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Distribution and characterization of helix-loop-helix enhancer-binding proteins from pancreatic β cells and lymphocytes

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ABSTRACT

Transcription of a number of mammalian genes is controlled in part by closely-related DNA elements sharing a CAxxTG consensus sequence (E boxes). In this report, we survey cell extracts from a variety of mammalian cell lineages for ability to bind to the E box denoted IEB1/κE1, which plays an important role in expression of both insulin and immunoglobulin κ genes. Insulin enhancer factor 1 (IEF1), a binding activity previously identified in β cells, was also present in pituitary endocrine cells but absent in 7 other mammalian cell lines tested. A distinct binding activity, lymphoid enhancer factor 1 (LEF1), was observed in several lymphoid cell lines, but was absent from all non-lymphoid cells tested. IEF1 and LEF1 were distinct according to electrophoretic mobility, and DNA binding specificity. As previously reported, both β cell and lymphoid cell factors are recognized by antibodies to helix-loop-helix (HLH) proteins, indicating that they may contain functional helix-loop-helix dimerization domains. To directly demonstrate this, we showed that the binding factors are able to interact *in vitro* with the HLH domain of a characterized HLH protein. These results support the notion that HLH proteins play a key role in cell-specific transcriptional regulation in cells from endocrine and lymphocyte lineages.

INTRODUCTION

Expression of the insulin gene is restricted to pancreatic endocrine β cells via transcriptional control mechanisms operating through well defined cis-acting DNA elements located in the 5′ flanking DNA of the gene (1, 2, 3). Of particular importance are two 9 bp elements (IEB1 and IEB2) (Figure 1) located at −104 and −233 in the rat insulin I gene (4, 5, 6). *In vitro* analyses have shown that a β-cell complex (insulin enhancer factor 1—IEFl) absent from fibroblasts, is capable of recognizing these sequences (7). Upon transfection of plasmids bearing point mutations throughout the region, characteristic transcription activities were observed in an *in vivo* assay: these activities closely parallel the *in vitro* binding properties of IEF1 (6). This strongly implicates IEF1 as a transcriptional activator of the insulin gene: its preferential occurrence in β cells offers a plausible explanation for the cell specific expression pattern. IEB1 and 2 show close similarity (8) to the 'E box' sequences (Figure 1), located in the μ heavy chain and κ light chain enhancer of immunoglobulin genes and also present in the enhancers of the chymotrypsin and muscle creatine kinase genes. The IEB1 and 2 sequences are most closely related to κE1 (Figure 1). E boxes have been shown by transfection experiments (9, 10, 11) to be essential for optimal enhancer activity. Furthermore, *in vivo* footprint experiments have indicated that the heavy chain E boxes are occupied by proteins in lymphoid cells but not fibroblasts (12, 13).

To better understand the nature of the factors which interact with the E boxes, several groups have screened expression cDNA libraries with E box probes. This led initially to the identification of the human lymphoid cDNA clones E12 and E47 (14) which appear to represent altered splice products of a single gene (E2A) (15). An E47-like clone (A1), was isolated from a mouse pancreatic β cell library (16) and two cDNAs, Pan-1 (E47-like) and Pan-2 (E12-like) were isolated from a rat pancreatic exocrine library (17). Sequence comparisons indicate that the rodent cDNAs probably represent the mouse and rat homologs of E12/E47 (14, 16, 17, 18). The E2A gene products were recognized to possess a potential helix-loop-helix (HLH) structure, apparently also present in several other proteins involved in transcriptional/developmental processes eg myc, MyoD, daughterless (14). However the presence of E2A gene transcripts in multiple cell types (14, 16) and the ability of the encoded proteins to bind E boxes of enhancers active in divergent cell types (lymphocytes, exocrine pancreas, endocrine pancreas) were not properties predicted for cell-specific transcription factors. This raises the question of whether the E2A gene products are, in fact, involved in cell-specific gene expression in the above cell types.

Murre *et al.* recently showed that an E box binding activity BCF1/2 present in nuclear extracts of lymphoid cells is recognized by antibody to E12/E47 (19). Likewise IEB1-binding activity present in β cells is also recognized by anti E12/E47 antibodies

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(20, 21) implying that these binding activities contain E12/E47-related proteins, possibly containing a helix-loop-helix dimerization domain. In this report, we confirm and extend the above findings by demonstrating that these characteristic binding activities are distinct from one another according to several parameters, including electrophoretic mobility, temperature stability, and DNA binding specificity. To show that these complexes contain a functional helix-loop-helix dimerization domain, we demonstrated their ability to interact *in vitro* with the HLH domain of the A1 protein. The β cell binding activity, previously seen only in pancreatic endocrine cells (6, 7) is shown to be present also in endocrine cells of pituitary origin.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts were prepared from established cell lines using the procedure of Schreiber *et al.* (22) modified to include the following protease inhibitors in the nuclear resuspension buffer: leupeptin (10 μg/ml), pepstatin A (1 μg/ml), p-amino benzoic acid (0.1 mM), aprotinin (1 mg/ml), and PMSF (1 mM).

