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Normal Peripheral T-Cell Function in c-Fos-Deficient Mice

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The ubiquitous transcription factors Fos and Jun are rapidly induced in T cells stimulated through the T-cell antigen receptor and regulate transcription of cytokines, including interleukin 2, in activated T cells. Since positive and negative selection of thymocytes during T-cell development also depends on activation through the T-cell receptor, Fos and Jun may play a role in thymocyte development as well. Fos and Jun act at several regulatory elements in the interleukin 2 promoter, including the AP-1 and NFAT sites. Using antisera specific to individual Fos and Jun family members, we show that c-Fos as well as other Fos family members are present in the inducible AP-1 and NFAT complexes of activated murine T cells. Nevertheless, c-Fos is not absolutely required for the development or function of peripheral T cells, as shown by using mice in which both copies of the c-*fos* gene were disrupted by targeted mutagenesis. c-Fos-deficient mice were comparable to wild-type mice in their patterns of thymocyte development and in the ability of their peripheral T cells to proliferate and produce several cytokines in response to T-cell receptor stimulation. Our results suggest that other Fos family members may be capable of substituting functionally for c-Fos during T-cell development and cytokine gene transcription in activated T cells.

Members of the AP-1 (Fos/Jun) family of transcription factors have been implicated in the inducible transcription of a wide variety of genes involved in cell growth and differentiation (reviewed in references 10, 24, and 25). At least four Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) and at least three Jun proteins (c-Jun, JunB, and JunD) have been described (25). The simplest mechanism for transcriptional regulation by AP-1 proteins involves the binding of Jun dimers or Jun/Fos heterodimers to AP-1 (TGAC/GTCA) sites in DNA (25); however AP-1 proteins have also been shown to interact with other transcription factors (notably the glucocorticoid receptor and the T cell transcription factor NFAT) at composite DNA elements containing weak AP-1 sites (17, 26, 32). The DNA binding and transcriptional activities of AP-1 dimers on such composite AP-1 sites may be substantially influenced by the specific Fos and Jun family members involved (32). The diverse combinatorial interactions of AP-1 proteins provide a powerful mechanism for modulating transcriptional activity, especially when complex promoter/enhancer regions are involved (31).

The question of whether individual Fos and Jun family members mediate specific, nonoverlapping functions is of considerable interest. The specific roles of c-Fos and c-Jun during embryogenesis and tissue differentiation have been assessed by using mice in which both copies of the corresponding gene have been disrupted by targeted mutagenesis (21, 22, 46). Mice lacking the c-*jun* gene die at midgestation, indicating that c-Jun is essential at a critical step of embryonic development (21). In contrast, mice lacking c-Fos are viable but show a multitude of developmental and neurological defects, with osteogenesis, gametogenesis, and the circadian response to light being notably impaired (22, 23, 46). The absence of widespread cellular dysfunction in c-Fos-deficient mice indicates that although c-Fos is ubiquitously expressed, it is not absolutely required for many functions of developing and differentiating cells.

It is likely that AP-1 proteins play a major role in T-lymphocyte development and function. Fos and Jun family members are induced in immature $CD3^+$ thymocytes stimulated with anti-CD3 (33) and thus may be critical regulators of positive and negative selection in the thymus. In mature peripheral T cells, mRNAs for several Fos and Jun family members are expressed within 15 to 30 min of stimulation through the T-cell receptor (19) and may participate in the later induction of cytokine and activation-specific genes. However, the importance of individual Fos and Jun family members in developing and differentiated T cells remains to be investigated.

Fos/Jun proteins are likely to regulate the induction of the interleukin 2 (IL-2) gene in activated T cells (2, 16–19, 35, 41). The IL-2 gene promoter may contain as many as four regulatory elements capable of binding Fos/Jun complexes, alone or in association with other transcription factors (2, 16–19, 35, 41). A strong AP-1 site, located at −150 bp relative to the transcription start site, binds Fos/Jun complexes and is essential for IL-2 promoter activity (19, 41). The IL-2 promoter also contains two binding sites for the cyclosporin-sensitive factor NFAT (11, 44) which are actually composite binding sites for AP-1 proteins in association with the preexisting subunit, NFATp (2, 16, 17, 35). Lastly, the NFIL2A region of the IL-2 promoter may also contain a composite AP-1 site; this element binds Oct-1 and Oct-2 together with an inducible Oct-associated protein (OAP-40) which appears to contain JunD (3, 45). Thus, T-cell-specific transcription of the IL-2 gene and other

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cytokine genes may be mediated by the functional interactions of ubiquitous transcription factors such as Fos and Jun with lymphoid cell-specific proteins such as NFATp and Oct-2.

Using mice in which both copies of the c-*fos* proto-oncogene have been disrupted by targeted mutagenesis (22), we show that c-Fos is not absolutely required for T-cell development and function. Thymocytes from c-Fos-deficient mice appear similar to those of normal mice in their patterns of CD3, CD4, and CD8 expression, and peripheral T cells from these mice proliferate comparably to T cells of normal mice in response to strong T-cell receptor stimuli. Although c-Fos is clearly present in the inducible nuclear AP-1 and NFAT complexes of wild-type murine T cells, T cells from normal and c-Fos-deficient mice produce equivalent levels of IL-2 and other cytokines. The most likely explanation is that Fos family members other than c-Fos are capable of compensating for the lack of c-Fos in mature and developing T cells.

MATERIALS AND METHODS

Mice. c-Fos-deficient mice were bred and maintained, and their genotypes were ascertained, at the Tufts University facility as previously described (22). (129/SvJ × C57BL/6J)F_2 mice between 10 and 18 weeks of age, segregating for the targeted c-*fos* allele, as well as normal C57BL/6J and 129/Sv control mice were used.

