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Stem Cell Factor Binding to Retrovirus Primer Binding Site Silencers

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Using modified nuclear lysis and binding conditions, we have examined the binding of an embryonal carcinoma (EC) cell factor, binding factor A, to a stem cell-specific silencer which acts at the DNA level and overlaps the Moloney murine leukemia virus (M-MuLV) proline primer binding site (PBS). Following our protocol, we found that in vitro binding of factor A correlated with the in vivo activity of the M-MuLV silencer. Factor A bound specifically to the wild-type silencer element at room temperature and 30°C, but not at 4°C, and bound 10-fold better to the full-length silencer than to a minimal silencer core element. The factor was enriched in nuclear compared with cytosolic extracts and in undifferentiated EC cells compared with differentiated cells in which the silencer is nonfunctional. Salt and ion requirements for factor A binding were investigated, and partial purification steps indicated the factor to be a heparin-Sepharose-binding moiety of greater than 100 kDa. To examine possible relationships between silencer and PBS activities, sequences representing phenylalanine, isoleucine, lysine-1,2, lysine-3, methionine, and tryptophan PBS DNA fragments were tested in vivo for stem cell-specific repression of M-MuLV expression and in vitro in DNA binding assays. Of these PBS elements, only the lysine-1,2 PBS DNA fragment showed consistently high levels of repression. Interestingly, the lysine-1,2 PBS DNA fragment also formed a complex with an EC cell factor with characteristics similar to those of factor A. However, the two factors did not cross-compete in binding studies, suggesting that they may be different but related factors. Our results suggest that expression of Mason-Pfizer monkey virus, visna virus, and spumavirus, which use the lysine-1,2 PBS, may be inhibited in undifferentiated stem cells.

Embryonal carcinoma (EC) cells, such as F9 cells (4), derive from spontaneous gonadal tumors or from the inner cell mass of embryos at the 64- to 128-cell stage (24). They are undifferentiated and are developmentally the earliest culturable mammalian cell line. Moloney murine leukemia virus (M-MuLV) infects EC cells with extremely low efficiency (3, 10–12, 15), while differentiated cell lines such as 3T3 fibroblasts are readily infected by M-MuLV (33). The repression of M-MuLV in EC cells occurs after the viral integration step of the replication cycle (3, 5, 10, 11, 30). Infection of EC cells with M-MuLV leads to integration of proviral DNA into the genome at normal levels, but virus-specific RNA is detected at less than 1% of the level of infected fibroblast cells (10, 11, 30). A wild-type (wt) DNA element referred to as the negative regulatory element (19) or the repressor binding site (RBS) (25) has been demonstrated to be critical for part of the repression effect (3, 5, 9, 16–19, 25, 32). Previous experiments with recombinant M-MuLV vectors showed that the RBS is sufficient to repress expression from M-MuLV, simian virus 40 early, and adenovirus major late promoters in EC cells in an orientation- and position-independent (upstream or downstream) fashion (25). However, there appears to be a diminution of the repression effect when the RBS is separated from its target promoter by more than 1 to 2 kbp (5, 16). Interestingly, stem cell-specific silencing mediated by the M-MuLV RBS has been difficult to demonstrate in transient transfections but has been readily observed in transient infections, stable infections, and stable transfections (16, 19, 25). One possible explanation for these results is that the amount of DNA used in transient transfections is greater than the amount of repressor present in the cell. Alternatively, there may be a requirement for integration into the host chromosome in order for the repression effect to occur. Regardless, after an initial 10- to 100-fold repression exerted by the RBS, RBS-associated promoter regions gradually become methylated (5, 16) and are repressed an additional 10- to 100-fold. Consequently, the eventual level of repression can be 100- to 10,000-fold relative to M-MuLV expression in differentiated 3T3 fibroblast cells (3).

With regard to the RBS sequence, the element was identified first by a single point mutation (G to A at M-MuLV nucleotide [nt] 160 according to the numbering of Shinnick et al. [28]) in a recombinant M-MuLV-derived retrovirus which was not repressed in EC cells (3). This mutation (RBS_{B2}) is located in the M-MuLV primer binding site (PBS) (M-MuLV nt 146 to 163), which binds a cellular tRNA ($tRNA^{Pro}$ for wt M-MuLV) to prime first-strand synthesis during reverse transcription (33). However, the silencer function of this region was shown to be distinct from the reverse transcription role, since silencing occurs at the DNA level, in the absence of viral proteins, and a recombinant retrovirus with a PBS-glutamine (PBS_Q) (7) substituted for the wt PBS-proline (PBS_P) was replication competent but lacked the repressor function (13, 25). By using a PBS_Q recombinant retrovirus backbone, PBS-derived DNAs were tested for repressor function, and it was shown that the minimal repressor site (RBS_{WT-18}) overlapped the M-MuLV PBS_P for 17 of its 18 bp and that few point mutations were compatible with repression (16). However, it was also found that the addition of the 11-bp wt sequence onto the 3′ end of the wt RBS (RBS_{WT-28}; M-MuLV nt 147 to 174) appeared to increase repression levels by a variable amount (16).

The above-described data suggested that an EC cellular factor mediated the M-MuLV repression via the RBS at the

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DNA level, and a putative repressor factor called the negative regulatory element binding factor (19) or binding factor A (25) was identified in exonuclease III protection assays (19) and DNA mobility shift assays (25). However, the RBS binding factor (referred to here as factor A) was extraordinarily sensitive to binding conditions and was refractory to standard DNase protection and methylation interference mapping protocols. Because of its sensitivity, it previously had been difficult to assess factor A with regard to a number of parameters, such as cell specificity, optimal binding conditions, factor size, and binding site size. In our current work we are investigating the optimization and characterization of factor A binding conditions, and we now show that in vitro factor A binding correlates well with in vivo results. We also have investigated the repression effects of other PBS variants and have found that the lysine-1,2 PBS sequence (PBS_{K2}) acts as a stem cell-specific silencer, binding a factor which is related to but distinct from factor A.

MATERIALS AND METHODS

Cell extracts. Nuclear extracts were prepared from tissue culture cells essentially as described by Dignam et al. (8) with the modifications of Baeuerle and Baltimore (1, 2), which permit the fractionation of nuclear and cytosolic extracts, as well as a postnuclear fraction, obtained as resuspended pellet material from centrifugations used to clear cytosolic extracts. Cell lysis was carried out with 10 strokes of a Wheaton Dounce homogenizer (type A pestle) in Dignam buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg of leupeptin per ml, 2.5 μg of pepstatin A per ml, 2 μg of benzamidine per ml, and 10 μg of aprotinin per ml). The nuclear-D, cytosolic, and postnuclear fractions were dialyzed quickly (twice for 2 h each at 4°C) against Dignam buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) in order to retain active protein. To increase yields of binding factor A DNA binding activity, some nuclear extracts (nuclear-C extracts) were prepared without dialysis against modified Dignam buffer D and were left in Dignam buffer C (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). Protein concentrations were measured by the method of Bradford (6).

