 5% acrylamide gel was used to separate complexed DNA from the free probe. Incubations included no added protein; (lane 1), 10 μl of extract from peak A of pLSVINS minichromosomes (lane 2), 10 μl of extract from peak B of pLSVINS (lane 3), 10 μl of extract from the region of peak A in uninfected cells (lane 4), 10 μl of extract from the region of peak B in uninfected cells (lane 5), 10 μl of extract from peak A of pLSVPC (lane 6), or 10 μl of extract from peak B of pLSVPC minichromosomes. Arrow indicates the position of the slowest-migrating band, termed the SVI complex.

specific DNA sequences was tested by the electrophoretic-mobility assay (46, 47). Proteins extracted from minichromosomes of peak B (but not peak A [Fig. 1C, lanes 3 and 7]) retarded the mobility of the SV40 enhancer probe (nucleotides 270 to 128) (53). The two retarded bands disappeared upon treatment of the minichromosome extracts with proteinase K (data not shown). Thus, protein interactions are responsible for the observed shift in mobility. The slowest-migrating band, termed the SVI complex, was specifically competed for by unlabeled SV40 enhancer sequences (data not shown).

The DNA sequences occupied by proteins in this complex were then mapped. Both strands of the SV40 enhancer P element (57), also shown to be the AP-1-binding region (1, 27, 28) (nucleotides 194 to 184; 5′AATTAGTCAG3′), were preferentially protected from cleavage by DNase I (Fig. 2). In addition, binding of the protein(s) of the SVI complex could be competed for by a synthetic DNA fragment containing the AP-1-binding site (data not shown). Therefore, it seems that the major protein bound to the SV40 enhancer late in viral infection was AP-1 (27, 28) or a protein with similar binding characteristics.

Previous in vitro studies have shown multiple protein-binding sites in the SV40 enhancer (11, 56). Our in vivo experiments revealed a single binding site at the P element (57) late in infection. Thus, AP-1 can be the dominant factor in activating SV40 late-gene expression (11a). This possibility suggests that different binding combinations of *trans*-acting factors may lead to alternate functional states of the SV40 enhancer (6–8, 15). Other proteins may participate and not be detected because they are present in low concentra-

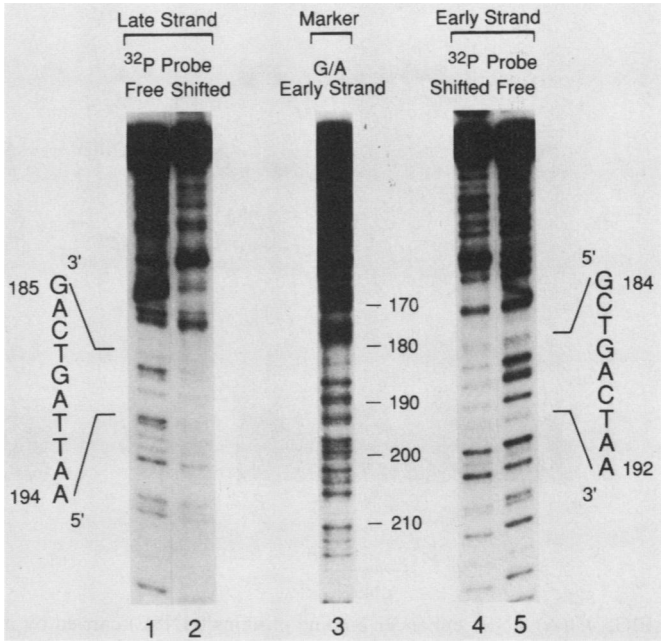

FIG. 2. DNase I footprints of the SVI complex. After incubation, the binding reaction mixture was treated with 0.7 μg of DNase I per ml for 1 min at room temperature. Lanes: 1, DNase I digestion products of the late-strand end-labeled SV40 uncomplexed probe extending from nucleotides 270 to 128; 2, DNase I digestion products of the late-strand end-labeled complexed probe; 3, G+A cleavage products (30) of the end-labeled early-strand fragment from 270 to 128 (numbers indicate nucleotide locations according to the SV40 late-gene numbering system [48]); 4, DNase I digestion products of the complexed probe end labeled on the early strand; 5, DNase I digestion products of the uncomplexed probe end labeled on the early DNA strand.