Electrophoretic mobility shift assay (EMSA)

Radioactive probes were generated by incubation of the appropriate annealed oligonucleotides in the presence of [^{32}P]dATP and DNA polymerase I (Klenow fragment). Reaction products were fractionated on 10% acrylamide gels and radioactive DNA was eluted from the gel by overnight incubation in 1 mM EDTA. Specific activity was typically $2-3\times10^3$ cpm/fmol. In all cases oligonucleotides used corresponded to the sequences shown in Figure 1 with an additional 5 bases comprising a 5′ BamHI overhang (top strand) and a 6 base 5′ BglII overhang (bottom strand)(7). Protein extract (4 μg) was incubated for 10 min in binding buffer (7) containing 300–600 ng poly d(IC) and 300–600 ng poly d(AT) in a final assay volume of 15 μl. Subsequently probe was added and incubation allowed to continue for an additional 25 min. For competition experiments, preannealed unlabeled oligonucleotides were mixed with the radioactive probe prior to addition to the extracts. Samples were subsequently resolved on 6% polyacrylamide gels (44:0.8 acrylamide:bis-acrylamide) at 4°C in 40 mM Tris-HCl, 195 mM glycine pH 8.5. All binding and electrophoresis steps were performed at 0–4°C, except where otherwise indicated.

Antibodies

Bacterial fusion protein containing the 281 amino acid C-terminal portion of the A1 protein linked to trpE (16) was purified by preparative SDS polyacrylamide gel electrophoresis (PAGE) and used to elicit polyclonal antibodies in rabbits. The IgG fraction was prepared from sera using *S.aureus* protein-A coupled to Sepharose. The effect of IgG fractions on DNA binding activity was tested using the above EMSA procedure by mixing purified IgG (8 μg) with protein extract (4 μg) for 10 min at 4°C prior to addition of the probe.

In vitro transcription/translation

A 998 bp fragment encoding the 281 amino acid C-terminus of the A1 protein (16) was subcloned to the plasmid pBS.ATG (23). In parallel, a DNA fragment encoding a 92 amino acid segment of A1 spanning the HLH region (amino acids 156–247 in (16)) was generated using PCR and subcloned to pBS.ATG. *In vitro* transcription was performed using 0.5 μg of DNA in the presence of T3 RNA polymerase. Typically, 20% of the transcription reaction was used for *in vitro* translation with rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Lysate products (10% of each reaction) were used for EMSA reactions in the absence or presence of nuclear extracts. To favor the generation of hybrid species, lysate and nuclear extracts were mixed and incubated at 23°C for 10 min prior to incubation (10 min 23°C) with the DNA probe and gel electrophoresis at 4°C.

Sequence	Designation
GCCATCTGC	IEB1 Insulin
GCCATCTGG	IEB2 Insulin
GCCATCTTG	μE1 Immunoglobulin
GCCAGCTGC	μE2 Immunoglobulin
GCCACATGA	μE3 Immunoglobulin
ACCACCTGG	μE4 Immunoglobulin
AACACCTGC	μE5 Immunoglobulin
GCCATCTGG	κE1 Immunoglobulin
GCCACCTGC	κE2 Immunoglobulin
CCCATGTGG	κE3 Immunoglobulin
AACACCTGG	muscle creatine kinase
GGCACCTGT	chymotrypsin
GCCAATCTGC	CCAAT
CAxxTG	consensus

Figure 1. Comparison of E box elements. Base pairs showing divergence from the IEB1/2 sequence are underlined. The sequences shown are from the rat insulin I gene (4), the mouse immunoglobulin μ and κ genes (9), the rat chymotrypsin gene (17), and the mouse muscle creatine kinase gene (10).