Cell lines. The IL-2- and IL-4-dependent cell line CTLL-20 (12), the IL-3-dependent cell line FL5.12 (36), and the IL-4-dependent cell line CT.4s (15) were used for assays of IL-2, IL-3, and IL-4, respectively. The B-lymphoma cell line WEHI 279, whose proliferation is inhibited by gamma interferon (38), was used as an indicator cell line for gamma interferon assays. The Ar-5 T-cell clone (37) was used for preparation of nuclear extracts and Northern (RNA) analysis of cytoplasmic RNA. All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), penicillin, streptomycin, and additives as previously described (37, 47), with growth factor supplements as follows: 5 to 10 U of crude rat IL-2 (CR-rTCGF; Collaborative Research, Bedford, Mass.) per ml for Ar-5 and CTLL-20 cells; 20 U of partially purified IL-3 (a kind gift of J. Ihle) per ml or 10% IL-3-containing WEHI-3B cell supernatant (48) for FL5.12 cells; and 500 U of recombinant murine IL-4 (Genzyme, Framingham, Mass.) per ml or 5% IL-4-containing LT-1 cell supernatant (43) (a kind gift of R. Tepper) for CT.4s cells. The medium used for proliferation and lymphokine assays was identical to culture medium except that it lacked growth factors and contained 5% fetal calf serum.

Flow cytometry. An R-phycoerythrin (PE)-conjugated monoclonal antibody (MAb) specific for mouse CD4 (GK1.5 [9]) and a fluorescein isothiocyanate (FITC)-conjugated MAb specific for mouse CD8 (Lyt2 [29]) were purchased from Becton Dickinson (Mountain View, Calif.). A PE-conjugated MAb specific for B220/CD45R (RA3-6B2 [1]) and an FITC-conjugated MAb specific for murine CD3 ε chain (145-2C11 [30]) were purchased from Pharmingen (San Diego, Calif.). Suspensions of cells from thymus, spleen, and lymph nodes were stained with a mixture of 1 μg each of PE- and FITC-conjugated MAb for 30 min on ice in 100 μl of phosphate-buffered saline containing 25% fetal calf serum. Cells were washed and analyzed on a FACScan flow cytometer (Becton Dickinson), using the FACScan software program. Dead cells were excluded from analysis by staining with propidium iodide and gating. Results are displayed as two-color dot plots of 10,000 cells analyzed, showing the percentage of cells staining in pertinent quadrants.

Proliferation assays. Spleen and lymph node cells from c-Fos-deficient mice and from their homozygous wild-type and heterozygous littermates were cultured at the indicated concentrations with 0.2 to 0.5 μg of concanavalin A (ConA; Sigma) per ml or 0.1 to 1% anti-CD3 in 96-well microtiter plates in a final volume of 200 μl. Two days later, the wells were pulsed for 4 h with 1 μCi of [^{3}H]thymidine and thymidine incorporation was determined (47).

Lymphokine assays. Spleen and lymph node cells (10^6) from c-Fos-deficient mice and from their homozygous wild-type and heterozygous littermates were cultured in 48-well Costar plates in 1 ml of medium containing 0.5 to 2 μg of ConA per ml. Supernatants were removed at 16 to 20 h for IL-2 assays or at 40 to 44 h for IL-3, IL-4, and gamma interferon assays and tested for the ability to support (or inhibit) proliferation of the indicator cells. Indicator cells (5,000 to 8,000 CTLL-20 cells per well, 10,000 CT.4s cells per well, and 20,000 FL5.12 or WEHI 279 cells per well) were cultured in 96-well microtiter plates with the indicated supernatant concentrations in a final volume of 100 to 200 μl. The number of cells present in the wells 2 to 3 days later was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction (34). Standard curves using recombinant lymphokines were included for each experiment.

Fos and Jun antibodies. The affinity-purified antiserum against the c-Fos M peptide, which recognizes all four Fos family members, has been described (19). A c-Fos-specific antibody raised against a hexahistidine-tagged protein comprising amino acids 1 to 131 of c-Fos (6) and rabbit antisera to Fra-1 (7) and Fra-2 proteins were kindly provided by T. Curran (Roche Institute, Nutley, N.J.). A second c-Fos-specific MAb was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit antisera specific for FosB, c-Jun, JunB, and JunD and a second Fra-1 antibody (27, 28) were a gift from R. Bravo (Bristol-Myers Squibb, Princeton, N.J.). Another set of rabbit antisera reacting specifically with c-Jun, JunB, or JunD were a kind gift of J. Chen (Children's Hospital, Boston, Mass.). The specificity of most antisera was evaluated by testing their ability to recognize recombinant c-Fos and c-Jun proteins synthesized in baculovirus extracts (14); the extracts were kindly provided by S. Agarwal (Dana-Farber Cancer Institute, Boston, Mass.). The specificity of both c-Fos antisera was further confirmed by lack of reaction with the inducible AP-1 complexes of c-Fos-deficient mice.

The antisera obtained from various sources for different Fos and Jun members were neither affinity purified nor matched for potency and were therefore used at different concentrations compared with each other. The antisera were used for qualitative determination of the presence or absence of specific Fos/Jun family members. Although it sometimes appeared that any given antiserum was capable of supershifting or inhibiting the upper complex more than others, we have not used these results for making quantitative assessments of the levels of corresponding Fos and Jun family members in the AP-1 and NFAT complexes.

Nuclear extracts. Small-scale nuclear extracts were made from short-term T-cell lines derived from spleen cells of homozygous c-Fos-deficient mice or their homozygous wild-type littermates (20). Briefly, spleen cells were stimulated with 2 μg of ConA per ml for 3 days, and the resulting T-cell blasts were maintained in IL-2-containing medium for 7 to 10 days. At the end of this period, the cell lines were >98% $CD3^+$ and 66 to 98% $CD8^+$. ConA blasts or Ar-5 T cells (2×10^7) were stimulated for 2 h with anti-CD3ε (cross-linked by attachment

to immobilized rabbit anti-hamster second antibody), and nuclear extracts from unstimulated and stimulated T cells were prepared as previously described (17, 19). Electrophoretic mobility shift assays were performed with oligonucleotides corresponding to the NFAT and AP-1 sites of the murine IL-2 promoter (17, 19) and the AP-1 site corresponding to the human metallothionein promoter (19).

Kinetics of mRNA induction. Northern analysis of cytoplasmic RNA from Ar-5 T cells stimulated for various times with anti-CD3 was performed as previously described (19). Rat Fra-1 and Fra-2 cDNA probes were kindly provided for these experiments by T. Curran.