Electrophoretic mobility shift assay (gel shift assay). The preparation of double-stranded (ds) radioactive oligonucleotide probes and the gel shift procedure were essentially those of Thornell et al. (31), with modifications. For probes, sense strand oligonucleotides were end labeled for 45 min at 37°C with [γ-^{32}P]ATP and T4 polynucleotide kinase (Gibco) in 20 μl of 50 mM Tris (pH 7.4)–10 mM $MgCl_2$–1 mM spermidine–100 μg of bovine serum albumin (BSA) per ml–10 mM DTT (the reaction volume was adjusted to 20 μl with distilled water). After kinase inactivation (5 min at 70°C), a 10% excess of complementary strands was added, and strand pairs were incubated at 50°C for 30 min and then allowed to cool to room temperature. The ds radioactive probes were gel purified on a 12% native acrylamide gel in Tris-borate, and fragments were eluted (16 to 20 h at room temperature in 1 M NaCl–20 mM Tris [pH 7.5]–1 mM EDTA). The eluted probes were ethanol precipitated, dried, resuspended (25,000 cpm/μl) in distilled water, and stored at −20°C. Oligonucleotide probes were as follows (only one strand for each is designated): a wt RBS probe (RBS_{WT-28}) (5′ GGG GGCTCGTCCGGGATCGGGAGACCCC 3′), an RBS point mutant (RBS_{B2}) (5′ GGGGGCTCGTCCGaGATCGGGAGACCCC 3′), a control Sp1 transcription factor probe (Sp1) (5′ GCTCGCCCCGCCCCGATCGAAT 3′), a 28-mer primer PBS lysine-1,2 probe (PBS_{K2-28}) (5′ GGCGCCCAACGTGGGGCCGG GAGACCCC 3′) (20), and 18-mer probes representing the wt M-MuLV PBS (RBS_{WT-18}) (5′ GGGGGCTCGTCCGGGATC 3′), a phenylalanine PBS (PBS_F) (5′ TCCCGGGTTTCGGCACCA 3′) (29), an isoleucine PBS (PBS_I) (5′ TCCC CGTACGGGCCACCA 3′) (20), lysine-2 (PBS_{K2-18}) (5′ GCCCCACGTTGG GCGCCA 3′) (20) and lysine-3 (PBS_{K3}) (5′ GTCCCTGTTCGGGCGCCA 3′) (33) PBSs, a methionine PBS (PBS_M) (5′ TCCTCACACGGGGCACCA 3′) (29), and a tryptophan PBS (PBS_W) (5′ ATCACGTCGGGGTCACCA 3′) (33).

Typical binding reactions were carried out with an excess of probe and 5 to 10 μl of cellular extract (the amount of extract varied because of the addition of various other components which were tested [see below]) in a total volume of 20 μl of Thornell binding buffer (25 mM HEPES [pH 7.9], 1 mM EDTA, 10% [vol/vol] glycerol, 5 mM DTT, 0.5 mM PMSF) with an additional 25 ng of poly(dI-dC) per μl, 5 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. Some reaction mixtures contained 3 μl of fetal calf serum (FCS), which was added to increase the signal of the factor A band, and various concentrations of salts (0 to 1 M NaCl and 0 to 90 mM KCl), divalent cations (0 to 10 mM [each] $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnCl_2$, and $NiSO_4$), nucleotides (0 to 10 mM [each] cyclic AMP [cAMP], ATP, GTP, and $GTP_{\gamma S}$), and detergents (0 to 1% [each] deoxycholate, Nonidet P-40, Sarkosyl, sodium dodecyl sulfate, and Triton X-100) were tested during our investigations. For competitions, various amounts (15 to 200 ng) of unlabeled ds and single-stranded forms of the oligonucleotides used for probes were added 20 min prior to the addition of the radioactive probe. Unless indicated otherwise, binding reaction mixtures were preincubated at 30°C for 20 minutes, and then ds probes were added (50,000 cpm, 0.5 ng per reaction mixture) and mixtures were incubated at 30°C for an additional 20 min. After incubations, binding reactions were run on 6% native acrylamide gels (31.0 cm by 38.5 cm by 0.4 mm) at 800 V for approximately 3 h in a Tris-glycine buffer (5 mM Tris [pH 8.5], 38 mM glycine, 0.2 mM EDTA). After electrophoresis, gels were transferred to used X-ray films for support and were exposed for 3 to 7 days at −80°C with intensifying screens.

Partial protein purifications. Binding factor A was partially purified from F9 nuclear extracts by Sephadex G-200 (Pharmacia), heparin-Sepharose (Pharmacia), and DNA-affinity chromatographies. Sephadex gel filtration was at 4°C with a 20-ml (bed volume) column and a 9-ml void volume. Crude nuclear extract was loaded in Dignam buffer D (without glycerol) at a gravity flow rate of 0.5 ml/min. Sephadex G-200 fractions (1 ml) were concentrated rapidly by centrifugation on Microcon-30 microconcentrators (Amicon) at 4°C in an Eppendorf 5415 microcentrifuge at 12,000 rpm for 30 min, and fractions were snap frozen on dry ice and stored at −80°C.

For heparin-Sepharose chromatography, crude nuclear extracts were applied to a 10-ml (bed volume) heparin-Sepharose column in Dignam buffer D (without glycerol) as the loading buffer, and proteins were eluted from the resin by increasing the KCl concentration of the running buffer in steps (0 mM, 100 mM, 300 mM, 600 mM, and 1 M KCl). Heparin-Sepharose fractions were desalted and concentrated with Centricon-100 microconcentrators by adding the fractions to the concentrators and spinning at 4°C in a Sorvall SS-34 rotor at 6,500 rpm for 30 min. The concentrated heparin-Sepharose fractions were then diluted with Dignam buffer D (with glycerol) and reconcentrated as described above. The dilution-reconcentration step was performed three times, each time with approximately a 1:5 dilution of concentrated sample to buffer D. The final volume of each fraction was approximately one-half of the starting fraction volume.

For DNA affinity chromatography, a 5′ biotinylated RBS_{WT-28} oligonucleotide was used. The DNA affinity resin was prepared by annealing 2.5 μg each of the antisense strand biotinylated RBS_{WT-28} oligonucleotide and its complementary strand and binding the ds product to streptavidin-agarose resin (Sigma) equilibrated in MY running buffer [25 mM HEPES (pH 7.9), 0.5 mM EDTA, 10% glycerol, 5 mM DTT, 0.5 mM PMSF, 3 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 25 ng of poly(dI-dC) per μl]. Chromatography binding reactions were performed in a total volume of 2.5 ml [500 μl (bed volume) of DNA affinity resin, 500 μl of F9 nuclear extract, 400 μl of 5× Thornell binding buffer, 300 μl of bandshift salts, 300 μl of FCS, 100 μl of 500-ng/μl poly(dI-dC), 50 μl of PMSF, and 350 μl of H_2O], and reaction mixtures were incubated at 30°C with periodic agitation. The binding mixture was centrifuged (15 s at 90 × *g*), and the supernatant was collected as the flowthrough fraction. The resin was washed with MY buffer containing increasing amounts of KCl (0 mM, 200 mM, 400 mM, 600 mM, 800 mM, and 1 M). Fractions were desalted and concentrated in the same manner as for heparin-Sepharose fractions. The resin was washed with 5 ml of MY running buffer plus 0.02% sodium azide and stored at 4°C.