RESULTS

Cell-specific IEB1-binding proteins

To investigate the possible relationships among factors involved in insulin and immunoglobulin gene expression, we surveyed nuclear extracts from a number of established cell lines, by incubation with a labelled IEB1 probe and analysis using electrophoretic mobility shift assay (EMSA) (Figure 2A). As previously reported (6, 7), extracts from β cell lines show a characteristic binding activity, IEF1 (lanes 1–6), absent from fibroblasts (lanes 13–16). Our current results extend this analysis to include myoblast (C2) and exocrine pancreas (AR4–2J) cells (lanes 17–20) which likewise show no significant specific binding activity i.e. binding which can be competed by excess unlabeled competitor. On the other hand, extracts from three independent lines of B lymphoid origin (S194, WEHI 231 and L10A) do show specific binding (lanes 7–12): the complex observed (designated LEF1 for lymphocyte enhancer factor 1) migrates significantly slower than IEF1 on EMSA. All extracts show a faster-migrating non-specific complex ('X') which does not compete with excess IEB1. The quality of the extracts was shown by their ability to bind (Figure 2A, bottom) to a mutant E box sequence 'CCAAT' (Figure 1) which has previously been shown to interact with proteins from a range of cell types (7). To test whether IEF1 is restricted to endocrine cells of pancreatic origin, we tested two pituitary endocrine lines GH1 and GH3: in both cases, binding

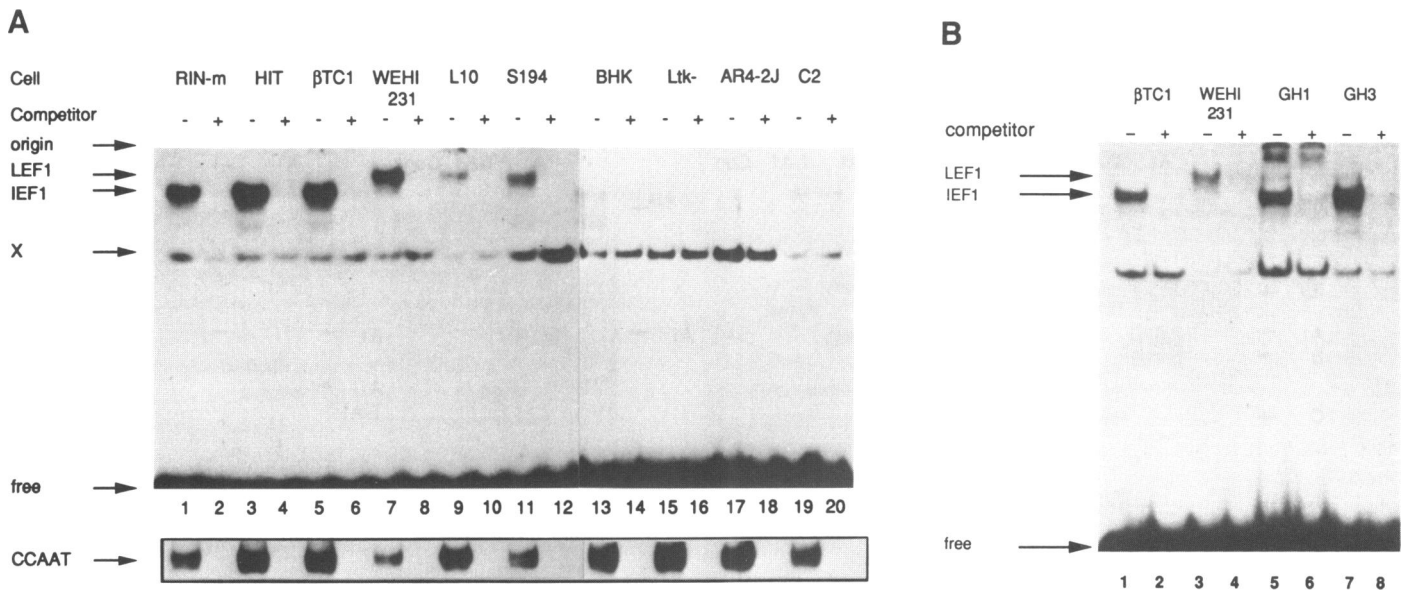

Figure 2. Cell type distribution of IEB1 binding activity. Cell extracts were mixed with radioactively labelled IEB1 probe in the absence (−) or presence (+) of 25-fold excess unlabeled IEB1 and subjected to EMSA analysis. **A.** The cell lines used are RIN-m, HIT, βTC1: pancreatic endocrine β cells from rat, hamster and mouse respectively (lanes 1−6), WEHI 231, L10A, S194: mouse B lymphoid cells (lanes 7−12), BHK and Ltk$^-$: fibroblast from hamster and mouse respectively (lanes 13−16), AR4−2J rat exocrine pancreas (lanes 17−18), C2: mouse myoblast (lanes 19−20). The inset at bottom shows the retarded bands resulting from interaction of the same extracts with a 'CCAAT' probe (odd numbered lanes only). This complex shows greater mobility than IEF1 (see Figure 3). **B.** The cell lines used were βTC1 (lanes 1−2), WEHI 231 (lanes 3−4), GH1 (lanes 5−6) and GH3 (lanes 7−8). The latter two lines are rat pituitary endocrine cells.

