lished results). Briefly, a heterozygote was grown in 50 μg/ml of hygromycin for 10 passages, and LPG$^+$ cells were removed by agglutination with monoclonal antibody CA7AE. Several clonal lines containing homozygous *HYG/HYG* replacements were recovered (termed *lpg2*$^-$ knockouts).
20. In vitro LPG synthesis was assayed with uridine diphosphate–Gal, guanosine diphosphate–^{14}C-labeled Man, and *Leishmania* microsomal membranes as described (*6*). In the presence of 0.1% Triton X-100, 1580 ± 220 cpm was incorporated with the wild type and 950 ± 350 cpm with C3P0 (65%). Incorporation (1.3%) was observed previously with R2D2 (*6*). Without detergent, 4760 ± 660 cpm was incorporated with the wild type and 500 ± 80 cpm (11%) with C3P0.
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Interaction of Tyrosine-Based Sorting Signals with Clathrin-Associated Proteins

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Tyrosine-based signals within the cytoplasmic domain of integral membrane proteins mediate clathrin-dependent protein sorting in the endocytic and secretory pathways. A yeast two-hybrid system was used to identify proteins that bind to tyrosine-based signals. The medium chains (μ_1 and μ_2) of two clathrin-associated protein complexes (AP-1 and AP-2, respectively) specifically interacted with tyrosine-based signals of several integral membrane proteins. The interaction was confirmed by in vitro binding assays. Thus, it is likely that the medium chains serve as signal-binding components of the clathrin-dependent sorting machinery.

Targeting of integral membrane proteins to endosomes, lysosomes, the basolateral plasma membrane, and the trans-Golgi network (TGN) is largely mediated by sorting signals contained within the cytoplasmic domain of the proteins [reviewed in (*1*)]. Many of these sorting signals consist of continuous sequences of four to six amino acids containing a critical tyrosine residue. A subset of tyrosine-based signals conforms to the canonical motif YXXØ, where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain (*1*). Although much has been learned in recent years about the structure and function of tyrosine-based signals, the molecular mechanisms involved in their recognition are still poorly understood. Previous studies have provided evidence for an association of cytoplasmic domains bearing tyrosine-based signals with clathrin-associated protein complexes (*2*). However, the exact identity of the signal-binding proteins and the molecular details of the recognition event remain to be established.

We decided to search for proteins that interact with tyrosine-based sorting signals, using a yeast two-hybrid approach (*3*). As a "bait" in the two-hybrid system, we used a triple repeat of the tyrosine-containing sequence SDYQRL (*4*, *5*) from the cytoplasmic tail of the integral membrane protein TGN38 (*6*). This sequence has the characteristics of a YXXØ motif and mediates both internalization from the cell surface and localization to the TGN (*7*). Screening of a mouse spleen complementary DNA (cDNA) library (~2.5×10^6 clones) resulted in the isolation of two clones that interacted specifically with the $(SDYQRL)_3$ bait sequence (*8*). The two clones (termed 3M2 and 3M9) corresponded to the medium chain (μ_2) of the plasma membrane, clathrin-associated protein complex AP-2 (*9*). In addition to μ_2, the AP-2 complex contains two large chains (α- and β-adaptin) and one small chain (σ_2) (*10*).

Using growth on histidine-deficient (–His) plates as an assay (*11*), we found that proteins encoded by both 3M2 and 3M9 interacted not only with the $(SDYQRL)_3$ repeat but also with a single SDYQRL sequence and with the full-length TGN38 cytoplasmic tail (Fig. 1A). Mutation of the tyrosine (Y) residues in all three contexts abolished interaction with the μ_2 clones (Fig. 1A). The binding specificity of μ_2 was further characterized by mutation of each residue of the SDYQRL sequence individually to alanine. Only the Y and L residues were absolutely required for interaction with 3M9, whereas mutation of the S, D, and Q residues had no detectable effect, and mutation of the R residue decreased but did not completely abolish the ability to grow on –His plates (Fig. 1B). Thus, μ_2 was capable of interacting with the sequence SDYQRL in various contexts and under sequence requirements that were consistent with those defined in studies in vivo (*7*).