Recombinant retroviruses. BlankIsle is a plasmid derived by inserting the 702-bp *Eco*RV-to-*Sca*I fragment of pBR322, with *Bam*HI linkers, into the unique *Bam*HI site of PBS_Q (16, 25). WTIsle is a subclone of BlankIsle containing M-MuLV nt 147 to 174 at the old pBR322 *Ssp*I site. B2Isle is similar to WTIsle but has the previously described single-base-pair RBS_{B2} mutation (3). The PBSIsle and miniWTIsle constructs are subclones containing the following different tRNA PBSs at the old pBR322 *Ssp*I sites (only the sense strand is shown): PBS_F, 5′ TCCCGGGTTTCGGCACCA 3′ (29); PBS_I, 5′ TCCCCGTA CGGGCCACCA 3′ (from RTVL-1) (20); PBS_{K2}, 5′ GCCCCACGTTGGGCG CCA 3′ (from Mason-Pfizer monkey virus, visna virus, and spumavirus) (20); PBS_{K3}, 5′ GTCCCTGTTCGGGCGCCA 3′ (from human immunodeficiency virus) (33); PBS_M, 5′ TCCTCACACGGGGCACCA 3′ (29); and PBS_W, 5′ AT CACGTCGGGGTCACCA 3′ (from Rous sarcoma virus and avian myeloblastosis virus) (33). The F9 EC and NIH 3T3 cell lines were grown as described previously (3, 32), and virus infections, selections with G418 (Gibco), and titer determinations (given as 3T3/F9 ratios [restriction indices], where higher numbers indicate greater viral repression) were performed as described previously (3, 23). Cloning and sequencing were by standard protocols (22, 27).

RESULTS

Characteristics of factor A binding to the M-MuLV RBS. The stem cell-specific silencer element called the RBS or negative regulatory element originally was identified as a *cis*-active element in M-MuLV that repressed expression at the DNA level in EC or embryonal stem cells (19, 25). Subsequently, an EC cell factor, binding factor A, was identified by exonuclease III protection assays and gel mobility shift assays with a wt RBS probe (RBS_{WT}) (19, 25). However, by either assay, factor A

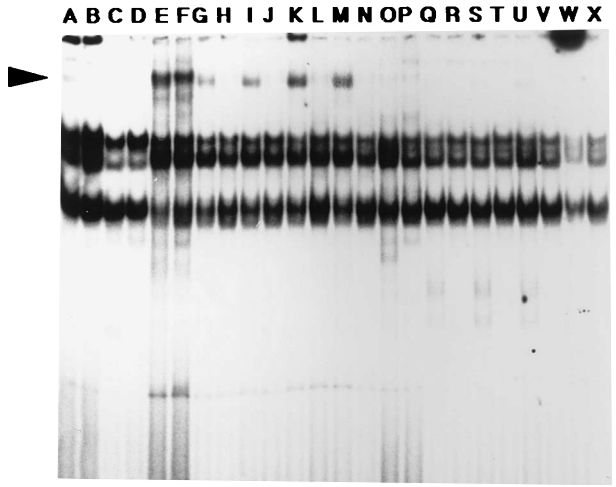


FIG. 1. RBS binding factors in F9 and 3T3 cells. The ds RBS_{WT-28} and RBS_{B2} (28-mer) probes were prepared as described in Materials and Methods and used at 50,000 cpm (approximately 2 ng) per binding reaction. The sequences of these probes were as follows.

RBS_{WT-28}: 5′ GGGGG CTCGT CCGGG ATCGG GAGCA CCC 3′
3′ CCCCC GAGCA GGCCC TCGCC CTCGT GGG 5′

RBS_{B2}: 5′ GGGGG CTCGT CCGaG ATCGG GAGCA CCC 3′
3′ CCCCC GAGCA GGCtC TAGCC CTCGT GGG 5′

RBS_{WT-28} (lanes A, C, E, G, I, K, M, O, Q, S, U, and W) or RBS_{B2} (lanes B, D, F, H, J, L, N, P, R, T, V, and X) was incubated for 20 min with extracts at room temperature (lanes A to D, G to N, and Q to X) or at 4°C (lanes E, F, O, and P). Reaction mixtures contained extracts from F9 cytosol (lanes A and B) (6.5 mg/ml), 3T3 cytosol (lanes C and D) (2.8 mg/ml), F9 nuclei (lanes E to N) (1.3 mg/ml), or 3T3 nuclei (lanes O to X) (1.5 mg/ml). In some cases, DTT was added to a final concentration of 1 mM (lanes I, J, S, and T), and $ZnCl_2$ was added to a final concentration of 10 μM (lanes A to J and O to T) or 100 μM (lanes K to N and U to X). Binding reactions were terminated by addition of loading dye, and free and complexed probes were separated by electrophoresis on a 6% native acrylamide–Tris-glycine gel and autoradiographed. Because large gels (31.0 cm by 38.5 cm by 0.4 mm) were used for electrophoresis, free probe is not shown. The factor A band is indicated by the arrowhead, and the nonspecific B and C complexes appear as bands of increasing mobilities.

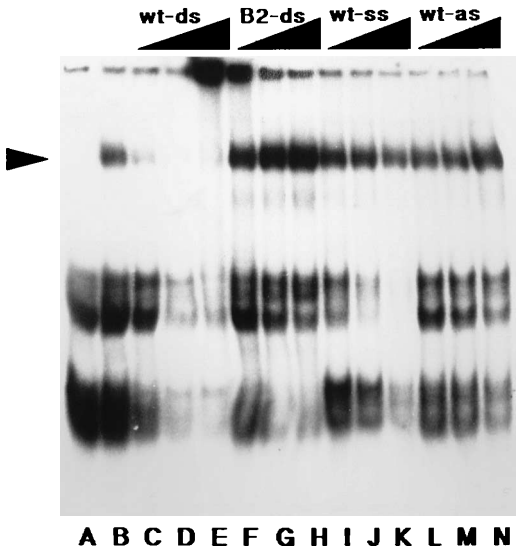


FIG. 2. Factor A binding competition studies. RBS_{WT-28} (lanes B to N) or RBS_{B2} (lane A) probe was incubated for 20 min at room temperature with F9 nuclear extract (1.3 mg/ml). Binding reactions were performed without competitor DNA (lanes A and B) or with increasing amounts of the following unlabeled competitors: RBS_{WT-28} (ds) (5 ng [lane C], 15 ng [lane D], or 45 ng [lane E]), RBS_{B2} (ds) (5 ng [lane F], 15 ng [lane G], or 45 ng [lane H]), RBS_{WT-28} (sense strand [ss] only) (5 ng [lane I], 15 ng [lane J], or 45 ng [lane K]), and RBS_{WT-28} (antisense strand [as] only) (5 ng [lane L], 15 ng [lane M], or 45 ng [lane N]). Binding reactions, electrophoresis, and autoradiography were as described in the legend to Fig. 1. The factor A band is indicated by the arrowhead.

binding was very sensitive and required specific binding and gel electrophoresis conditions (19, 25). Because of these difficulties, additional attempts to characterize factor binding to the RBS and related sequences were hampered.