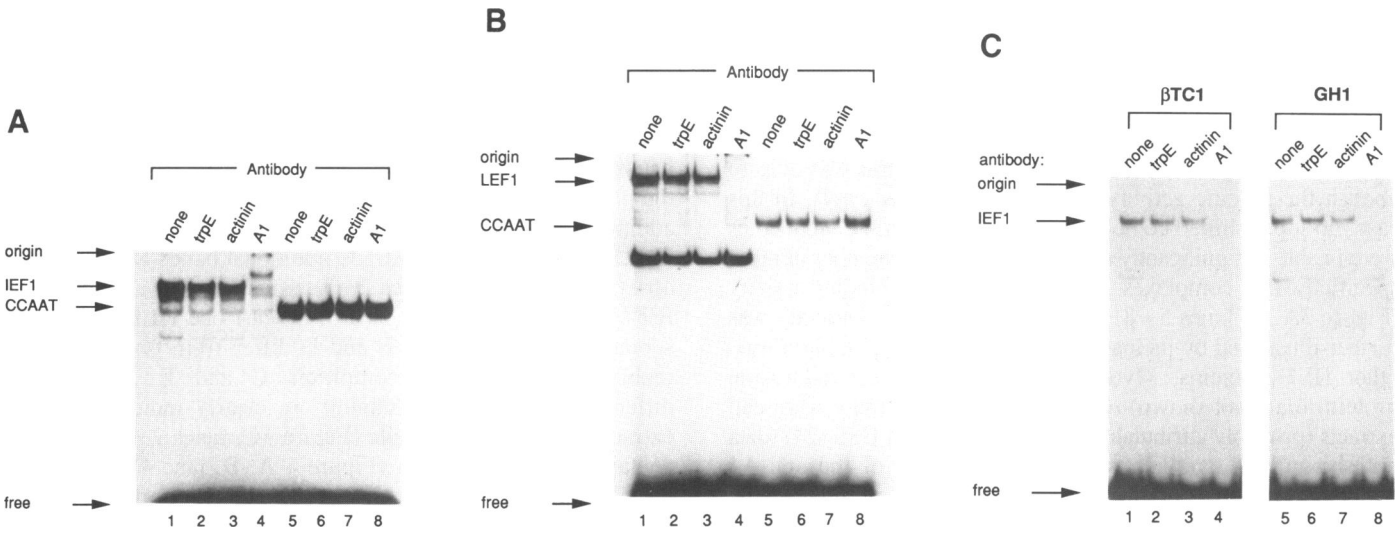

Figure 3. IEF1 of β cell and pituitary endocrine cells and LEF1 of lymphocytes are recognized by antibodies directed against the HLH protein A1 (16). Extracts from β cells (βTC1) (Figure 3A, C), B cells (WEHI 231) (Figure 3B) and GH1 cells (Figure 3C) were incubated alone or in the presence of IgG fractions derived from rabbit serum directed against trpE, actinin or trpE-A1 prior to EMSA analysis. In Figures 3A, B, lanes 1−4 contain IEB1 probe; lanes 5−8 CCAAT probe. In Figure 3C, lanes 1−4 show βTC1 extract with IEB1 probe and lanes 5−8 show GH1 extract with IEB1 probe. Above each lane is indicated the antibody used for preincubation. The mobility of IEF1 and LEF1 is indicated.

activity showing mobility identical to IEF1 was observed (Figure 2B lanes 5−8).