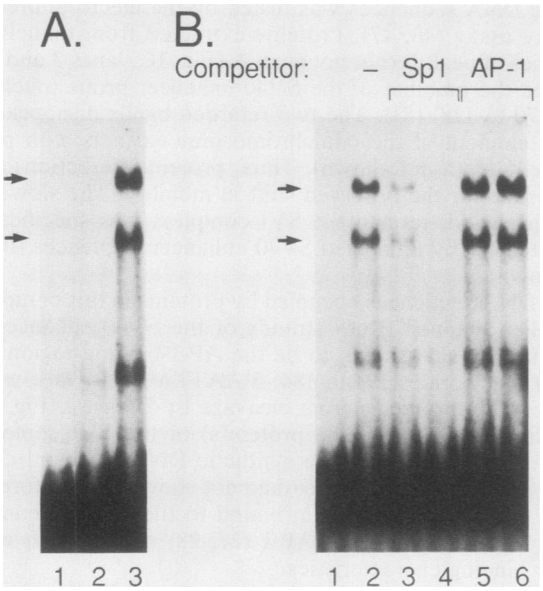

FIG. 3. (A) Sp1 molecules carried by pLSVPC minichromosomes. Polynucleotide kinase end-labeled synthetic Sp1-binding site was incubated with no protein (lane 1), with 5 μl of the protein extract from peak A of pLSVPC (lane 2), or with 5 μl of extract from peak B of pLSVPC minichromosomes (lane 3). (B) Specificities of proteins binding to the synthetic Sp1-binding site. End-labeled Sp1-binding site was incubated with no protein (lane 1), with 3 μl of protein extract from peak B of pLSVPC minichromosomes (lane 2), with 3 μl of extract in the presence of a 20-fold molar excess of unlabeled Sp1-binding site (lane 3), with 3 μl of extract in the presence of a 100-fold molar excess of Sp1-binding site (lane 4), or in the presence of a 20- or 100-fold molar excess of unlabeled synthetic AP-1-binding site (lanes 5 and 6, respectively). Arrows denote the shifted probe.

tion or have low binding affinity. By quantitating our gel shift assay, we found that about 1% of the minichromosomes (16, 29) carried AP-1 molecules, presumably one at each 72-base-pair (bp) repeat (data not shown). With this system, the recovery of protein is sufficient for detection and quantitation, but the system does not appear practical for the isolation and characterization of the bound proteins. Approximately 10^8 cells would be required to isolate 1 ng of AP-1 with a molecular weight of 47,000 (28).

Sp1 binding to SV40-derived minichromosomes late in viral infection. Three of the six Sp1-binding sites in the 21-bp repeat of the SV40 early promoter regulate late transcription in vitro (17). Therefore, we tested whether the purified minichromosomes isolated late in infection carry Sp1 molecules (9). Proteins isolated from peaks A and B (Fig. 1B)