In order to circumvent the above-described problem, we have undertaken efforts to optimize and analyze factor A binding conditions. Because we found that factor A did not tolerate extensive Dounce homogenization or dialysis, we modified our original protocol for extract preparation as described in the Materials and Methods. By using this protocol, lysates were prepared from F9 EC cells, which demonstrate the RBS-mediated repression, and from 3T3 fibroblasts, which are unaffected by the RBS (3, 9, 19, 25). As shown in Fig. 1, three major cellular complexes form with the 28-bp RBS_{WT} probe (RBS_{WT-28}): a pair of fast-migrating nonspecific complexes (referred to as complexes B and C) and the low-mobility factor A complex, indicated by the arrowhead. At 4°C, factor A appeared to bind equally well to the RBS_{WT-28} probe (Fig. 1, lane E) and a 1-bp mutant probe (RBS_{B2}) (lane F), but at room temperature or 30°C, the factor A specificity for the RBS_{WT-28} probe was apparent (lanes G to N). In comparison with complexes B and C, factor A appeared to be enriched in nuclear versus cytosolic extracts (compare lane A with lanes G, I, K, and M in Fig. 1), as might be expected for a factor which acts at the DNA level. Consistent with the observation that RBS repression is specific for EC or embryonal stem cells, factor A was enriched greatly in F9 (Fig. 1, lanes G, I, K, and M) versus 3T3 (lanes Q, R, U, and W) nuclear extracts. Similarly, we have observed factor A in extracts of the PCC4 EC cell line (25), in which the RBS is active, but not in other cell lines which do not demonstrate RBS repression (16), such as mouse SLK.3 and 10T1/2, rat PC12, and human HeLa and 293 cells (data not shown).

Previous competition and interference assays demonstrated the specificity of factor A binding to the RBS_{WT-28} probe, as did assays with RBS_{WT-28} versus RBS_{B2} probes (Fig. 1). Nevertheless, it was of interest to test competitors in binding assays with lysates produced by our modified protocol. Results of these experiments are shown in Fig. 2. Unlabeled ds RBS_{WT-28} DNA effectively competed for factor A binding to the RBS_{WT-28} probe (Fig. 2, lanes C to E), whereas unlabeled ds RBS_{B2} (lanes F to H) and single-stranded RBS_{WT-28} (lanes I to N) DNAs were poor competitors. Because the RBS sequence overlaps the M-MuLV tRNA PBS, it also was of interest to investigate whether factor A might recognize some feature of a tRNA molecule. However, neither total calf liver tRNA (up to 400 ng, a 200-fold excess of tRNA to probe) nor purified $tRNA^{Pro}$ (up to 500 ng, a 250-fold excess of tRNA to probe) (kindly provided by D. Dignam) competed for factor A binding (data not shown). Similarly, purified proline tRNA synthetase (40 μg/ml) (kindly provided by D. Dignam) showed no binding to the RBS_{WT-28} probe on gel shifts (data not shown).

With regard to optimization of factor A binding, the presence of 100 μM zinc (Fig. 1, lanes K and M) versus 10 μM zinc (lanes G and I) appeared to improve binding, while a reducing agent such as DTT had no apparent effect (Fig. 1, compare lanes I and G and compare lanes M and K). The effects of other salts, divalent cations, chelators, phosphorylation inhibitors, nucleotides, and detergents also were tested. In particu-

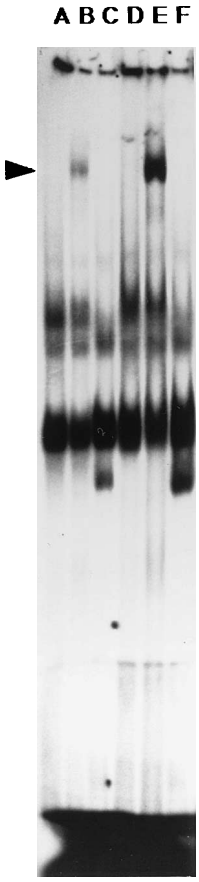


FIG. 3. Serum effects on factor A detection. RBS_{WT-28} (lanes B and E), RBS_{B2} (lanes A and D), or JCV, a control dsDNA (sense, 5′ GAGCT CATGC TTGGC TGGCA GCCAT CCCT) (lanes C and F) probes were incubated at 30°C (following preincubations for 20 min at 30°C without probe) with F9 nuclear extract (0.28 mg/ml) in the absence (lanes A to C) or presence (lanes D to F) of 3 μl of FCS (Gibco). Electrophoresis and autoradiography were as described in the legend to Fig. 1. The factor A complex is indicated by the arrowhead, and free probe is shown at the gel bottom.

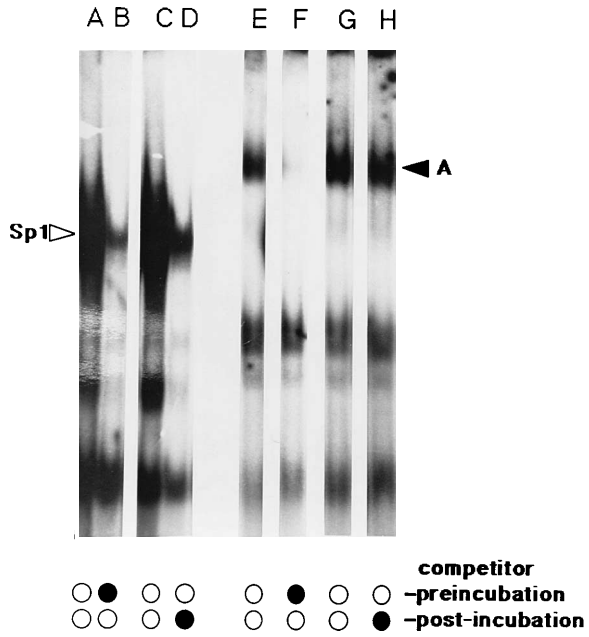
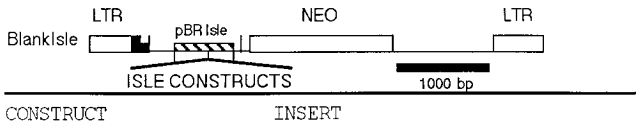


FIG. 4. Binding factor dissociation rates. Sp1 (sense, 5′ GCTCG CCCCG CCCCG ATCGA AT 3′ (lanes A to D) or RBS_{WT-28} (lanes E to H) probe was incubated with F9 nuclear extract (2.1 mg/ml) for 20 min at 30°C. Fifteen nanograms of unlabeled Sp1 or RBS_{WT-28} competitor was either not added (lanes A, C, E, and G), added 5 min prior to probe addition (lane B [Sp1 competitor] and lane F [RBS_{WT-28} competitor]), or added 20 min after probe addition (lane D [Sp1 competitor] and lane H [RBS_{WT-28} competitor]). For lane D, binding continued for 5 min after competitor addition, while for lane H, binding continued for 20 min after competitor addition. Gel electrophoresis and autoradiography was as described in the legend to Fig. 1.

lar, we found that factor binding occurred at 0 to 150 mM NaCl but was abolished at 250 mM NaCl. Divalent cations provided as $MgCl_2$ and $CaCl_2$ permitted detection of the factor A complex at 3 mM (with $MgCl_2$ enhancing the factor A band at this concentration), but they inhibited binding of factor A at 10 mM (data not shown). Binding was eliminated by 6 mM EDTA, detergents (0.1% Sarkosyl or deoxycholate), and 10 mM *N*-ethylmaleimide (unless it was preblocked by DTT) but was detected at only slightly reduced levels with 10 mM EGTA [ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and was unaffected by up to 1 mM sodium vanadate or sodium fluoride, 1 to 10 mM cAMP, 0.1 to 1 mM ATP or GTP, or 0.1 mM $GTP_{\gamma S}$ (data not shown).