IEF1 and LEF1 are recognized by anti-A1 antibodies

We previously used an IEB1 DNA probe to isolate a cDNA clone from a β cell cDNA library (16). The cDNA encodes a protein (A1) which is a member of the HLH family of transcription factors and is closely related to E47, a cDNA cloned by screening human lymphoid libraries using κE2 (14) or μE2/μE5 (18) DNA probes. To determine whether the A1 protein is related to IEF1 and/or LEF1, we generated polyclonal antibodies to a trpE-A1 fusion protein (16). Nuclear extracts from β cells were preincubated with antibodies, mixed with labelled IEB1, and then analyzed by EMSA (Figure 3A). Anti-A1 antibodies caused a dramatic reduction in the intensity of the IEF1 complex and led to the appearance of a weak, slower migrating 'super-shifted' band (lane 4). This indicates recognition of the IEF1 complex by the antibodies. Antibodies directed against trpE, or actinin

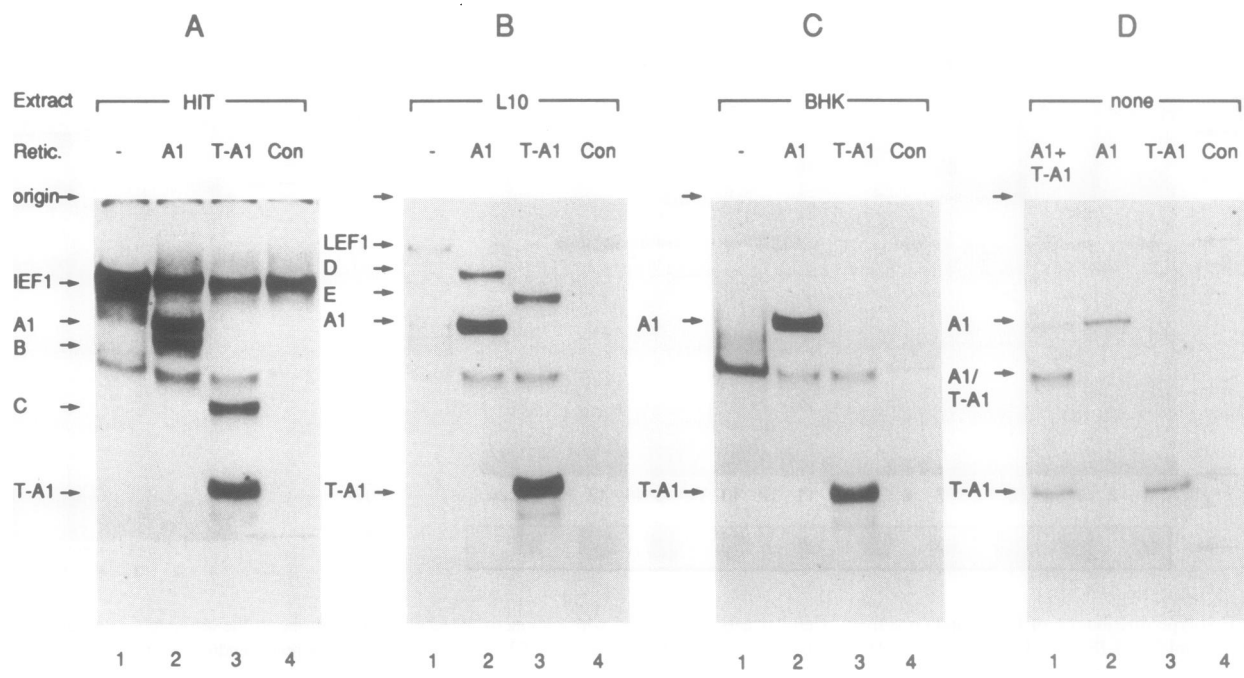

Figure 4. EMSA analysis of interaction of *in vitro* translated A1 with IEF1 and LEF1. A1 protein (281 amino acids) and T-A1 protein (92 amino acids) were translated in the reticulocyte lysate cell-free system and incubated for 10 min at 23°C with nuclear extracts from β cells (HIT) (panel A), B cells (L10A) (Panel B), and fibroblasts (BHK) (Panel C). Samples were then incubated in the presence of radiolabeled IEB1 as described. Panels A–C show the pattern of nuclear extract alone (lane 1) or in the presence of translated A1 (lane 2), T-A1 (lane 3) and control lysate (no mRNA added) (lane 4). Panel D shows lysate with no added nuclear extract: mixed A1 and T-A1 translation products (lane 1), A1 translation products (lane 2), T-A1 (lane 3) and no added RNA (lane 4).

showed no effect on the binding complexes (lanes 2, 3). A similar experiment was performed with lymphocyte extract (Figure 3B): anti-A1 antibody, but not anti-trpE or anti-actinin was able to abolish the binding activity of the extract (lanes 1–4). In this case, no super-shifted complex was observed. Anti-A1 antibodies were unable to significantly inhibit formation of a number of other specific EMSA complexes, such as the CCAAT-binding activity (Figure 3A, B; lanes 5–8). The specificity of the antibody was further illustrated by its inability to inhibit DNA binding of two other HLH proteins, MyoD (expressed as a bacterial fusion protein) (data not shown) or the μE3 binding activity from cell extracts (probably attributable to the HLH protein USF (24) (data not shown). In parallel, we tested the ability of the anti-A1 antibody to recognize the IEF1–like activity of GH3 cells: substantial inhibition of binding was observed (Figure 3C lanes 5–8).