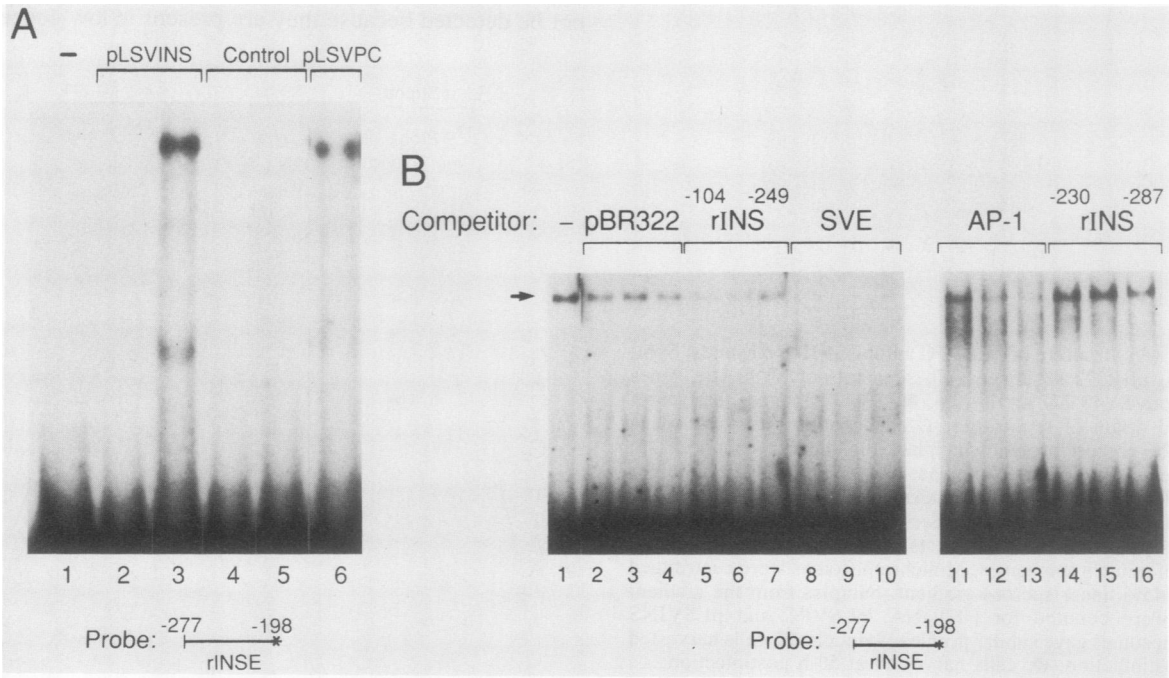

FIG. 4. (A) rINS1 enhancer binding proteins (rINSE) carried by minichromosomes. Polynucleotide kinase end-labeled rINS1 enhancer fragment (rINSE) from −198 (*DdeI*) to −277 (*MnlI*) (3, 9) was incubated with no protein (lane 1), with 10 μl of protein extract from peak A of pLSVINS (lane 2), with 10 μl of protein extract from peak B of pLSVINS (lane 3), with 10 μl of extract from the region of peak A from noninfected cells (lane 4), with 10 μl of extract from the region of peak B from noninfected cells (lane 5), or with 10 μl of protein extract from peak B of pLSVPC (lane 6). (B) Specificity of protein(s) binding to rINS1 enhancer fragment from −198 to −277. Labeled rINS1 enhancer fragment (rINSE) from −198 to −277 was incubated with 3 μl of protein extract from peak B of pLSVINS minichromosomes (lane 1); with protein extract in the presence of a 100-, 50-, or 25-fold molar excess of unlabeled *Hin*fI (220-bp) fragment of pBR322 (lanes 2 to 4, respectively); with protein extract in the presence of a 100-, 50-, or 25-fold molar excess of rINS1 enhancer fragment extending from −104 to −249 (rINS; lanes 5 to 7, respectively); with protein extract in the presence of a 100-, 50, or 25-fold molar excess of the SV40 enhancer fragment (SVE) from 270 to 128 (lanes 8 to 10, respectively); with protein extract in the presence of a 20-, 50-, or 100-fold molar excess of unlabeled synthetic AP-1-binding site (lanes 11 to 13, respectively); or with protein extract in the presence of a 20-, 50-, or 100-fold molar excess of rINS1 enhancer fragment from −230 to −287 (rINS; lanes 14 to 16, respectively). Arrow denotes the shifted probe.

were incubated with end-labeled synthetic oligonucleotide comprising a high-affinity Sp1-binding site, 5'GGGGC GGGGC3' (24). This synthetic probe, like the enhancer probe, bound protein(s) carried by peak B of the pLSVPC minichromosomes and not by peak A (Fig. 3A). Two mobility-shifted bands were detected (Fig. 3A, lane 3). These bands could reflect two different Sp1 molecules (9), two binding modes of one Sp1 species, or the binding of Sp1 and another non-Sp1 factor(s) (25, 34). The formation of the two labeled bands was specifically prevented by competition with an unlabeled synthetic Sp1-binding site but not by a synthetic AP-1-binding site composed of the sequence 5'TCTCAATTAGTCAGCAACCA3' extending from nucleotides 198 to 179 (1, 28, 48, 56) (Fig. 3B). These results suggest that two species are bound to the Sp1-binding site late in viral infection.

Binding of two proteins to rINS1 enhancer in COS-7 cells. To test the selective binding of nuclear factors to a foreign DNA fragment carried by the pLSVPC vector, we constructed a plasmid, pLSVINS, that contained a 460-bp fragment comprising the 5' flank of the rat insulin 1 (rINS1) gene DNA including the rINS1 tissue-specific enhancer (Fig. 1A) (13, 51). Proteins isolated from both pLSVPC and pLSVINS minichromosomes were tested for binding to the rINS1 enhancer fragments in regions −198 to −277 and −85 to −165 (13). These fragments are the two most important regions for enhancer activity (24a). The rINS1 fragment from −198 to −277 bound protein(s) carried by peak B of both pLSVPC and pLSVINS minichromosomes (Fig. 4A, lanes 3 and 6). The SV40 enhancer fragment competes for the bound proteins about 10-fold more effectively than does the insulin enhancer fragment (Fig. 4B, lanes 8 to 10). An oligonucleotide containing the AP-1-binding site (1, 28) also competed for the protein(s) bound to the rINS1 fragment from −198 to −277 (Fig. 4B, lanes 11 to 13). The insulin fragment from −230 to −287 did not compete for this protein (Fig. 4B, lanes 14 to 16). We inferred that sequences between nucleotides −198 and −230 bind AP-1 itself or an AP-1-related protein. This region is involved in negative regulation of the rINS1 enhancer in COS-7 cells (37) and contains the sequence 5' ATAATCT3', which is related to the AP-1-binding sequences (1, 27). A parallel sequence in rat insulin 2 enhancer is 5'AGACTCT3'. The mismatches between the insulin sequences and the consensus AP-1-binding site could explain the 10-fold difference between the AP-1-binding affinities of rINS1 and the SV40 enhancer. It is possible that AP-1 or another protein with similar binding characteristics is involved in negative regulation in COS-7 cells (37) and perhaps in regulation of the rINS1 enhancer activity in pancreatic B cells.