RESULTS

Sizes of lymphoid organs. The spleens and thymuses of c-Fos-deficient mice showed somewhat greater variability in size and content of viable lymphocytes than the spleens and thymuses of their heterozygous or wild-type littermates. Whereas 90×10^6 to 110×10^6 lymphocytes were routinely recovered from normal spleens ($n = 7$), the spleens of c-Fos-deficient mice yielded 10×10^6 to 77×10^6 cells ($n = 8$). The variation in thymic size was smaller, with c-Fos-deficient mice yielding between 40 and 90% of the thymocytes obtained from their heterozygous or wild-type littermates. In general, the heterozygous mice were indistinguishable from their wild-type littermates in all the characteristics described in this report.

Expression of T- and B-cell markers in spleen, thymus, and lymph nodes. The lymphoid organs of c-Fos-deficient mice and their heterozygous and wild-type counterparts were examined for their content of T and B cells in two independent experiments. Cells from the thymus, spleen, and lymph nodes of 10- to 14-week-old mice were stained simultaneously with an FITC-conjugated MAb specific for the CD3 ε chain, a marker for T cells that have successfully rearranged and expressed the TCR/CD3 complex, and a PE-conjugated MAb specific for the B220 determinant of CD45R, a marker for immature and mature B cells (Fig. 1A). The patterns of surface CD3 expression were similar in thymocytes of c-Fos-deficient mice, their heterozygous littermates, and homozygous wild-type mice in that three distinct cell populations ($CD3^{high}$, $CD3^{low}$, and $CD3^{neg}$) were observed in all cases; this pattern is characteristic of cells undergoing normal thymic development. Similarly, spleen and lymph node cells of all three types of mice expressed equivalent levels of $CD45R^+$ and $CD3^+$ B and T cells. The number of thymocytes expressing high levels of CD3 varied in independent experiments, but no systematic differences between Fos $-/-$ and Fos $+/+$ mice were observed. However Fos $-/-$ mice showed some tendency toward a selective reduction in the number of $CD45R^+$ cells in spleen and lymph nodes (Fig. 1A); this B-cell deficit was especially obvious in the mice with the smallest spleens (not shown).

In parallel, the development and distribution of $CD4^+$ and $CD8^+$ T-cell subsets was assessed in c-Fos-deficient mice. Cells from thymus, spleen, and lymph nodes were stained simultaneously with a PE-conjugated MAb specific for CD4 and an FITC-conjugated MAb specific for CD8 (Fig. 1B). Mice lacking c-Fos were similar to their heterozygous littermates and to wild-type mice in their levels of double-negative ($CD4^-$ $CD8^-$), single-positive ($CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$), and double-positive ($CD4^+$ $CD8^+$) thymocytes. Likewise, the spleen and lymph nodes of mice lacking c-Fos were abundantly populated with mature single-positive T cells.

Cytokine production. The c-Fos-deficient mice were equivalent to their heterozygous and wild-type littermates and to nonlittermate controls in their ability to proliferate and produce lymphokines in response to T-cell receptor stimulation (Fig. 2 and data not shown). Spleen and lymph node cells from c-Fos-deficient ($-/-$) mice and their homozygous wild-type ($+/+$) littermates exhibited the same level of proliferation in response to minimal levels of ConA and anti-CD3ε over a wide range of cell concentrations (Fig. 2A). Similarly, they produced equivalent levels of the four T-cell-specific lymphokines IL-2, IL-3, IL-4, and gamma interferon in response to stimulation with moderate concentrations of ConA and anti-CD3ε (Fig. 2B to D). Highly purified $CD4^+$ T cells prepared from spleen and lymph node cells of both Fos-minus and wild-type mice were also equivalent in their responses to allogeneic antigen presented by BALB/c spleen cells (data not shown).

Induction of NFAT and AP-1 in nuclear extracts. T cells from c-Fos-deficient mice were equivalent to wild-type T cells in their ability to induce nuclear factors containing Fos and Jun proteins after T-cell receptor stimulation (Fig. 3). Short-term splenic T-cell lines derived from c-Fos-deficient mice and their homozygous wild-type littermates were stimulated for 2 h with ConA and anti-CD3ε, and nuclear extracts were assessed for the presence of factors capable of binding to the AP-1 site of the murine IL-2 promoter (Fig. 3A, lanes 5 to 8; Fig. 3B, lanes 8 to 12). An oligonucleotide corresponding to this site formed a single inducible DNA-protein complex with nuclear extracts from both the heterozygous (Fig. 3A, lanes 5 and 6) and c-Fos-deficient (Fig. 3A, lanes 7 and 8) cell lines. In both cases, the complexes contained c-Fos or Fos-related proteins, since an affinity-purified antiserum which cross-reacts with all four Fos family members (17, 19) inhibited their formation (Fig. 3B, lanes 11). The specificity of this antiserum has been demonstrated previously by blocking its effect with its cognate peptide (17, 19).

The same nuclear extracts were tested for binding to an oligonucleotide spanning the distal NFAT site of the murine IL-2 promoter (Fig. 3A, lanes 1 to 4; Fig. 3B, lanes 1 to 7). Nuclear extracts from both the heterozygous ($+/-$) and c-Fos-deficient ($-/-$) cell lines formed two inducible DNA-protein complexes with this NFAT oligonucleotide (Fig. 3A, lanes 1 to 4); the lower complex contains NFATp, whereas the upper complex contains NFATp in association with Fos and Jun (or related) proteins (17, 18). There was no difference in the binding specificities of the complexes in the $-/-$ and $+/-$ cell lines (Fig. 3B), as judged by competition with unlabelled oligonucleotides; the NFAT oligonucleotide competed effectively, whereas the M3 oligonucleotide, which is mutated in four bases essential for binding of NFATp (17, 18), did not compete at all (Fig. 3B, lanes 2 and 3). In both $-/-$ and $+/-$ cell lines, the upper NFAT complex contained AP-1 proteins, since its formation was inhibited by excess unlabelled AP-1 oligonucleotide (Fig. 3B, lanes 4). Moreover, it contained c-Fos or related proteins, as judged by inhibition with the affinity-purified pan-Fos antiserum (Fig. 3B, lanes 6). We conclude that under these conditions of stimulation, c-Fos-deficient mice are comparable to normal mice in their ability to induce nuclear factors capable of binding to the AP-1 and NFAT sites of the IL-2 promoter.