IEF1 and LEF1 contain functional HLH dimerization domains

To determine whether the complexes recognized by the antibody contain functional HLH dimerization motifs, we mixed cell extracts with *in vitro* translated A1 protein. If IEF1 and LEF1 contain an HLH motif, additional retarded complexes should be observed, corresponding to novel heteromeric species. For this experiment, we used *in vitro* translated proteins comprising either the C-terminal 281 amino acids of the 649 amino acid full length protein (A1) or a truncated 92 amino acid version (T-A1) spanning the 61 amino acid HLH domain. These proteins generated characteristic bands on EMSA analysis with labelled IEB1 (Figure 4D, lanes 2–3). When A1 and T-A1 containing lysates were mixed, a single additional EMSA band was observed (Figure 4D, lane 1). This is consistent with the idea that A1 and T-A1 bind DNA as homodimers: upon mixing, a single heterodimeric species of intermediate size is generated. When the *in vitro* translated proteins were mixed with β cell extracts, additional bands were seen (B and C in Figure 4A, lanes 2 and 3). Likewise with B cell extract, additional bands D and E were observed (Figure 4B, lanes 2–3). This strongly suggests that IEF1 and LEF1 are composed of at least one HLH-containing subunit. Since complexes B and D differ from one another in mobility, and likewise complexes C and E, cell-specific differences in this HLH subunit are clearly indicated. When extracts from fibroblast cells (Figure 4C, lanes 2–3) or lysate containing no added RNA (Figure 4 A, B, lane 4) were used, no additional bands were observed. This supports the above interpretation that the novel bands do indeed result from interaction with subunits of IEF1 and LEF1, rather than some other HLH protein present in the extracts. Interestingly, the complex B migrates faster than both IEF1 and A1. A simple explanation for this would be that IEF1 consists of a heterodimeric complex, one subunit of which is smaller than the 281 amino acid A1 and therefore generates a heterodimer with A1 that migrates faster than the A1 homodimer. (This assumes that the primary determinant of electrophoretic mobility in EMSA analysis is molecular weight as suggested previously (22)).

DNA binding specificity of IEF1 and LEF1

In order to compare the DNA-binding specificity of IEF1 and LEF1, we used the EMSA assay with radioactive probes corresponding to the IEB1 sequence and the sequences corresponding, to the E boxes of immunoglobulin genes μE1–5 and κE1–3) (see Figure 1 for sequences—note the sequence of