To corroborate the results obtained with the two-hybrid system, we tested whether in vitro–translated, ^{35}S-methionine–labeled μ_2 was capable of interacting with various sequences appended to glutathione-S-transferase (GST) (Fig. 2). We observed that both the 3M2 and 3M9 forms of μ_2 bound to $GST\text{-}(SDYQRL)_3$ but not to $GST\text{-}(SDGQRL)_3$ or to GST (Fig. 2A). In vitro–translated luciferase, used as a negative control, did not interact with any of the GST fusion proteins tested (Fig. 2A). Binding of

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μ_2 to GST-(SDYQRL)$_3$ was inhibited by C(SDYQRL)$_3$ peptide but not by C(SDAQRL)$_3$ (Fig. 2B) or by C(DKQTLL)$_3$ peptide (Fig. 2C), further demonstrating the specificity of the μ_2 interactions. Inhibition by C(SDYQRL)$_3$ was dose-dependent and occurred at micromolar concentrations of peptide (Fig. 2C). These results were thus consistent with the existence of a specific interaction between the sequence SDYQRL and μ_2.

The assays described above involved expression of μ_2 without simultaneous expression of the other subunits of the AP-2 complex. To test whether the complete AP-2 complex could also interact with tyrosine-based signals, we examined the binding of AP-2, purified from bovine brain coated vesicles (*12*), to the different GST fusion proteins described above. Immunoblot analyses showed that AP-2 complexes indeed bound to GST-(SDYQRL)$_3$ but not to GST-(SDGQRL)$_3$ or to GST (Fig. 3A). Analyses by surface plasmon resonance (SPR), a spectroscopic technique used to study protein-protein interactions (*13*), confirmed a tyrosine-dependent association of GST-(SDYQRL)$_3$ with purified AP-2 (Fig. 3B).

Further analyses using the two-hybrid system showed that the triple repeat (SDYQRL)$_3$ was also capable of interacting with mouse μ_1 (*14*), the medium chain of the TGN-localized, clathrin-associated protein complex AP-1 (*10*) (Fig. 1C). Triple repeats of other cytoplasmic tyrosine-based signals from the integral membrane proteins lamp-1 [AGYQTI (*15*)], CD68 [STYQPL (*16*)], and H2-Mb [SSYTPL (*17*)], which mediate internalization from the cell surface and transport to lysosomal compartments, were similarly capable of interacting with both μ_2 and μ_1 (Fig. 1C). The tyrosine-based internalization signal YTRF, from the transferrin receptor cytoplasmic tail (*18*), did not interact with either μ_2 or μ_1 when expressed as the triple repeat (LSYTRF)$_3$ (Fig. 1C). However, when the sequence YTRF was placed at the end of a 26–amino acid sequence derived from the cytoplasmic tail of TGN38, it interacted with μ_2 but not with μ_1 (Fig. 1C). This observation suggests that the context of the tyrosine-based signals or their spacing from the GAL4 DNA binding domain (GAL4bd) can affect their recognition by the medium chains. The interaction of all of these signals with μ_2 is likely involved in internalization of the proteins from the plasma membrane, whereas the interaction with μ_1 might mediate sorting at the TGN. For lamp-1, CD68, and H2-Mb, binding to μ_1 could mediate delivery to lysosomal compartments. The significance of TGN38 binding to μ_1 is less clear; a possible explanation is that TGN38 might be able to leave the TGN toward a prelysosomal compartment, from which it could be retrieved to the TGN.

In contrast to tyrosine-based signals, the di-leucine–based signal DKQTLL (*4*) from CD3-γ (*19*), which also functions as an internalization and lysosomal-targeting signal, failed to show an interaction with either μ_2 or μ_1 in various sequence contexts [Fig. 1C and (*20*)]. This observation suggests that the functions of di-leucine–based signals are mediated by interaction with proteins other than μ_2 or μ_1.