In other studies, on the basis of the observation that extensive dialysis either diminished the factor A band signal or abolished it outright, we modified the lysis procedure by leaving the extracts in Dignam buffer C and eliminating the dialysis against Dignam buffer D, as described in Materials and Methods. The nuclear extracts which were kept in Dignam buffer C (nuclear-C extracts) yielded a more reproducible factor A band than did the nuclear extracts which were dialyzed against Dignam buffer D (nuclear-D extracts) (data not shown). Surprisingly, we also found that the addition of serum to our standard binding reaction mixtures increased the signal of the factor A band approximately 10-fold. Serum itself showed no RBS_{WT-28} or RBS_{B2} binding activity (data not shown), but as illustrated in Fig. 3, FCS enhanced the factor A complex (lane E versus lane B), while it had no effect on the B or C complex (Fig. 3) or on the F9 nuclear Sp1 site binding activity (data not shown). The enhancement of the factor A band also was observed with rabbit serum, horse serum, and FCS that had been extensively dialyzed or that had been heat treated for 1 h at 50°C, suggesting that the enhancement effect was a nonspecific one. However, factor A signals were not increased by up to 9 mg of BSA per ml, and 9 mg of gelatin per ml produced only a twofold increase in the signal. These results imply that factor A is stabilized by either a specific heat-resistant component or a nonspecific mixture of nondialyzable components in serum, and we currently are testing these hypotheses.

One possible explanation for the repeatedly observed sensitivity of factor A is that it might possess an unusually high rate of dissociation from its DNA-binding site. To test this, rates were examined by adding ds competitor DNAs to binding reaction mixtures either 5 min before or 20 min after probe addition. As shown in Fig. 4, the F9 Sp1 site binding activity (14, 26) was inhibited effectively both when competitor was added before the probe (lane B) and when it was added after the probe (lane D). However, ds RBS_{WT-28} competitor DNA competed with RBS_{WT-28} probe for factor A only when the competitor was added before the probe (lane F) and not when it was added after the probe (lane H). These results suggest that the Sp1 site binding activity dissociates from its probe within 5 min after competitor addition (lane D), while factor A remains probe bound for over 20 min (lane H). Thus, it does



BlankIsle	none
B2Isle	T GGGGG CTCGT CCGaG ATCGG GAGAC CCCAT
WTIsle	T GGGGG CTCGT CCGGG ATCGG GAGAC CCCAT
miniWTIsle	T GGGGG CTCGT CCGGG AT
PBSIsleF	T GGTGC CGAAA CCCGG GA
PBSIsleI	T GGTGG CCCGT ACGGG GA
PBSIsleK2	T GGCGC CCAAC GTGGG GC
PBSIsleK3	T GGCGC CCGAA CAGGG AC
PBSIsleM	T GGTGC CCCGT GTGAG GA
PBSIsleW	T GGTGA CCCCG ACGTG AT

FIG. 5. Recombinant retrovirus constructs. BlankIsle (16) is a recombinant retrovirus construct based on the vectors PBSQ (25) and MP10 (3). As a provirus it is 5,370 bp and contains the following elements: an intact M-MuLV 5′ long terminal repeat (LTR) up to the *Kpn*I site at proviral nt 480 (viral nt 32); 5′ noncoding sequences corresponding to M-MuLV viral nt 32 to 212 from an endogenous murine retrovirus (7), which alters the M-MuLV PBS from one for proline (PBSP) to one for glutamine (PBSQ); M-MuLV nt 212 to 563; a 702-bp *Eco*RV-to-*Sca*I "island" fragment from pBR322, which was *Bam*HI linkered and inserted into a created *Bam*HI site; the M-MuLV splice acceptor (viral nt 5409 to 5768); the neomycin gene (NEO) from Tn*5*, including the bacterial promoter; simian virus 40 (viral nt 160 to 0 to 5154) and pBR322 (nt 3102 to 2521) origins of replication; and the 3′ end of M-MuLV from viral nt 7197 through the 3′ LTR. As a virus, BlankIsle uses a glutamine tRNA primer and is not repressed in undifferentiated EC cells (16). The constructs WTIsle and B2Isle differ from BlankIsle in that fragments from wt M-MuLV (nt 147 to 174) or from the nonrepressed B2 mutant were inserted into the unique *Ssp*I site in the BlankIsle pBR322 island. The miniWTIsle construct is similar to WTIsle except that it contains an insert of only M-MuLV PBS sequences and does not possess M-MuLV nt 164 to 174. PBSIsleF, PBSIsleI, PBSIsleK2, PBSIsleK3, PBSIsleM, and PBSIsleW were derived from BlankIsle by insertion into the *Ssp*I site of PBS sequences corresponding to tRNAs for phenylalanine, isoleucine, lysine-1,2, lysine-3, methionine, and tryptophan, respectively. For each insert the indicated initial T residue is derived from the *Ssp*I juncture sequence, as is the 3′ AT for the B2Isle, WTIsle, and miniWTIsle constructs.

not appear that the sensitivity of the factor A complex is due to an inordinately high dissociation rate.

To further characterize factors which bind to the M-MuLV RBS, F9 nuclear lysates were passed over a Sephadex G-200 gel filtration column and binding activity from the column was monitored by gel shift assay. By following this protocol, we found that binding activity eluted from the column near the void volume, implying a size of greater than 100 kDa for factor A (data not shown). In a separate enrichment step, crude F9 nuclear extracts were applied to a heparin-Sepharose column, from which factor A eluted in the 600 mM KCl wash with a 5- to 10-fold enrichment (data not shown). In addition to Sephadex G-200 and heparin-Sepharose chromatographies, alternate factor A enrichment steps have been tested. Factor A was present in the flowthrough fraction of nuclear lysate-loaded DEAE-Sepharose columns, and precipitates in the 40 to 50% ammonium sulfate fractionation cut, but these steps yielded only a slight enrichment in specific binding activity. Initial attempts at affinity chromatography with RBS_{WT-28} multimers covalently attached to Sepharose 4B via a cyanogen bromide catalyst showed little enrichment of factor A in high-salt washes. Subsequent attempts with a 5′ biotinylated RBS_{WT-28} sequence in conjunction with streptavidin-agarose beads showed that factor A bound and eluted in a 600 mM KCl wash (data not shown), although these attempts so far have yielded too little protein to permit accurate estimation of enrichment levels. With all our purification efforts, we have found that two characteristics of factor A have hampered purification. First, binding activity rapidly decays in high-salt solutions. Second, partially purified factor A binding activity in dilute solutions is sensitive to freeze-thaw episodes and diminishes over time, even at −80°C. Nevertheless, immediate Centricon desalting and concentration of partially purified fractions have improved retention of factor A binding activity, suggesting that further purification should be possible.

Effects of other PBS sequences. The fact that the M-MuLV RBS could not be delineated more than 1 bp smaller than the wt PBS_P (16) suggested that there might be something about a tRNA PBS which affects repression. It was shown that a PBS_Q substitution for the PBS_P eliminated RBS-mediated repression of M-MuLV in EC cells (16, 25), but other PBS sequences were not tested for the repression phenomenon. To examine potential effects of other PBS sequences on EC cell repression of M-MuLV, we employed a parental construct (BlankIsle) which uses the nonrepressed PBS_Q and possesses a unique *Ssp*I site for insertion of test sequences (16) (Fig. 5). For test sequences we inserted PBS sequences which can anneal to tRNA acceptors for phenylalanine (PBS_F) (29), isoleucine (PBS_I) (present in RTVL-1) (20), lysine-1,2 (PBS_{K2}) (present in Mason-Pfizer monkey virus visna virus, and spumavirus) (20), lysine-3 (PBS_{K3}) (present in human immunodeficiency virus) (33), methionine (PBS_M) (29), and tryptophan (PBS_W) (present in Rous sarcoma virus and avian myeloblastosis virus) (33), as well as the RBS_{WT-18}, RBS_{WT-28}, and RBS_{B2} sequences (Fig. 5).