Presence of Fos and Jun family members in AP-1 and NFAT complexes. To determine whether Fos family members other than c-Fos participated in the formation of AP-1 and NFAT complexes in stimulated T cells, we assessed the composition of these complexes by using antisera specific for different Fos and Jun family members and nuclear extracts made from the untransformed murine T-cell clone Ar-5 (37) and from short-term splenic T cells of the wild-type ($+/+$) and c-Fos-deficient ($-/-$) mice (Fig. 4 and 5). The inducible AP-1 complex contained at least c-Fos, Fra-1, and Fra-2, as judged by the fact

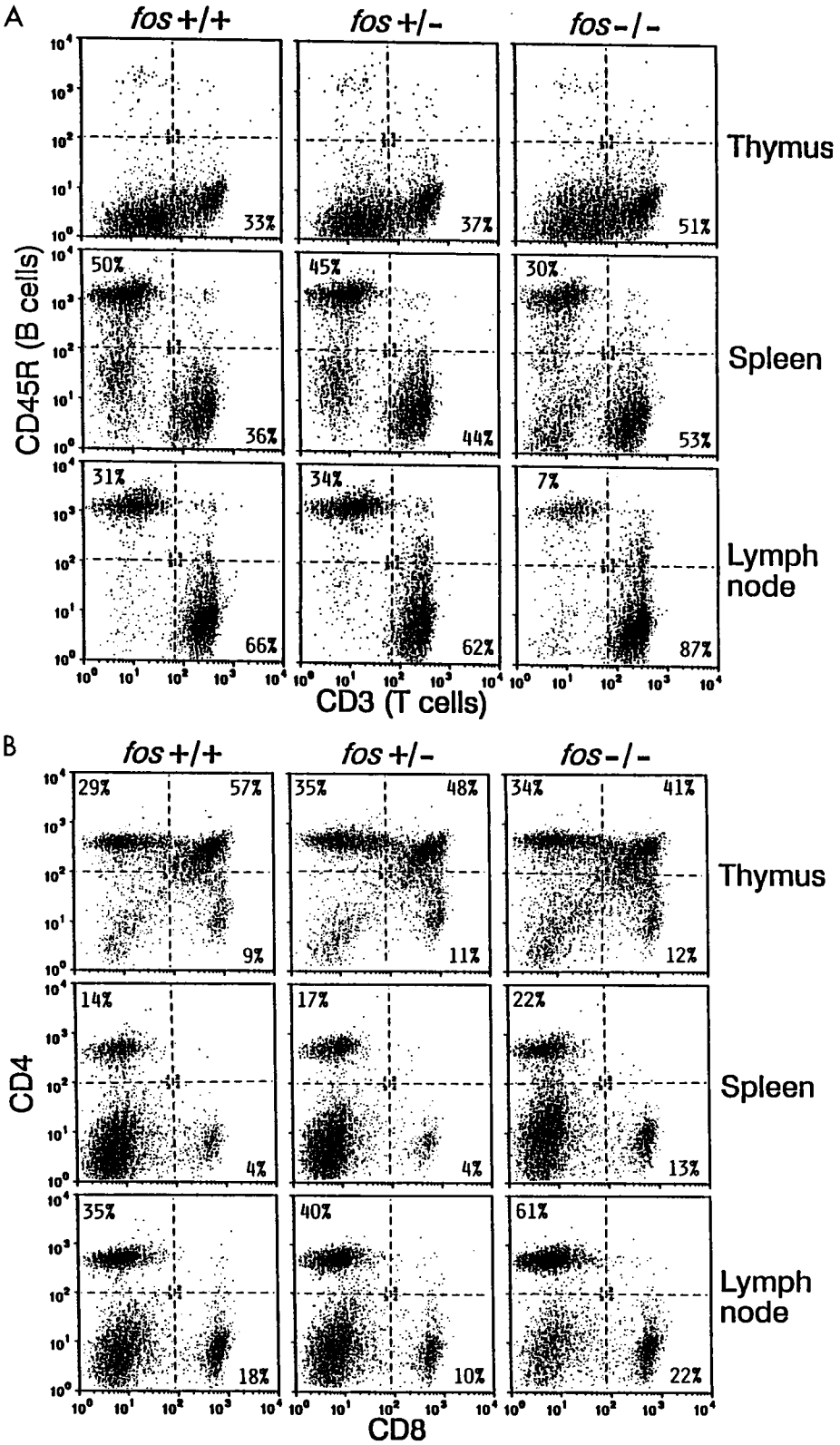

FIG. 1. Equivalent expression of T- and B-cell markers in lymphocytes of c-Fos-deficient and wild-type mice. Cells from thymus, spleen, or lymph nodes of c-Fos-deficient mice (−/−) and their heterozygous littermates (+/−) or homozygous controls (+/+) were stained simultaneously with MAb specific for T and B cells (A) or T-cell subsets (B) and analyzed by two-color flow cytometry. In panel A, cells were stained with an FITC-conjugated MAb specific for CD3 expressed on T cells and a PE-conjugated MAb specific for the B220 determinant of CD45R expressed on B cells. In panel B, cells were stained with an FITC-conjugated MAb specific for CD8 and a PE-conjugated MAb specific for CD4. Results are displayed as dot plots on a four-decade logarithmic scale of 10,000 living cells analyzed. The percentages of single- or double-positive cells in relevant quadrants are indicated.