The results presented here demonstrate

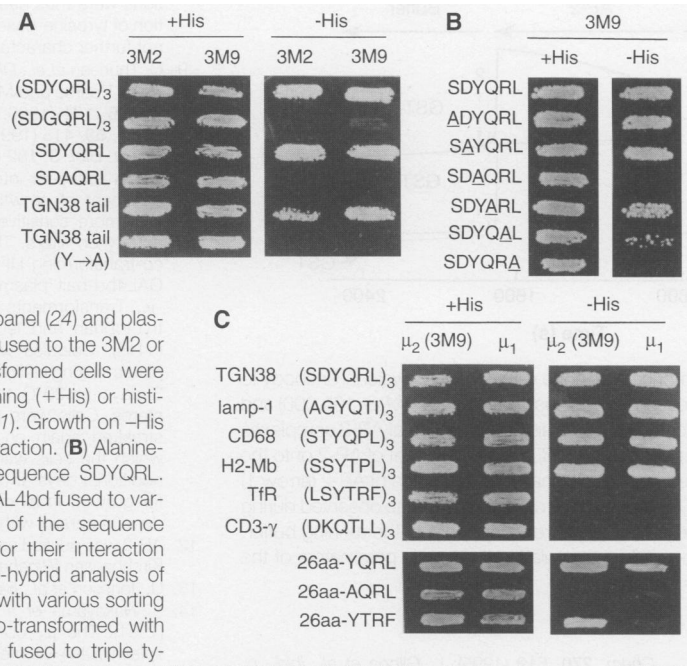

Fig. 1. Specific interaction of tyrosine-based signals with the medium chains of clathrin-associated protein complexes. (**A**) Interaction of μ_2 with sequences derived from the TGN38 cytoplasmic tail (*6*). Yeast cells were co-transformed with plasmids encoding GAL4bd fused to the sequences indicated on the left of each panel (*24*) and plasmids encoding the GAL4ad fused to the 3M2 or 3M9 forms of μ_2 (*8*). Transformed cells were streaked on histidine-containing (+His) or histidine-deficient (–His) plates (*11*). Growth on –His plates is an indication of interaction. (**B**) Alanine-scan mutagenesis of the sequence SDYQRL. Constructs containing the GAL4bd fused to various alanine-scan mutants of the sequence SDYQRL (*24*) were tested for their interaction with GAL4ad-3M9. (**C**) Two-hybrid analysis of the interaction of μ_2 and μ_1 with various sorting signals. Yeast cells were co-transformed with plasmids encoding GAL4bd fused to triple tyrosine-containing motifs from TGN38 (*6*), lamp-1 (*15*), CD68 (*16*), H2-Mb (*17*), or the transferrin receptor (TfR) (*18*), or to a triple di-leucine motif from CD3-γ (*19*) and plasmids expressing GAL4ad-μ_2(3M9) or -μ_1. Similar analyses were done on cells expressing the sequences YQRL, AQRL, or YTRF at the end of a 26–amino acid (26aa) sequence derived from TGN38 (*24*).