Analysis of stem cell-specific repression effects of test sequences followed our previously established methods (3, 16, 25, 32). Virus stocks were made in Psi2 packaging cells (23) and used for parallel infections of 3T3 fibroblasts (as a control

TABLE 1. Viral infectivities[a]

Expt and construct	Titer			% WTIsle repression
	3T3	F9	3T3/F9	
1				
WTIsle	137,500	6	22,917	100
B2Isle	75,000	1,213	62	0.3
MiniWTIsle	600,000	502	1,195	5.2
PBSIsleF	35,500	296	110	0.5
PBSIsleI	65,000	963	67	0.3
PBSIsleK2	101,000	15	6,733	29
PBSIsleK3	27,750	245	113	0.5
PBSIsleM	65,000	2,388	27	0.2
2				
WTIsle	165,000	18	9,167	100
B2Isle	105,000	1,260	83	0.9
MiniWTIsle	212,000	95	2,232	24.5
PBSIsleF	72,500	75	966	10.5
PBSIsleI	47,500	1,140	42	0.5
PBSIsleK2	165,000	15	11,000	120
PBSIsleK2	240,000	90	2,667	29
PBSIsleK3	120,000	170	706	7.7
PBSIsleK3	82,500	120	688	7.5
PBSIsleM	50,000	640	78	0.9
PBSIsleW	23,000	340	68	0.7
PBSIsleW	27,500	420	65	0.7

[a] DNA constructs were converted into recombinant retrovirus stocks by transient transfection followed by infection of into Psi2 packaging cells (23). Identical Psi2 supernatants were used to infect NIH 3T3 and F9 cells. Titers are expressed as the number of G418-resistant colonies formed per milliliter of viral supernatant. The ratio of 3T3 to F9 titers (the restriction index) is given to compare F9 restriction of different constructs within the same experiment, with 3T3 titers as a standard. The percentage of WTIsle repression was calculated as 100 times the restriction index of the experimental construct divided by the restriction index of WTIsle in the same experiment.

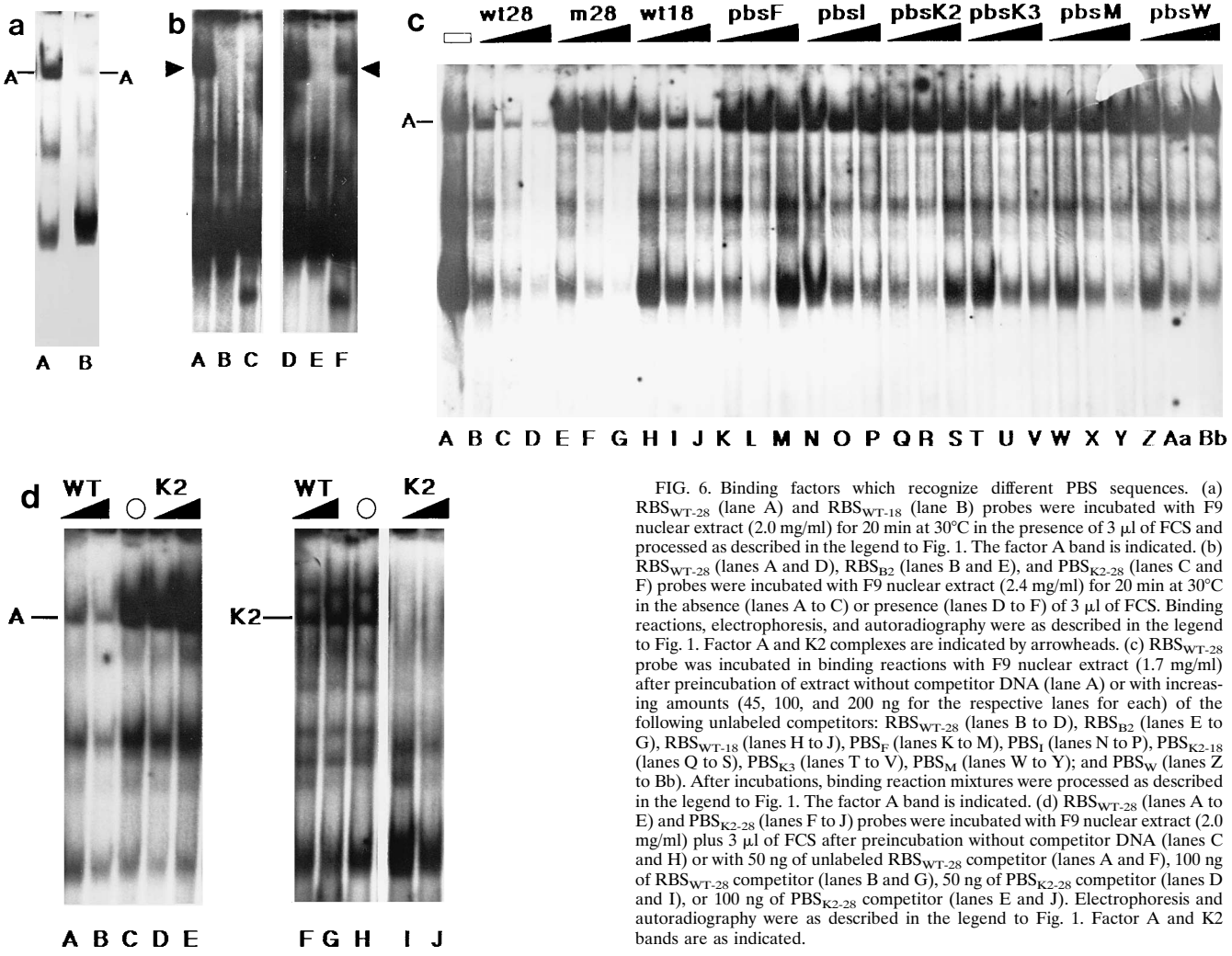


FIG. 6. Binding factors which recognize different PBS sequences. (a) RBS_{WT-28} (lane A) and RBS_{WT-18} (lane B) probes were incubated with F9 nuclear extract (2.0 mg/ml) for 20 min at 30°C in the presence of 3 μl of FCS and processed as described in the legend to Fig. 1. The factor A band is indicated. (b) RBS_{WT-28} (lanes A and D), RBS_{B2} (lanes B and E), and PBS_{K2-28} (lanes C and F) probes were incubated with F9 nuclear extract (2.4 mg/ml) for 20 min at 30°C in the absence (lanes A to C) or presence (lanes D to F) of 3 μl of FCS. Binding reactions, electrophoresis, and autoradiography were as described in the legend to Fig. 1. Factor A and K2 complexes are indicated by arrowheads. (c) RBS_{WT-28} probe was incubated in binding reactions with F9 nuclear extract (1.7 mg/ml) after preincubation of extract without competitor DNA (lane A) or with increasing amounts (45, 100, and 200 ng for the respective lanes for each) of the following unlabeled competitors: RBS_{WT-28} (lanes B to D), RBS_{B2} (lanes E to G), RBS_{WT-18} (lanes H to J), PBS_F (lanes K to M), PBS_I (lanes N to P), PBS_{K2-18} (lanes Q to S), PBS_{K3} (lanes T to V), PBS_M (lanes W to Y); and PBS_W (lanes Z to Bb). After incubations, binding reaction mixtures were processed as described in the legend to Fig. 1. The factor A band is indicated. (d) RBS_{WT-28} (lanes A to E) and PBS_{K2-28} (lanes F to J) probes were incubated with F9 nuclear extract (2.0 mg/ml) plus 3 μl of FCS after preincubation without competitor DNA (lanes C and H) or with 50 ng of unlabeled RBS_{WT-28} competitor (lanes A and F), 100 ng of RBS_{WT-28} competitor (lanes B and G), 50 ng of PBS_{K2-28} competitor (lanes D and I), or 100 ng of PBS_{K2-28} competitor (lanes E and J). Electrophoresis and autoradiography were as described in the legend to Fig. 1. Factor A and K2 bands are as indicated.