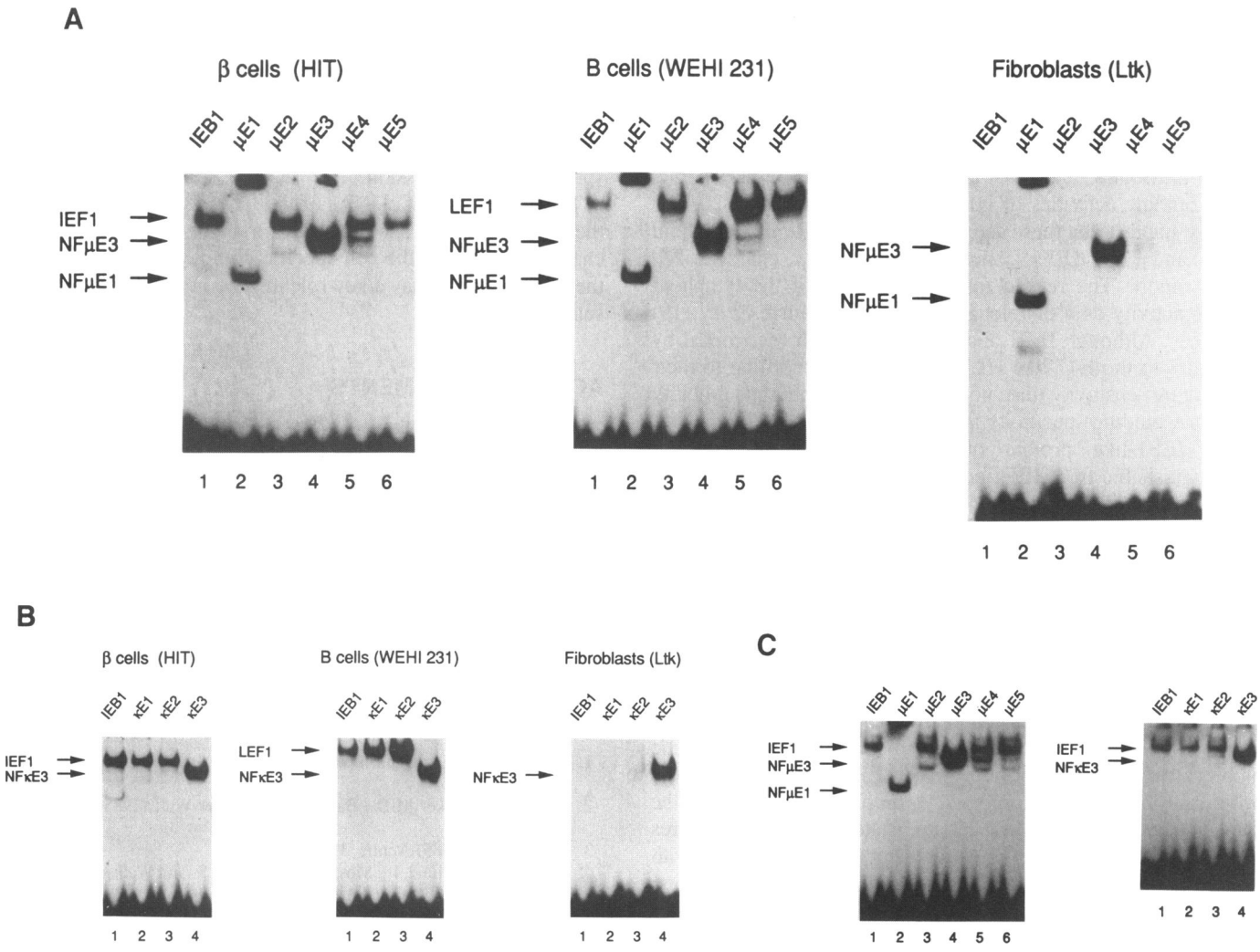

Figure 5. DNA binding specificity of IEF1 and LEF1. Figure 5A, B: extracts from β cells (HIT) (left panel), B cells (WEHI 231) (center panel) and fibroblasts (Ltk$^-$) (right panel) were allowed to interact with radioactive probes derived from IEB1, μE1, μE2, μE3, μE4, μE5 (Figure 5A), and IEB1, κE1, κE2, κE3 (Figure 5B). The mobility of IEF1 and LEF1 is indicated together with the mobility of the μE1-, μE3- and κE3-binding activities. Figure 5C shows the binding specificity of extracts from GH1 cells. The specific activities of the probes used was comparable in all cases. The binding profile for βTC1 cells was indistinguishable from that seen for HIT cells (data not shown).

IEB2 is identical to that of κE1). IEF1 binds efficiently to IEB1, μE2, μE4, κE1 and κE2; less efficiently to μE5 and not at all to, μE1, μE3 and κE3 (β cell extracts, Figure 5A, B). In contrast, LEF1 binds most efficiently to μE2, μE4, μE5 and κE2, less efficiently to IEB1 and κE1 and not at all to μE1, μE3, and κE3 (B cell extracts Figure 5A, B). With both β cell and lymphoid extracts, the μE1-, μE3- and κ-E3-binding complexes were observed: these complexes migrated faster than both IEF1 and LEF1 and probably correspond to the previously identified constitutive factors NFμE1 (25) and NFμE3 (26). The presence of these activities in fibroblasts (fibroblast extracts Figure 3A,B) is consistent with this interpretation. Furthermore, the ability to detect these E box-binding activities but not IEF1 or LEF1 in fibroblast extracts, supports our suggestion that IEF1 and LEF1 are indeed distributed in cell-specific fashion. In addition, we tested the ability of several unlabeled E box sequences to compete with labelled IEB1 in the EMSA analysis. The results (data not shown) were consistent with the conclusions reached above regarding the preferences of IEF1 and LEF1 for binding different E boxes. In parallel we tested the ability of GH1 cell extracts to bind the E box probes. The results (Figure 5C) show that the IEF1-like activity seen in these cells has a binding specificity very similar to that of IEF1 of β cells.

