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Fidelity of a BAC-EGFP transgene in reporting dynamic expression of IL-7R α in T cells

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Abstract

Interleukin-7 receptor α chain (IL-7R α)-derived signals are critical for normal T cell development, mature T cell homeostasis, and longevity of memory T cells. IL-7R α expression in T cells is dynamically regulated at different developmental and antigen-responding stages. However, the molecular mechanism underlying the dynamic regulation is not completely understood. Here we describe generation of a bacterial artificial chromosome (BAC)-based reporter transgenic mouse strain, which contains 210 kb DNA sequence flanking the *Il7r* locus. We used *in vitro* validated EGFP reporter and insulator sequences to facilitate the reporter transgene expression. Consistent with endogenous IL-7R α expression, the BAC transgene was expressed in mature T cells, a portion of natural killer cells but not in mature B cells. In the thymus, the EGFP reporter and endogenous IL-7R α showed synchronized silencing in CD4⁺CD8⁺ double positive stage, were both upregulated in CD4⁺ or CD8⁺ single positive thymocytes, and both continued to be co-expressed in naïve T cells in the periphery. Upon encountering antigen, the antigen-specific effector CD8⁺ T cells downregulated both endogenous IL-7R α and the EGFP reporter, which were upregulated in synchrony in antigen-specific memory CD8 T cells. These results indicate that the BAC-EGFP transgene reports endogenous IL-7R α regulation with high fidelity, and further suggest that the 210 kb sequence flanking the *Il7r* locus contains sufficient genetic information to regulate its expression changes in T lineage cells. Our approach thus represents a critical initial step towards systematic dissection of the *cis* regulatory elements controlling dynamic IL-7R α regulation during T cell development and cellular immune responses.

Keywords

IL-7R α ; bacterial artificial chromosome; T cell development; effector T cells; memory T cells

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Introduction

Interleukin 7 (IL-7) receptor α chain (IL-7R α) chain is a receptor subunit for 2 different cytokines. It can either pair with common cytokine receptor γ chain to respond to IL-7 or pair with thymic stromal lymphopoietin (TSLP) receptor to respond to TSLP (Liu et al., 2007; Rochman et al., 2009). Whereas mice deficient in either TSLP or TSLPR do not manifest apparent disturbance of lymphocyte development and function (Leonard, 2002; Liu et al., 2007), the IL-7/IL-7R α axis is known to play critical roles in both T cell development and mature T cell function (Mazzucchelli and Durum, 2007). The development of $\alpha\beta$ T cells in the thymus proceeds through well-defined stages. The most immature CD4⁻CD8⁻ double negative (DN) thymocytes progress through a CD4⁺CD8⁺ double-positive (DP) stage and then become single positive (SP) CD4⁺ or CD8⁺ T cells. The DN thymocytes can be further fractionated into DN1-DN4 subsets based on CD25 and CD44 expression, with DN1 (CD44⁺CD25⁻) being the least differentiated subset maintaining the potential to become natural killer (NK) and dendritic cells and DN3 being committed to T lineage cells (Rothenberg and Taghon, 2005). IL-7R α is expressed in all DN subsets and at slightly decreased levels in DN4 thymocytes. IL-7R α expression is completely silenced in DP thymocytes whereas SP T cells regain IL-7R α expression (Sudo et al., 1993). The dynamic IL-7R α expression changes are considered to correlate with functional requirements of IL-7-derived signals, which protect DN and SP thymocytes from apoptosis and promote their proliferation (Mazzucchelli and Durum, 2007). Downregulation of IL-7R α in DP cells is thought to avoid over-consumption of IL-7 that is present in limited concentrations in the thymus (Mazzucchelli and Durum, 2007). The pivotal roles of IL-7/IL-7R α in T cell development are substantiated by the observations that ablation of the *Il7r* gene in mice diminishes thymic cellularity with early block of T cell development at the DN stage (Peschon et al., 1994). Furthermore, mutations in the human *IL7R* locus resulted in T(-)B(+)NK(+) severe combined immunodeficiency with the affected patients having no mature T cells in the periphery (Puel et al., 1998).