for absolute levels of infectious virus) and F9 cells. By comparison of virus titers (number of G418-resistant colonies per milliliter of virus), it was possible to assess repression levels in EC cells. Consistent with previous results (16), we found that the RBS_{WT-28} element in WTIsle caused a 100- to 300-fold reduction in virus titers in EC cells relative to that caused by the 1-bp RBS_{B2} mutant sequence in B2Isle (Table 1). Additionally, the minimal 18-bp RBS_{WT-18} sequence in miniWTIsle repressed expression about 20-fold relative to repression by B2Isle in parallel experiments but 4- to 20-fold less than the 28-bp RBS_{WT-28} element (Table 1). Of the alternative 18-bp PBS sequences, the isoleucine, methionine, and tryptophan PBS elements in PBSIsleI, -M, and -W consistently showed no repression effect, while those in PBSIsleF and -K3 demonstrated moderate (2- to 10-fold) but variable levels of repression. Follow-up experiments using a recombinant M-MuLV with a PBS_{K3} replacement for the natural PBS_P (tvPBS-Lys-3; kindly provided by F. S. Pedersen) (21), also showed the lysine-3 PBS to have a small but detectable (threefold) repression of M-MuLV expression in EC cells (data not shown). However, in contrast to the other alternative PBS inserts, the lysine-1,2 tRNA PBS of PBSIsleK2 consistently gave titers in EC cells that were repressed over 30-fold relative to those with B2Isle (Table 1). These results indicate that the PBS_{K2} present in Mason-Pfizer monkey virus, visna virus, and spumavirus can repress expression in EC cells, at least in the context of the BlankIsle backbone.

To extend our in vivo results, factor binding to variant RBS and PBS sequences was examined by gel shift assay (Fig. 6). An RBS_{WT-18} probe corresponding to the insert in miniWTIsle formed three complexes with F9 nuclear lysates (Fig. 6a, lane B), one of which had the same mobility as the corresponding RBS_{WT-28} factor A band (lane A). However, the factor A complex detected by RBS_{WT-18} was about 10-fold less intense than that detected by RBS_{WT-28}, a reduction which could be due to the difference in probe fragment sizes but nonetheless correlates well with in vivo results.

With regard to alternative PBS sequences, we found no evidence of a factor A-like complex with any of our PBS_F, PBS_I, PBS_{K3}, PBS_M, and PBS_W probes (data not shown). However, with 18- and 28-bp PBS_{K2} probes, a complex with a mobility similar to that of factor A was observed (Fig. 6b, lane C). Interestingly, PBS_{K2} levels of the low-mobility complex detected by the PBS_{K2} probe were increased by addition of serum to binding reaction mixtures (compare lanes F and C) in a fashion similar to what we have seen for factor A (lanes A

and D). Despite this similarity, competition studies suggest that factor A and the K2 factor which complexes with PBS_{K2} are not identical. As shown in Fig. 6c, factor A binding to the RBS_{WT-28} probe was inhibited efficiently by the RBS_{WT-28} competitor (lanes B to D), less well by RBS_{WT-18} (lanes H to J), and not at all by the 1-bp mutant RBS_{B2} (lanes E to G), PBS_{K2} (lanes Q to S), or any of the other ds PBS competitors. This result was confirmed in cross-competition studies between RBS_{WT-28} and the 28-bp PBS_{K2} sequence, PBS_{K2-28} (Fig. 6d). Factor A (Fig. 6d, lane C) was inhibited well by RBS_{WT-28} (lanes A and B) but not by PBS_{K2-28} (lanes D and F), while the K2 factor (lane H) was inhibited by PBS_{K2-28} (lanes I and J), but not by RBS_{WT-28} (lanes F and G). The fact that RBS_{WT} and PBS_{K2} did not cross-compete but represent PBS sequences which repress M-MuLV expression in EC cells (Table 1) and bind to factors with similar mobilities leaves open the possibility that factors A and K2 may be different, but related, stem cell binding factors.

DISCUSSION

The aim of our studies was to investigate the RBS-mediated repression phenomenon of M-MuLV in undifferentiated EC cells. Specifically, experiments were designed to characterize the RBS binding factor A and to determine the relationship between stem cell-specific RBS sequences and retrovirus tRNA PBS sequences. Previous studies (16, 19, 25) showed that factor A bound specifically to the M-MuLV RBS, but no other correlations between factor binding and in vivo repression were observed. However, after optimization of factor isolation and binding protocols, additional data support the notion that factor A is the cellular factor which mediates repression at the M-MuLV RBS. First, factor A was enriched in F9 nuclear extracts versus cytosolic extracts (Fig. 1), consistent with its action at the DNA level (3, 5, 16–19, 25). Second, the factor was enriched in F9 cells versus 3T3 cells (Fig. 1), consistent with the cell type specificity of RBS repression (16, 19, 25). Additionally, our radiolabeled RBS_{WT-28} ds oligonucleotide bound factor A approximately 10-fold better than the radiolabeled RBS_{WT-18} ds oligonucleotide (Fig. 6a). This observation is in agreement with infectivity studies which showed that the full-length sequence in WTIsle was 10- to 20-fold more efficient in stem cell-specific silencing than the short version in miniWTIsle (Table 1). The fact that sequences present in WTIsle but not in miniWTIsle (M-MuLV nt 164 to 174) can influence the observed repression phenomenon may explain why the myeloproliferative sarcoma virus does not appear to be expressed in EC cells (32): myeloproliferative sarcoma virus is identical to M-MuLV at nt 147 to 163 but possesses two T nucleotides in place of the one C nucleotide at M-MuLV nt 164, immediately 3′ of the M-MuLV PBS.

It has been shown that factor A is very unstable (19, 25), and thus some standard studies have not been possible to perform. To combat this instability, we have varied conditions both for preparation of protein extracts and for DNA binding. Specifically, we have observed that by shortening the times of dialysis of the crude protein extracts against Dignam buffer D or eliminating dialysis altogether, factor A bands on gel shift assays were enhanced. We have shown that performance of binding reactions at 30°C increased the RBS_{WT-28} versus RBS_{B2} probe specificity of factor A binding but did not decrease the intensity of the band (Fig. 1), that $tRNA^{Pro}$ did not compete with and tRNA synthetase did not bind the RBS_{WT} probe, that addition of serum to reaction mixtures enhanced the binding of factor A (Fig. 3) but that gelatin or BSA had little or no effect on binding (data not shown), and that 3 mM Mg^{2+} and 100 μM Zn^{2+} enhanced binding (Fig. 1), while other cations, nucleotides, or detergents either had no effect or impaired factor A binding. Furthermore, Sepharose G-200 column chromatography results indicated that factor A is larger than 100 kDa and is enriched in the 600 mM KCl washes of both heparin-Sepharose and DNA affinity columns (data not shown). These results suggest that purification and identification of factor A should be possible.