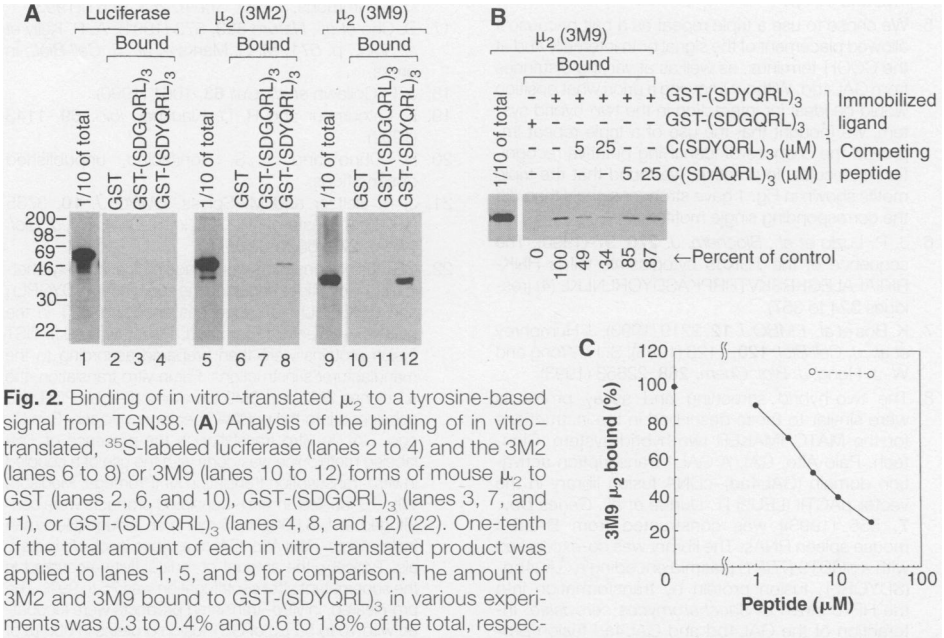

Fig. 2. Binding of in vitro–translated μ_2 to a tyrosine-based signal from TGN38. (**A**) Analysis of the binding of in vitro–translated, ^{35}S-labeled luciferase (lanes 2 to 4) and the 3M2 (lanes 6 to 8) or 3M9 (lanes 10 to 12) forms of mouse μ_2 to GST (lanes 2, 6, and 10), GST-(SDGQRL)$_3$ (lanes 3, 7, and 11), or GST-(SDYQRL)$_3$ (lanes 4, 8, and 12) (*22*). One-tenth of the total amount of each in vitro–translated product was applied to lanes 1, 5, and 9 for comparison. The amount of 3M2 and 3M9 bound to GST-(SDYQRL)$_3$ in various experiments was 0.3 to 0.4% and 0.6 to 1.8% of the total, respectively. The positions of relative molecular mass (M_r) markers (expressed as $10^{-3} \times M_r$) are indicated on the left. (**B**) Analysis of the binding of in vitro–translated, ^{35}S-labeled 3M9 to GST-(SDYQRL)$_3$ or GST-(SDGQRL)$_3$, in the absence or presence of the competing peptides C(SDYQRL)$_3$ or C(SDAQRL)$_3$. The amount of radioactivity in each lane is expressed as a percentage of the control (no peptide added). (**C**) Competition by C(SDYQRL)$_3$ (●) and C(DKQTLL)$_3$ (○) peptides of 3M9 binding to GST-(SDYQRL)$_3$. Use of concentrations of C(SDYQRL)$_3$ peptide greater than 25 μM resulted in precipitation of the peptides and increased nonspecific binding.