In addition to its essential roles in lymphocyte development, IL-7R α is critical for the survival and homeostatic proliferation of mature peripheral T cells (Schluns et al., 2000). Upon encountering a foreign antigen, the naïve antigen-specific T cells are activated via T cell receptor (TCR) stimulation and become effector T cells whose proliferation and differentiation are driven by cytokines other than IL-7. The expression of IL-7R α is downregulated in effector T cells. In contrast, after the antigen is cleared, the majority of effector T cells succumb to apoptosis and only a small portion transition into antigen-specific memory CD4⁺ or CD8⁺ T cells, which re-express higher levels of IL-7R α (Kaeche et al., 2003; Kondrack et al., 2003; Li et al., 2003) and their survival and homeostatic proliferation require intact IL-7/IL-7R signaling pathways (Kondrack et al., 2003; Schluns et al., 2000; Seddon et al., 2003). In addition, IL-7R α -derived signals are recently found to be important for primary CD4 (but not CD8) responses (Osborne et al., 2007). Thus, IL-7R α expression is dynamically regulated in mature T cells, with its expression levels fine-tuned to functional needs during the cellular immune responses (Mazzucchelli and Durum, 2007).

The function-linked dynamic regulation of IL-7R α expression has been a subject of intense investigation. The *Il7r* promoter was initially defined to be located at about 950 bp upstream of the translation start codon (Pleiman et al., 1991), but later studies including our own found that the transcription was initiated 90 bp upstream of the “ATG” (DeKoter et al., 2007; Lee et al., 2005; Xue et al., 2004). An Ets binding motif (GGAA) located 95 bp upstream of the transcription initiation site (TIS) was found to be critical for *Il7r* promoter activity (DeKoter et al., 2002; DeKoter et al., 2007; Lee et al., 2005; Xue et al., 2004). We demonstrated that in T lineage cells, GA binding protein (GABP), a member of the Ets transcription factor family, binds to the Ets motif and transactivates *Il7r* transcription (Xue

et al., 2004; Yu et al., 2010). GABP expression was relatively stable during T cell development and activation (Xue et al., 2004; Zhao et al., 2010) and thus may not account for the dynamic changes of IL-7R α expression. Growth factor-independent 1 (Gfi-1) has transcription repressor activity and is considered to be associated with negative regulation of IL-7R α . Gfi-1 knockout mice showed an about 1.8-fold increase of IL-7R α expression on CD8 T cells when measured with “total fluorescent unit” by flow cytometry, however, such an increase was not observed on CD4 T cells (Park et al., 2004). During CD8 T cell responses to viral infection, a recent study showed that stable repression of IL-7R α in effector CD8 T cells requires Gfi-1, which binds directly to an intronic element approximately 5.5 kb downstream of the *Il7r* TIS (Chandele et al., 2008). However, the repression of IL-7R α by TCR stimulation in naïve T cells is independent of Gfi-1.

We have previously shown that IL-2 can downregulate IL-7R α expression in activated T cells via a PI3-kinase/Akt pathway (Xue et al., 2002). A serendipitous finding about IL-7R α regulation came about through analysis of mice deficient for a forkhead-box transcription factor Foxo1. Disruption of Foxo1 abrogated IL-7R α expression in mature T cells in the periphery, and Foxo1 acted through direct binding to a Foxo responsive element located approximately 3.5 kb upstream of the *Il7r* TIS (Kerdiles et al., 2009; Ouyang et al., 2009). Foxo transcription factors are known substrates for Akt, and phosphorylated Foxo proteins are translocated from nucleus to cytoplasm, followed by proteosomal degradation (Calnan and Brunet, 2008). Thus, changes in Foxo1 activity and binding to the *Il7r* locus help explain IL-2- and TCR-mediated downregulation of IL-7R α when naïve T cells are activated and become effector T cells. Importantly, IL-7 and IL-15 are known to be critical for longevity of memory T cells, and both cytokines can activate PI3-kinase-Akt pathway. It remains to be determined whether Foxo proteins are involved in regulation of IL-7R α expression in memory T cells. It is also of note that in contrast to mature CD4⁺ or CD8⁺ T cells in the periphery, Foxo1 deficiency had a much smaller impact on IL-7R α expression in SP thymocytes. The role of Foxo factors in the dynamic regulation of IL-7R α in developing thymocytes remains to be determined.