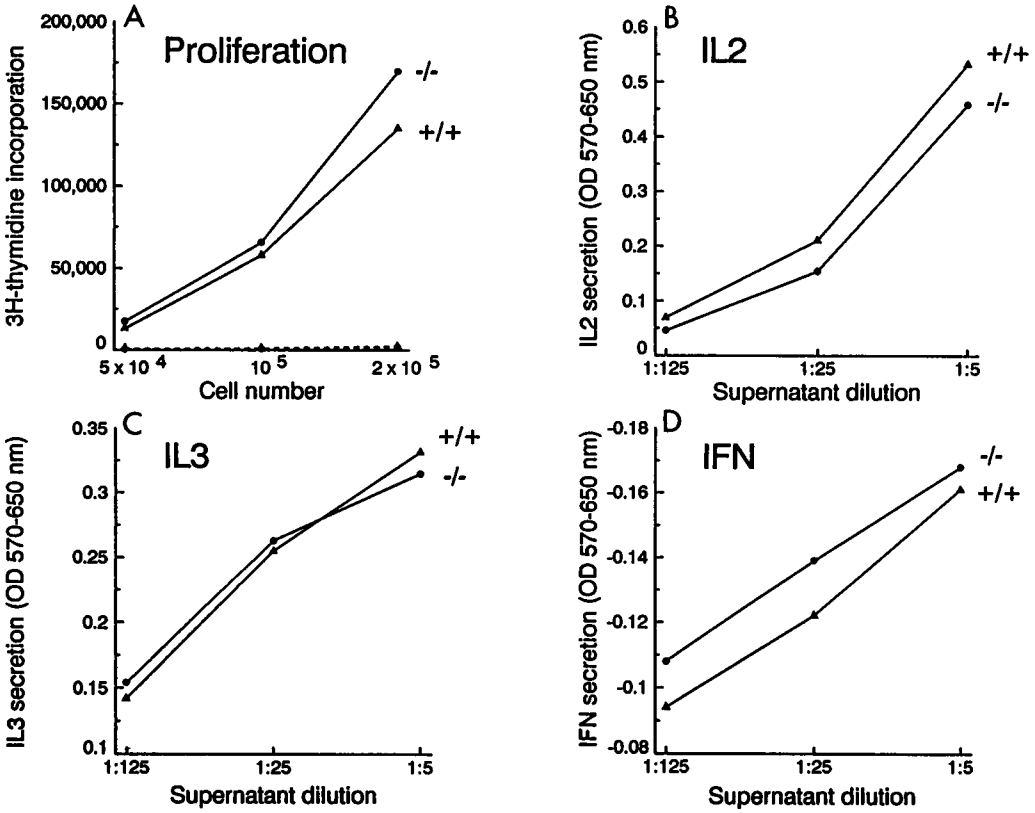

FIG. 2. Equivalent levels of lymphokine production by c-Fos-deficient and wild-type mice. (A) Increasing numbers of spleen cells from c-Fos-deficient (−/−) mice and their homozygous wild-type (+/+) littermates were stimulated with ConA (0.5 μg/ml), and proliferation was assessed by [^{3}H]thymidine incorporation. The dashed lines indicate proliferation in response to medium alone. (B to D) Spleen cells from c-Fos-deficient (−/−) mice and their homozygous wild-type (+/+) littermates were cultured with ConA (2 μg/ml), and supernatants were assayed at the indicated concentrations for IL-2, IL-3, and gamma interferon (IFN) as described in Materials and Methods. The negative absorbance values for the gamma interferon assay (D) reflect the inhibition of WEHI 279 cell proliferation by gamma interferon (38). The data are representative of three independent experiments with five Fos-deficient and five control mice.

that antisera to these proteins reproducibly diminished the intensity of the Ar-5 and wild-type homozygous (+/+) AP-1 DNA-protein complexes (Fig. 4A and B, upper panels). However, two different FosB antisera reacted only weakly with the AP-1 complexes of splenic T cells compared with their reaction with the Ar-5 T cell AP-1 complexes (Fig. 4A and B). The AP-1 complex in nuclear extracts made from c-Fos-deficient mice did not react with the c-Fos antiserum, as expected (Fig. 4A and B, lower panels; compare lanes 1 and 2); however, it showed a weak, variable reaction with the FosB and Fra-1 antisera (Fig. 4B, lower panel, lanes 5 and 8) and a significant reaction with the Fra-2 antiserum (Fig. 4A and B, lower panels, lanes 9 to 11). This decrease in intensity was not due to proteolysis, since faster-migrating complexes representing possible proteolytic products were not observed; moreover, the antisera specifically affected the intensity of the AP-1 complexes and not the intensity of NFκB and Sp1 DNA-protein complexes (data not shown). Finally, antisera specifically reactive to c-Jun, JunB, and JunD proteins diminished the intensity of the inducible AP-1 complexes of stimulated Ar-5 T cells, with the JunB and JunD antisera supershifting part of the AP-1 complex (Fig. 4C). These results indicate that all three Jun members are likely to participate in the formation of the AP-1 complexes of stimulated murine T cells.

Since the representation of Fos and Jun family members in the AP-1 complexes of Ar-5 T cells was generally comparable with that in normal splenic T cells (Fig. 4A and B), and since we had previously established the kinetics of induction of different Fos and Jun mRNAs in Ar-5 T cells by Northern analysis (19), we used nuclear extracts made from Ar-5 T cells to determine which Fos and Jun members were present in the NFAT complexes. The c-Fos and Fra-2 antisera significantly diminished the intensity of the upper NFAT complex, whereas the Fra-1 and FosB antisera had little or no effect over several experiments (Fig. 5), indicating that c-Fos and Fra-2 (or antigenically related proteins) could associate with NFATp in the nucleus of stimulated T cells. The simultaneous addition of both anti-c-Fos and anti-Fra-2 was sufficient to inhibit completely the formation of the upper NFAT complex, suggesting that only c-Fos and Fra-2 were present in these extracts (data not shown). It is possible that FosB and Fra-1 are genuinely absent from the upper nuclear NFAT complex; alternatively, they may be unable to react with their specific antisera when associated with NFATp.