The insulin fragment from −85 to −165 bound specifically (Fig. 5A, bands I and II) proteins extracted from peak B of pLSVINS minichromosomes. In contrast, this fragment did not bind proteins extracted from pLSVPC minichromosomes. Thus, we presumed that the proteins bound to the insulin-specific insert. This region contains the sequence 5'CGCCATCTG3', which is homologous to the immunoglobulin μ enhancer motif (43; L. Moss et al., manuscript in preparation), which is essential for enhancer activity in pancreatic B cells (24a). To delineate the specific binding site of these proteins, competition experiments with synthetic DNA oligomers were carried out. Oligomers with the sequence extending from −92 to −124 (Fig. 5B, lanes 3 and 4) eliminated the slowly migrating band (band I). In contrast, a similar synthetic fragment, in which nucleotides −104 to −112 were mutated, was much less efficient in competing for band I (Fig. 5B, lane 5). Thus, the region from −104 to −112 of the insulin enhancer may be involved in binding the protein(s) included in band I. Competition experiments suggest that bands I and II are produced by factors that are similar to or identical with those that bind to the E elements of the immunoglobulin heavy-chain enhancer, one of which is involved in rINS1 enhancer function in pancreatic B cells (L. Moss et al., in preparation).

Our results showed that isolated minichromosomes carried sequence-specific DNA-binding proteins from the cell nucleus. This system should be valuable in studying the

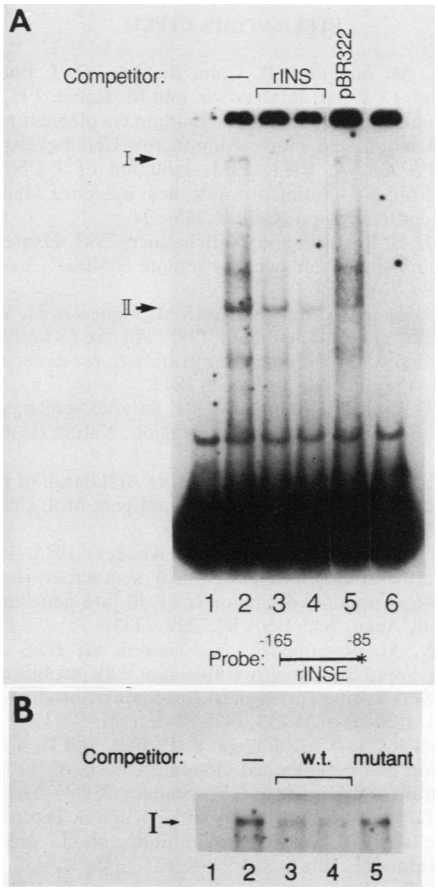

FIG. 5. (A) rINS1 enhancer binding proteins from −85 to −165 carried by minichromosomes. Labeled rINS1 enhancer fragment from −85 to −165 was incubated without proteins (lane 1); with 10 μl of protein extract from peak B of pLSVINS minichromosomes alone (lane 2), in the presence of a 50- or 150-fold molar excess of unlabeled fragment from −85 to −165 (rINS; lanes 3 and 4, respectively) or in the presence of a 150-fold molar excess of the 220-bp *Hin*fI fragment of pBR322 (lane 5) or with 10 μl of protein extract from peak B of pLSVPC minichromosomes (lane 6). Arrows indicate positions of bands I and II, which are discussed in the text. (B) Specificity of binding to rINS1 fragment from −85 to −165. Only band I (from panel A) is presented. Labeled rINS1 fragment from −85 to −165 was incubated without protein (lane 1) or with 10 μl of protein extract from peak B of pLSVINS minichromosomes alone (lane 2), in the presence of a 50- or 150-fold molar excess of double-stranded unlabeled synthetic fragment extending from nucleotides −92 to −124 of the rINS1 5'-flanking DNA sequences (lanes 3 and 4, respectively), or in the presence of a 150-fold molar excess of a similar synthetic fragment in which nucleotides −104 to −112 were changed (adenine to cytosine and guanine to thymidine; lane 5). w.t., Wild type. Arrow indicates position of band I, which is discussed in the text.

molecular nature of the complexes formed in vivo around enhancer and promoter elements (40, 52).

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