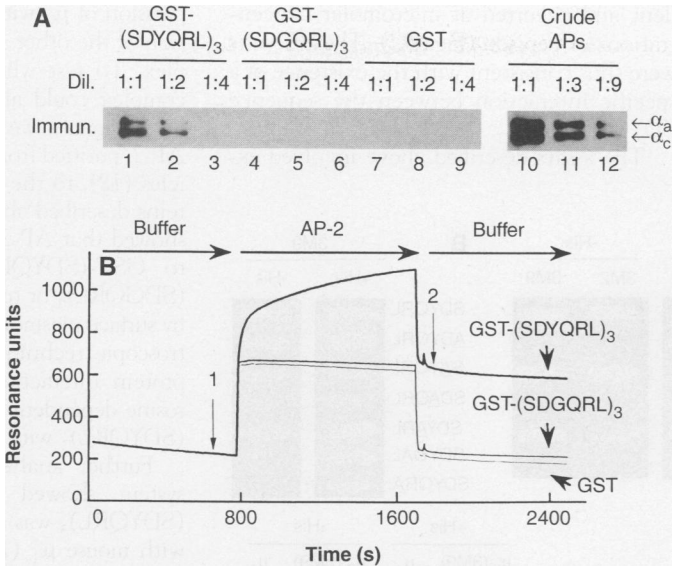

Fig. 3. Binding in vitro of purified AP-2 complexes to a tyrosine-based signal from TGN38. (**A**) Immunoblot analysis of the recruitment of AP-2 complexes by GST fusion proteins (*23*). Glutathione-agarose beads were incubated with equal amounts of GST-$(SDYQRL)_3$ (lanes 1 to 3), GST-$(SDGQRL)_3$ (lanes 4 to 6), or GST (lanes 7 to 9) and then with purified AP-2 complexes (*12*). Serial dilutions (dil.) of proteins bound to the beads were resolved by SDS-PAGE. Crude APs (100, 33, and 11 ng) were run alongside for determination of the electrophoretic migration of the α-adaptin chains (lanes 10 to 12). Immunoblot analysis (immun.) was done with a rabbit polyclonal antibody to α-adaptin, which was visualized by enhanced chemiluminescence. The positions of the α_a (M_r ~ 105,000) and α_c (M_r ~ 102,000) brain isoforms of α-adaptin (*25*) are indicated. (**B**) Analysis of the binding of AP-2 complexes to GST-$(SDYQRL)_3$, GST-$(SDGQRL)_3$, and GST by SPR spectroscopy (*13, 26*). Recruitment of AP-2 onto the GST fusion proteins was determined by comparison of the resonance signal before injection of AP-2 (arrow 1) with the signal after removal of the AP-2 solution (arrow 2). The temporary change in baseline observed during infusion of AP-2 is due to a different refractive index in the AP-2 sample relative to that of the running buffer. Dissociation of the bound AP-2 was very slow, preventing us from calculating the kinetic parameters of the interaction.

that the medium chains of AP complexes can interact with a subset of tyrosine-based sorting signals, which suggests a signal recognition function for the medium chains. Our results do not rule out the possibility that other subunits of AP-1 and AP-2 might participate in the recognition of tyrosine-based signals or interact with other elements of the cytoplasmic domains, as previously suggested (*21*).

The specificity of the interactions with the medium chains is underscored by the fact that μ_2 was selected from over two million clones in the initial screening of the library and by the fulfillment of several other specificity criteria, all of which are in good concordance with the requirements for function in vivo. Our observations also demonstrate that tyrosine-based signals can be directly recognized even when they are isolated from their original sequence contexts, which lends support to the notion that the signals function as discrete autonomous ligands for the sorting machinery (*1*).

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5. We chose to use a triple repeat as a bait because it allowed placement of the signal both internally and at the COOH-terminus, as well as at varying distances from GAL4bd. Without knowing a priori what position would be ideal for interaction in the two-hybrid system, we thought that the use of a triple repeat improved the chances of identifying putative recognition molecules. In practice, we found that the triple motifs shown in Fig. 1 gave stronger signals than did the corresponding single motifs.
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8. The two-hybrid screening and assay protocols were similar to those described in the instructions for the MATCHMAKER two-hybrid system (Clontech, Palo Alto, CA). A GAL4 transcription activation domain (GAL4ad)–cDNA fusion library in the vector pACTII (LEU2) [T. Durfee *et al.*, *Genes Dev.* **7**, 555 (1993)] was constructed from BALB/c mouse spleen RNAs. The library was co-expressed with a pGBT9 (*TRP1*) plasmid encoding a GAL4bd-$(SDYQRL)_3$ fusion protein by transformation into the HF7c strain of *Saccharomyces cerevisiae*. Interaction of the GAL4bd and GAL4ad fusion proteins resulted in GAL4-dependent transcription of *HIS3* or *lacZ* reporter genes, enabling cells to grow in the absence of histidine and to exhibit β-galactosidase (β-Gal) activity. Eight clones were identified that grew on plates lacking tryptophan, leucine, and histidine and that tested positive for β-Gal. The two clones that gave the strongest β-Gal signal encoded amino acid residues 2 to 435 (3M2) and 121 to 435 (3M9) of the 435–amino acid mouse μ_2 (deposited in GenBank under accession number U27106). Clone 3M2 lacks residues 142 and 143 and most likely represents a naturally occurring variant of μ_2. Interaction of both forms of μ_2 with $(SDYQRL)_3$ was strictly dependent on the presence of tyrosine residues in the bait sequence (Fig. 1A). The other six clones interacted with the bait sequence even when the critical tyrosine residues were mutated to glycine residues. These interactions were thus deemed unrelated to the recognition of tyrosine-based signals, and the clones were not further characterized.
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products were detected by fluorography. Quantitation was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