DISCUSSION

Despite the well-documented role of E box *cis*-elements in expression of insulin and immunoglobulin genes, little is known about the transcription factors which underlie their activity. Recent reports have described the presence of E box binding activities in lymphoid cells (19) and β cells (20, 21) which were recognized by antibodies to the HLH protein E12/E47. However, the relationship between these binding activities is not clear, an important issue given the postulated involvement of the E boxes in controlling cell-specific transcription. Nor was it determined whether the binding complexes contain a functional HLH dimerization domain.

In this report, we have surveyed a broader range of cell types for binding activity to IEB1/κE1 and further characterized these activities. The β cell binding activity IEF1 (7) was undetectable

in extracts from lymphocytes, pancreatic exocrine cells, and myoblasts. Interestingly, we observed a complex indistinguishable from IEF1 in endocrine cell lines of pituitary origin (GH1 and GH3 cells). These cells synthesize growth hormone (GH1) and growth hormone and prolactin (GH3) but no insulin. LEF1 was observed only in lymphoid cells. The available information on cellular distribution and DNA binding specificities of the reported E box binding activities of lymphoid (19) and β cells (20, 21) strongly suggest that these activities correspond to the lymphoid (LEF1) and β cell (IEF1) complexes respectively, characterized in this report. The reason for the migration of the lymphoid binding activity as a doublet in the study of Murre *et al.* (19) is unclear. Although IEF1 and LEF1 were both recognized by antibodies to the E47-like HLH protein A1, according to their temperature sensitivity (data not shown), electrophoretic mobility and DNA binding specificity the complexes are clearly distinct.

The IEF1-like protein of pituitary endocrine cells is indistinguishable from IEF1 according to all parameters tested. The identification of IEF1 in non-pancreatic cells is unexpected given its postulated role in cell-specific expression of the insulin gene (6, 7). Its absence from all non-endocrine cells tested is consistent with a role in expression of endocrine specific genes: restriction of insulin gene expression to endocrine β cells may involve combinatorial interaction between IEF1 and other, as yet uncharacterized, β cell-specific transcription factors.

In vitro mixing experiments show that IEF1 and LEF1 contain functional HLH dimerization domains. The complexes generated *in vitro* by mixing with IEF1 are distinct in electrophoretic mobility from those formed with LEF1, underscoring the differences between these species. A simple interpretation of the data is that IEF1 and LEF1 are heterodimeric structures possessing a constitutive HLH component (A1-like) together with as yet undiscovered β and B cell specific HLH subunits, analogous to the situation in muscle where the muscle-specific protein MyoD appears to require heterodimerization with E12/E47 to permit efficient DNA binding (27). Expression of the MyoD protein appears to be high in muscle cells (28); this presumably leads to sequestering of all available E12/E47 to heterodimers and an absence of homodimeric E12/E47 (19). Whether an analogous situation accounts for the apparent absence of homodimeric E12/E47 in β cells will require further investigation. Based on the electrophoretic migration of *in vitro* translated E47 on EMSA, it has been suggested that the lymphoid cell E box binding activity may, in fact, correspond to homodimers or homotetramers of E47 (19). If so, an open issue remains the apparent absence of this activity in non-lymphoid cells, given that the E2A gene is actively transcribed in most, if not all, cell types (14, 16). This may be explained by the presence of negatively-acting HLH proteins such as Id (29).

IEF1 and LEF1 display clearly distinguishable DNA binding specificities: IEF1 recognizes the insulin enhancer E boxes IEB1 and IEB2 in preference to most immunoglobulin E boxes, whereas LEF1 recognizes the insulin elements more weakly than most of the immunoglobulin elements. Nevertheless, the binding preference is not absolute, and it seems unlikely that this preference could explain the restriction of insulin enhancer activity to β cells and immunoglobulin enhancer activity to lymphoid cells. This issue of overlapping DNA binding specificities among cell-specific factors has been raised previously with regard to other transcription factors e.g. the paradox surrounding the ability of the transcription factors oct-1 and oct-2 to bind identical DNA target sequences, yet activate different sets of genes *in vivo* (30). The generation of cell-specific enhancers may involve negative elements within the enhancers (reviewed in (31)). Alternatively, protein−protein interactions between the IEB1/κE1 binding species and cell-specific species such as the lymphoid specific NF-κB (32) and oct-2 (33, 34, 35) and the endocrine specific Isl-1 (36) may define cell-specificity of expression.

The availability of cDNAs of the E2A gene and antibodies to the gene products should assist in more detailed molecular characterization of the cell-specific complexes described here; these species may play a key role in generation of transcriptional selectivity.

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