Antisera specifically reactive to c-Jun, JunB, and JunD were used to assess the presence of these proteins in the NFAT complex of stimulated Ar-5 T cells (Fig. 5B and data not shown). The JunB antiserum specifically diminished the intensity of the NFAT complex (Fig. 5B), indicating that JunB was present in the AP-1 complexes and could associate with NFATp. Despite the fact that c-Jun is induced upon stimulation and JunD is constitutively expressed at relatively high

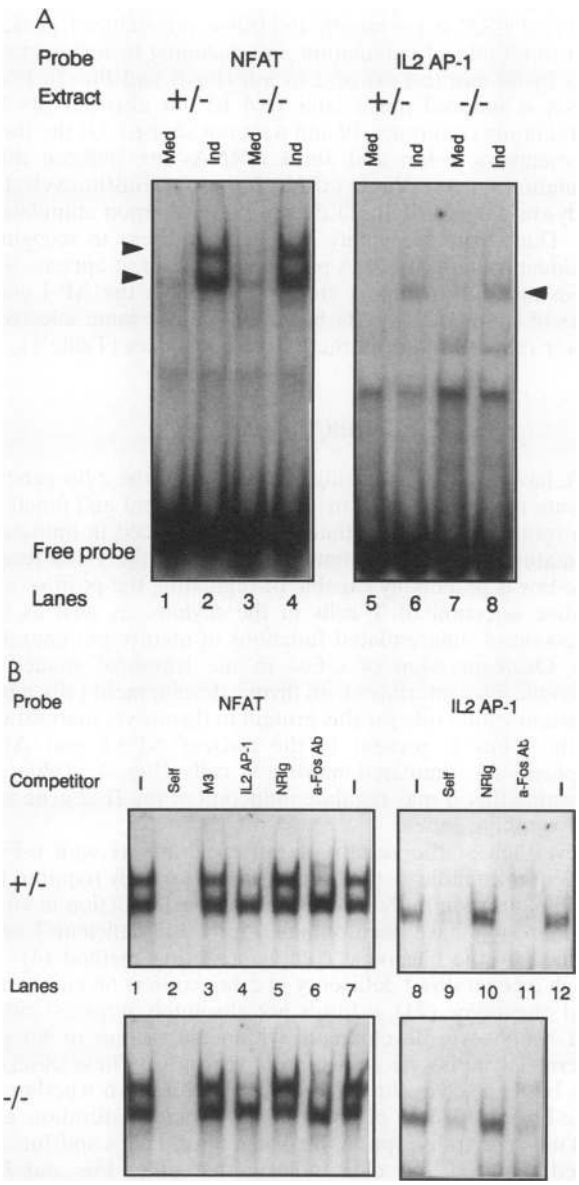

FIG. 3. Equivalent induction of NFAT and AP-1 nuclear complexes in c-Fos-deficient and wild-type mice. (A) Induction by anti-CD3. Short-term T-cell lines (>98% $CD8^+$ cells) from c-Fos heterozygous or null homozygous mice were left unstimulated (Med) or stimulated with anti-CD3 (Ind), and nuclear extracts were analyzed by electrophoretic mobility shift assays using the NFAT (lanes 1 to 4) or AP-1 (lanes 5 to 8) oligonucleotide. Two inducible DNA-protein complexes are observed with the NFAT oligonucleotide, and a single inducible complex is observed with the AP-1 oligonucleotide (arrowheads). The position of the free probe is indicated. (B) Analysis of complexes by competition with unlabelled oligonucleotides and reaction with anti-Fos antibodies. Nuclear extracts from stimulated T cells were incubated with the labelled NFAT (lanes 1 to 7) or AP-1 (lanes 8 to 12) oligonucleotide. Where indicated, a 200-fold excess of the indicated competitor oligonucleotides was included in the binding reaction mixtures (lanes 2 to 4 and 9). To test for the presence of Fos family members, nuclear extracts were preincubated with normal rabbit immunoglobulin (NRIg; lanes 5 and 10) or affinity-purified antibodies directed against a peptide from the basic domain of c-Fos (a-Fos Ab; lanes 6 and 11) prior to addition of the labelled oligonucleotides.