23. Glutathione-agarose beads (12.5 μl) were pre-incubated with 75 μg of each GST fusion protein (*22*) in 100 μl of phosphate-buffered saline. The beads were then washed twice in AP buffer [0.1 M MES (pH 7.0), 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% w/v NaN_3, and 0.6% w/v Triton X-100] and incubated with 30 μl of purified brain AP-2 (at 0.9 mg/ml) in AP buffer for 45 min at room temperature. After three more washes with AP buffer, bound proteins were resolved by SDS-PAGE on 12.5% acrylamide gels and processed for immunoblotting.
24. Synthetic oligonucleotides encoding $(SDYQRL)_3$, $(SDGQRL)_3$, SDYQRL, SDAQRL, ADYQRL, SAYQRL, SDYARL, SDYQAL, SDYQRA, $(AGYQTI)_3$, $(STYQPL)_3$, $(SSYTPL)_3$, $(LSYTRF)_3$, and $(DKQTLL)_3$ (*4*) were ligated in-frame to the 3′ end of GAL4bd coding sequences contained within the vector pGBT9. Constructs GAL4bd-TGN38 tail and GAL4bd-TGN38 tail (Y → A) were made by PCR with the use of the TGN38 cDNA as a template, and the products were cloned in-frame into pGBT9-GAL4bd. Gal4bd constructs having residues 324 to 347 of TGN38 followed by two alanine residues and the sequences YQRL, AQRL, or YTRF at the COOH-termini were constructed by PCR and ligation of synthetic oligonucleotides.
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Jak-STAT Signaling Induced by the v-*abl* Oncogene

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The effect of the v-*abl* oncogene of the Abelson murine leukemia virus (A-MuLV) on the Jak-STAT pathway of cytokine signal transduction was investigated. In murine pre–B lymphocytes transformed with A-MuLV, the Janus kinases (Jaks) Jak1 and Jak3 exhibited constitutive tyrosine kinase activity, and the STAT proteins (signal transducers and activators of transcription) normally activated by interleukin-4 and interleukin-7 were tyrosine-phosphorylated in the absence of these cytokines. Coimmunoprecipitation experiments revealed that in these cells v-Abl was physically associated with Jak1 and Jak3. Inactivation of v-Abl tyrosine kinase in a pre–B cell line transformed with a temperature-sensitive mutant of v-*abl* resulted in abrogation of constitutive Jak-STAT signaling. A direct link may exist between transformation by v-*abl* and cytokine signal transduction.

The v-*abl* oncogene of A-MuLV encodes a fusion protein of viral Gag sequences and the COOH-terminus of the c-Abl protein [including the tyrosine kinase and the Src homology 2 (SH2) domains] (*1*). Loss of the c-Abl SH3 domain, addition of viral Gag sequences, and changes in the subcellular localization of the protein from the nucleus to the cytoplasm have all been implicated in contributing to the oncogenic activity of v-Abl (*2*). The tyrosine kinase and the SH2 domains of v-Abl are required for transformation (*3*, *4*), but the mechanism by which v-*abl* transforms cells and allows cytokine-independent growth is unclear (*5*).