Although maximal RBS repression requires sequences outside the M-MuLV PBS and tRNA does not compete for factor A binding, the 17-of-18-bp overlap of the PBS and the core RBS has suggested that PBS and RBS functions may be intertwined. We (3) and others (32) have shown that recombinant M-MuLV vectors with glutamine tRNA PBSs do not suffer stem cell-specific repression. However, to ascertain whether PBS_P or PBS_Q is atypical, PBS sequences for phenylalanine (PBS_F), isoleucine (PBS_I), lysine-1,2 (PBS_{K2}), lysine-3 (PBS_{K3}), methionine (PBS_M), and tryptophan (PBS_W) were tested for in vivo silencing function and in vitro binding activity. Of these sequences, PBS_I, PBS_M, and PBS_W showed no silencing activity, while the phenylalanine and lysine-3 PBS sequences reduced viral infectivities 2- to 10-fold relative to that with the RBS mutant construct, B2Isle (Table 1). Interestingly, the PBSIsleK2 virus consistently showed reduced titers in EC cells (Table 1), and the PBS_{K2} probe formed a complex with a mobility and response to serum that were similar to those of factor A (Fig. 6). However, we observed that the RBS_{WT} and PBS_{K2} probes did not cross-compete (Fig. 6). These results suggest that factor A and the factor which binds to the PBS_{K2} probe (factor K2) may be different but related factors, and they raise the possibility that expression of viruses which use the tRNA lysine-1,2 PBS sequence (Mason-Pfizer monkey virus, visna virus, and spumavirus) (20) may also be repressed in undifferentiated stem cells.

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REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κβ transcription factor. Cell **53:**211–217.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. Iκβ: a specific inhibitor of the NF-κβ transcription factor. Science **242:**540–546.
3. **Barklis, E., R. C. Mulligan, and R. Jaenisch.** 1986. Chromosomal position or virus mutation permits retrovirus expression in embryonal carcinoma cells. Cell **47:**391–399.
4. **Bernstine, E., M. Hooper, S. Grandchamp, and B. Ephrussi.** 1973. Alkaline phosphatase activity in mouse teratoma. Proc. Natl. Acad. Sci. USA **70:**3899–3903.
5. **Berwin, B., and E. Barklis.** 1993. Retrovirus-mediated insertion of expressed and non-expressed genes at identical chromosomal locations. Nucleic Acids Res. **21:**2399–2407.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.
7. **Colicelli, J., and S. Goff.** 1987. Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. J. Virol. **57:**37–45.
8. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
9. **Feuer, F., M. Taketo, R. Hanecak, and H. Fan.** 1989. Two blocks in Moloney murine leukemia virus expression in undifferentiated F9 embryonal carcinoma cells as determined by transient expression assays. J. Virol. **63:**2317–2324.

10. **Gautsch, J., and M. Wilson.** 1983. Restriction of Moloney murine leukemia virus growth in tetratocarcinoma: involvement of factors other than DNA methylation. Cold Spring Harbor Conf. Cell Proliferation **10:**363–378.
11. **Gautsch, J., and M. Wilson.** 1983. Delayed *de novo* methylation in tetratocarcinoma suggests additional tissue-specific mechanisms for controlling gene-expression. Nature (London) **301:**32–37.
12. **Gorman, C., P. Rigby, and D. Lane.** 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell **42:**519–526.
13. **Grez, M., E. Akgun, F. Hilberg, and W. Ostertag.** 1990. Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. Proc. Natl. Acad. Sci. USA **87:**9202–9206.
14. **Jackson, S., and R. Tjian.** 1989. Purification and analysis of RNA polymerase II transcription factors by using wheat germ agglutinin affinity chromatography. Proc. Natl. Acad. Sci. USA **86:**1781–1785.
15. **Jaenisch, R., and A. Berns.** 1977. Tumor virus expression during mammalian embryogenesis, p. 267–314. *In* M. Sherman (ed.), Concepts in mammalian embryogenesis. MIT Press, Cambridge, Mass.
16. **Kempler, G., B. Freitag, B. Berwin, O. Nanassy, and E. Barklis.** 1993. Characterization of the Moloney murine leukemia virus stem cell-specific repressor binding site. Virology **193:**690–699.
17. **Loh, T., L. Sievert, and R. Scott.** 1987. Proviral sequences that restrict retroviral expression in mouse embryonal carcinoma cells. Mol. Cell. Biol. **7:**3775–3784.
18. **Loh, T., L. Sievert, and R. Scott.** 1988. Negative regulation of retrovirus expression in embryonal carcinoma cells mediated by an intragenic domain. J. Virol. **62:**4086–4095.
19. **Loh, T., L. Sievert, and R. Scott.** 1990. Evidence for a stem cell-specific repressor of Moloney murine leukemia virus expression in embryonal carcinoma cells. Mol. Cell. Biol. **10:**4045–4057.
20. **Löwer, L., J. Löwer, C. Tondera-Koch, and R. Kurth.** 1993. A general method for the identification of transcribed retrovirus sequences (R-U5 PCR) reveals the expression of the human endogenous retrovirus loci HERV-H and HERV-K in tetratocarcinoma cells. Virology **192:**501–511.
21. **Lund, A., M. Duch, J. Lovmand, P. Jorgensen, and F. S. Pedersen.** 1993. Mutated primer binding sites interacting with different tRNAs allow efficient murine leukemia virus replication. J. Virol. **12:**7125–7130.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Mann, R., R. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153–159.
24. **Martin, G. R.** 1980. Tetratocarcinomas and mammalian embryogenesis. Science **209:**768–776.
25. **Petersen, R., G. Kempler, and E. Barklis.** 1991. A stem cell-specific silencer in the primer-binding site of a retrovirus. Mol. Cell. Biol. **11:**1214–1221.
26. **Prince, V., and P. Rigby.** 1991. Derivatives of Moloney murine leukemia virus capable of being transcribed in embryonal carcinoma stem cells have gained a functional Sp1 binding site. J. Virol. **65:**1803–1811.
27. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Shinnick, T., R. Lerner, and J. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293:**543–548.
29. **Sprinzl, M., T. Hartmann, J. Weber, J. Blank, and R. Zeidler.** 1989. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. **17:**R1–R172.
30. **Stewart, C., H. Stuhlman, D. Jähner, and R. Jaenisch.** 1982. *De novo* methylation, expression and infectivity of retroviral genomes introduced into embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA **79:**4098–4192.
31. **Thornell, A., B. Hallberg, and T. Grundström.** 1988. Differential protein binding in lymphocytes to a sequence in the enhancer of the mouse retrovirus SL3-3. Mol. Cell. Biol. **8:**1625–1637.
32. **Weiher, H., E. Barklis, W. Ostertag, and R. Jaenisch.** 1987. Two distinct sequence elements mediate retroviral gene expression in embryonal carcinoma cells. J. Virol. **61:**2742–2746.
33. **Weiss, R., N. Teich, H. Varmus, and J. Coffin.** 1985. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.