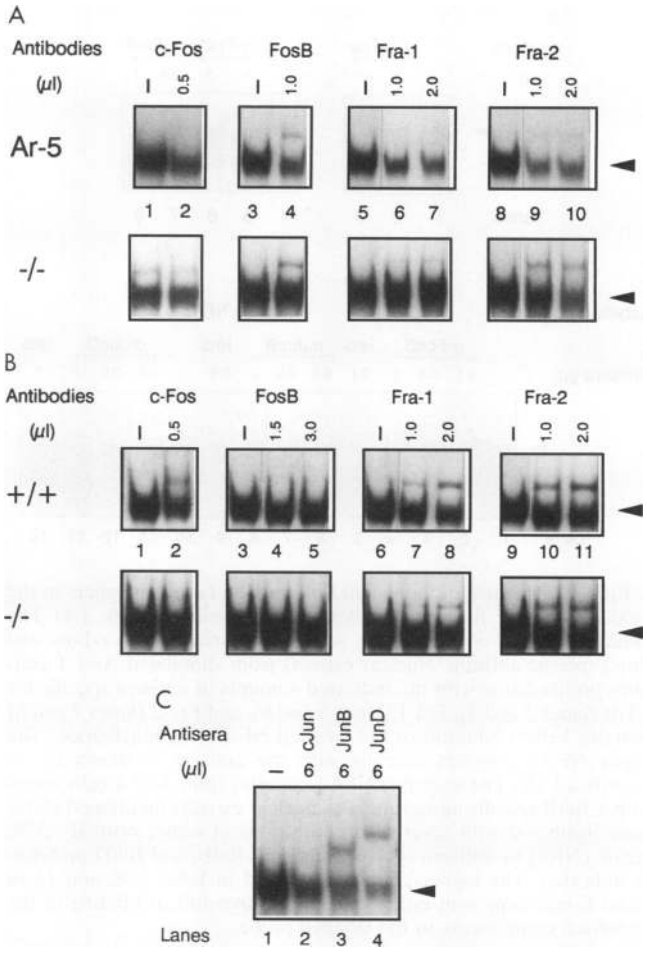

FIG. 4. Representation of individual Fos and Jun family members in the inducible AP-1 nuclear complexes of murine T cells. (A) Nuclear extracts from stimulated Ar-5 or c-Fos-deficient splenic T cells were preincubated with the indicated amounts of antisera specific for c-Fos (lane 2), FosB (lane 4), Fra-1 (lanes 6 and 7), and Fra-2 (lanes 9 and 10) before addition of a labelled oligonucleotide containing the AP-1 site of the metallothionein promoter. The single inducible AP-1 complex observed in the absence of any competing antisera (lanes 1, 3, 5, and 8) is indicated (arrowhead). (B) Nuclear extracts made from stimulated splenic T cells of wild-type homozygous (+/+) or c-Fos null (−/−) mice were preincubated with the indicated amounts of antisera specific to c-Fos (lane 2), FosB (lanes 4 and 5), Fra-1 (lanes 7 and 8), and Fra-2 (lanes 10 and 11) and then bound to the labelled oligonucleotide containing the AP-1 site of the metallothionein promoter. The FosB antiserum used in this experiment was different from that used in panel A; the other antisera were the same. (C) All three Jun family members are present in the inducible AP-1 complex. Nuclear extracts made from stimulated Ar-5 T cells were preincubated with the indicated amounts of the antisera reactive specifically to c-Jun (lane 2), JunB (lane 3), and JunD (lane 4) and assessed for DNA binding with the IL-2 AP-1 site oligonucleotide. The upper complex observed in lanes 4, 9, and 10 of panel A and lanes 2, 7, 8, 10, and 11 of panel B may represent either a genuine supershift or binding of the antiserum components to the labelled probe.

levels (19), and that all three Jun members are present in the AP-1 complex (Fig. 4C), antisera to c-Jun and JunD did not significantly decrease the intensity of the upper NFAT complex (Fig. 5B and data not shown), indicating either that c-Jun and JunD were minor components of NFAT complex or that they

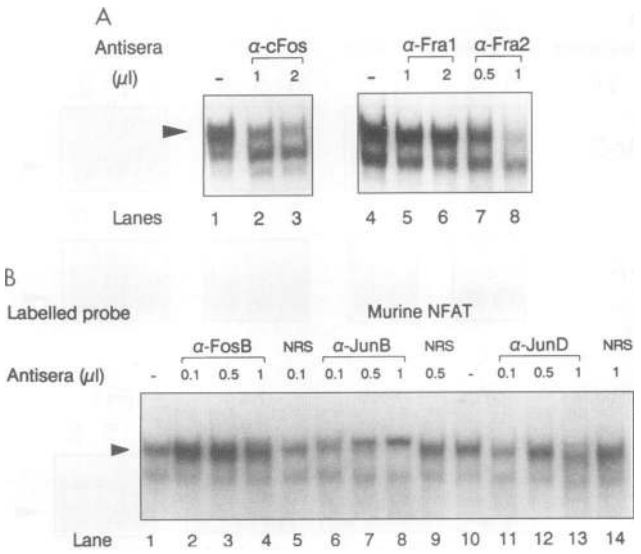

FIG. 5. Presence of individual Fos and Jun family members in the inducible NFAT nuclear complex of the murine T cells. (A) The inducible NFAT complex from Ar-5 T cells reacts with c-Fos- and Fra-2-specific antisera. Nuclear extracts from stimulated Ar-5 T cells were preincubated with the indicated amounts of antisera specific for c-Fos (lanes 2 and 3), Fra-1 (lanes 5 and 6), and Fra-2 (lanes 7 and 8) proteins before addition of the labelled NFAT oligonucleotide. The upper NFAT complex reacting with the antisera is shown by an arrowhead. (B) The inducible NFAT complex from Ar-5 T cells reacts with a JunB-specific antiserum. The nuclear extracts mentioned above were incubated with several concentrations of either normal rabbit serum (NRS) or antisera specific for FosB, JunB, and JunD proteins, as indicated. The higher complex observed in lanes 7, 8, and 13 of panel B may represent either a genuine supershift or binding of the antiserum components to the labelled probe.

were unavailable for reaction with the antibodies when present in the complex.

Table 1 summarizes the kinetics of induction of mRNAs encoding Fos and Jun family members in Ar-5 T cells (reference 19 and data not shown) and their presence in AP-1 and NFAT DNA-protein complexes as detected by gel shift analysis (Fig. 4 and 5). mRNAs for all four Fos members are undetectable in unstimulated Ar-5 T cells but are induced upon stimulation with anti-CD3ε (19). Of these, c-Fos, FosB, and Fra-2 mRNAs are rapidly and transiently induced, peaking at 30 to 60 min of stimulation and declining to undetectable levels by 60 min (c-Fos) or 2 to 3 h (FosB and Fra-2). Fra-1 mRNA is induced much later (~4 h) but also persists for several hours (reference 19 and data not shown). Of the three Jun members, c-Jun and JunB mRNAs are induced after stimulation, whereas JunD mRNA is present constitutively; the steady-state levels of JunD do not increase upon stimulation (19). Thus, from the ability of specific antisera to recognize individual proteins in DNA-protein complexes, it appears that all Fos and Jun members are represented in the AP-1 complexes of stimulated T cells, but there may be some selectivity in their representation in the NFAT complexes (Table 1).

TABLE 1. Fos and Jun family members present in T cells[a]

Protein	Induction of mRNA (time of peak)	Presence at 2 h in:	
		AP-1 complex	NFAT complex
c-Fos	30 min	+	+
FosB	30 min–1 h	+	−
Fra-1	4–8 h	+	−
Fra-2	30 min–1 h	+	+
c-Jun	30 min–1 h	+	−
JunB	30 min–2 h	+	+
JunD	Constitutive	+	+

[a] The data summarize results obtained with Ar-5 T cells stimulated with anti-CD3. The time of peak mRNA induction was assessed by Northern analysis of cytoplasmic RNA (19). The presence of Fos and Jun family members in AP-1 and NFAT complexes was evaluated as in Fig. 4 and 5, using nuclear extracts from cells stimulated for 2 h with anti-CD3. −, no reactivity was observed with antisera to FosB, Fra-1, and c-Jun proteins, possibly because of the genuine absence of the individual member in the NFAT complex or because the particular protein was unable to react with the given antiserum when present in the NFAT complex.