The growth of pre–B lymphocytes, the most common in vivo target of transformation by v-*abl*, normally requires cytokines such as interleukin-7 (IL-7) produced by bone marrow stromal cells (*6*). To understand the possible mechanism underlying the cytokine-independent growth of A-MuLV–transformed pre–B cell lines (*7*), we studied signal transduction by cytokines important for growth and proliferation of B lymphoid cells. Signal transduction by IL-4 and IL-7 involves the activation of Jak1 and Jak3 nonreceptor tyrosine kinases, which in turn are thought to phosphorylate latent cytoplasmic transcription factors referred to as STATs (signal transducers and activators of transcription) (*8*). Phosphorylation allows STATs to dimerize and translocate to the nucleus,

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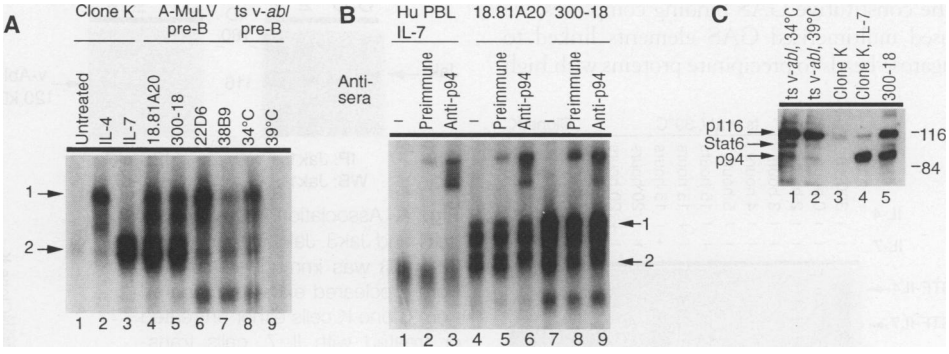

Fig. 1. Constitutive activation of STATs in pre–B cell lines transformed with v-*abl*. (**A**) Constitutive GAS binding activities in Abelson cell lines. EMSA was done with whole-cell extracts prepared from Clone K cells, either untreated or treated with IL-4 or IL-7 (lanes 1 to 3), A-MuLV–transformed pre–B cells derived from bone marrow cells (lanes 4 and 5) and fetal liver (lanes 6 and 7) (*25*), or cells expressing a temperature-sensitive (ts) mutant of v-*abl* grown at permissive temperature (34°C) (lane 8), or shifted to nonpermissive temperatures (39°C) for 15 hours (lane 9). Clone K cells were deprived of feeder cells for 4 hours before the addition of recombinant murine IL-4 (400 U/ml; DNAX) or IL-7 (10 ng/ml; Genzyme) for 15 min. The probe used was from the IRF-1 GAS element; 5′-gatcGATTTC-CCCGAAAT-3′ (*17*). For (A) and (B), arrow 1 indicates STF-IL4, and arrow 2 indicates STF-IL7. (**B**) Identification of DNA binding complexes in (A) with an antibody supershift assay. After incubation with the probe whole-cell extracts were incubated for 30 min with either preimmune serum or an antibody that recognizes the 94-kD isoform of Stat5 (anti-p94) (*14*) (1:20 dilution). Human peripheral blood lymphocytes (PBLs) were isolated by centrifugation on Ficoll-Hypaque (Sigma) and treated with IL-7 (lanes 1 to 3). Human STF-IL7 migrates faster than murine STF-IL7 (*14*). (**C**) Binding of proteins to GAS oligonucleotide. Cells extracts were incubated with multimerized GAS from the β casein gene linked to agarose beads (*17*). This GAS element binds STF-IL7 and STF-IL4 with high affinity (*11*). Bound material was eluted with 2M NaCl, fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (7% gel), blotted with antibody to phosphotyrosine (4G10, Upstate Biotechnology), and detected with enhanced chemiluminescence (ECL, Amersham). Prestained molecular size markers (Sigma) are shown on the right in kilodaltons.