DISCUSSION

We have used mice lacking both copies of the c-*fos* gene to evaluate the role of c-Fos in T-cell development and function. As a transcription factor that is rapidly induced in immature and mature T cells upon stimulation through the T-cell receptor, c-Fos is potentially capable of regulating the positive and negative selection of T cells in the thymus, as well as the expression of differentiated functions in mature peripheral T cells. Overexpression of c-Fos in the lymphoid tissues of transgenic mice interferes with thymic development (40), again consistent with a role for this protein in thymocyte maturation. Finally, c-Fos is present in the nuclear NFAT and AP-1 complexes of stimulated murine T cells (Fig. 4; Table 1), suggesting that it may regulate induction of the IL-2 gene and other cytokine genes.

Nevertheless, the results of our experiments with c-Fos-deficient mice indicate that c-Fos is not absolutely required for T-cell maturation in the thymus or for T-cell function in vitro. Similar results have been obtained for c-Jun-deficient T cells obtained by the blastocyst complementation method (4): although a generalized deficiency in c-Jun confers an embryonic lethal phenotype (21), c-Jun is not absolutely required either for T-lymphocyte development within the thymus or for peripheral T-lymphocyte activation in vitro (3a). These observations by themselves do not conclusively establish whether or not c-Fos and c-Jun play a role in thymic maturation and cytokine gene transcription in normal mice. If Fos and Jun are indeed required, our data indicate that other Fos and Jun family members can compensate for the lack of c-Fos and c-Jun in developing and differentiated T cells of c-Fos- and c-Jun-deficient mice, respectively.

The process of thymocyte development in c-Fos-deficient mice appears similar to that in normal mice, as judged by expression of surface markers characteristic of thymocytes at different stages of positive and negative selection. c-Fos-deficient and normal mice are also comparable in their ability to populate their peripheral lymphoid organs with B and T cells, indicated by the appropriate expression of B- and T-cell markers on lymphocytes from spleen and lymph nodes. The slight selective reduction in B-cell number in spleen and lymph nodes, most pronounced in the mice with the smallest spleens, probably reflects an insufficiency of bone marrow function secondary to the pronounced osteopetrotic phenotype of c-Fos-deficient mice (22, 46). A decrease in B-cell number in spleens of c-Fos-deficient mice was also observed in a previous study (46). However we did not observe the decrease in immature double-positive ($CD4^+$ $CD8^+$) thymocytes noted by these workers; the discrepancy may be due to the fact that the

mice were analyzed at 4 to 6 weeks of age (46) in contrast with 10 to 14 weeks in the present study.

Additionally, c-Fos-deficient mice showed no gross impairment in their ability to proliferate and produce lymphokines in vitro in response to strong T-cell receptor stimuli. T cells from c-Fos-deficient and wild-type mice were activated similarly by ConA and anti-CD3ε, as judged by equivalent production of at least three cytokines (IL-2, IL-3, and gamma interferon) by stimulated spleen cells. Moreover, both AP-1 and NFAT complexes were normally induced in c-Fos-deficient T cells stimulated with ConA or anti-CD3, and both contained Fos-related proteins, as judged by reaction with a pan-Fos antiserum (Fig. 3). The ligands used for these experiments, ConA and anti-CD3ε, bind to and cross-link a significant fraction of the T-cell receptor complexes present on each cell; the physiological response to antigen, which involves ligation of smaller numbers of T-cell receptor complexes (8), may be more sensitive to the absence of c-Fos. Indeed, analysis of collagenase and stromelysin mRNA induction in fibroblasts of c-Fos-deficient mice indicates that the requirement for c-Fos in inducible gene transcription varies depending on the particular stimulus used (epidermal growth factor, platelet-derived growth factor, serum, or phorbol esters [14a]).

Composite binding sites for NFATp and AP-1 have been described in the regulatory regions of several cytokine genes, including the IL-2 promoter and the granulocyte-macrophage colony-stimulating factor/IL-3 intergenic enhancer (2, 5, 16, 17, 35). Binding sites for NFATp are also present in the tumor necrosis factor alpha and IL-4 promoters (13, 39, 42). The cooperation of any given Fos/Jun member with NFATp during cytokine gene induction may be investigated at three levels: Which Fos and Jun members can physically interact with NFATp in vitro? Which members are actually present in inducible nuclear NFAT complexes? And finally, which members form transcriptionally active complexes during the immune response?

It is clear from our own studies and those of others that different Fos and Jun family members can associate with NFATp in vitro. We have shown that purified NFATp can form DNA-protein complexes with recombinant c-Fos/c-Jun, Fra-2/c-Jun and c-Jun/c-Jun dimers on the murine NFAT site (16, 18a); likewise, NFATp renatured from slices of a sodium dodecyl sulfate-gel was detected by its ability to bind to the human NFAT site in the presence of recombinant JunD (35). With regard to the members actually present in nuclear complexes, we have shown here that Ar-5 murine T cells stimulated for 2 h with anti-CD3ε express nuclear NFAT complexes which contain at least c-Fos, Fra-2, and JunB. A previous study suggested that the NFAT complex of human peripheral blood T cells, stimulated for 6 to 8 h with phorbol ester plus ionomycin, contained predominantly Fra-1 and JunB (2). The difference in time of stimulation is likely to be relevant: Fra-1 is induced later than c-Fos and Fra-2 in activated T cells and persists for a longer time (Table 1), suggesting that c-Fos and Fra-2 are present in the NFAT complex at early times after stimulation, whereas Fra-1 may be predominant at later times. According to this interpretation, three of four Fos family members (c-Fos, Fra-1, and Fra-2) and at least one Jun member (JunB) may be capable of interacting with NFATp in nuclear extracts. As mentioned earlier, the lack of reaction with antisera specific for FosB, JunD, and c-Jun may reflect either the absence of these proteins from the NFAT complex or the masking of their reactive epitopes upon complexing with NFATp. The presence of any particular Fos or Jun member in the nuclear NFAT complex, however, does not by itself imply that it regulates gene transcription in cooperation with NFATp. Further studies are needed to resolve whether different DNA-protein complexes containing NFATp and AP-1 proteins have different transcriptional activities and whether they are involved in transcription of different cytokines during the immune response.

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