Despite these advances, there are many unanswered questions about the dynamic regulation of IL-7R α and related biological processes. A systematic approach is necessary to better understand the molecular basis for gene regulation of the *Il7r* locus, i.e., identifying the critical *cis* regulatory elements and their responsiveness to various extracellular signals. In this report, we describe a strategy to generate bacterial artificial chromosome (BAC)-based reporter transgenic mice, and the fidelity of the transgene in reporting endogenous IL-7R α expression in developing and antigen-responding T cells.

Materials and methods

Reporter constructs, transient transfection, and retroviral infection transduction

The cDNA sequence encoding the first 16 amino acids of IL-7R α were fused in-frame to the 5'-end of EGFP cDNA in pEGFP-N1 vector (Clontech) to generate IL-7R α -EGFP fusion reporter. The fusion reporter, along with SV40 polyA sequence, was released and used to replace the original IRES-GFP sequence in an MSCV-backbone retroviral vector, pMIG (Addgene). IL-7R α -Stop-IRES-EGFP reporter was generated by replacing the original GFP with the EGFP-SV40 polyA sequence in the pMIG vector followed by subcloning of cDNA encoding 16 amino acids of IL-7R α + a stop codon upstream of the IRES. The reporter constructs were transiently transfected into 293 T cells along with pCL-Eco and pVSVG for retrovirus packaging (Xue et al., 2002). Nucleated bone marrow cells were isolated from C57BL/6 mice and cultured for 2 days in IMDM medium supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml), Flt-3 ligand (10 ng/ml), and stem cell factor (20 ng/ml). Splenic T cells were isolated and activated with plate-bound anti-CD3 and soluble anti-CD28 in the

presence of IL-2 as described (Xue et al., 2002). The bone marrow cells and pre-activated T cells were spinofected with retroviruses expressing either IL-7R α -EGFP fusion reporter or IL-7R α -Stop-IRES-EGFP reporter. Forty-eight hours later, the EGFP expression was determined by flow cytometry.

Building BAC-EGFP reporter transgene construct by recombineering

The PR23-365P6 BAC clone was obtained from Invitrogen, and the BAC DNA was introduced into SW102 cells by electroporation. The IL-7R α -Stop-IRES-EGFP reporter was PCR-amplified along with the targeting arms and targeted into the first exon of IL-7R α gene, and two copies of chicken β -globin insulator sequence were targeted to the 3' end of the BAC DNA. All site-directed recombineering followed the standard procedures as previously published (Liu et al., 2003) and recommended in the "recombineering website" (<http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>). Insertion sites and PCR-amplified sequences were all verified to be free of mutations by DNA sequencing.

Generation of BAC-EGFP reporter transgenic mice

The BAC-EGFP reporter transgene construct was released from the BAC vector by Not I digestion, and separated from the BAC cloning vector backbone by Sepharose 4B-CL chromatography. The integrity of purified BAC DNA was verified by pulse-field electrophoresis. The BAC DNA was resuspended in polyamine buffer and microinjected into (C57BL/6J X SJL/J)F2 fertilized eggs at the concentration of 0.25 ng/ μ l as described (Van Keuren et al., 2009). All the mouse experiments were performed under protocols approved by the Institutional Animal Use and Care Committee of the University of Iowa (Approval ID: 0911245) and the University Committee on the Use and Care of Animals at the University of Michigan (Approval ID: 09325). Mice were housed in an AAALAC accredited facility in accordance with the National Research Council's guide for the care and use of laboratory animals.

Characterization of the BAC-EGFP transgenic founders

PCR was used to detect 5' - and 3' -ends of the BAC transgene as well as the EGFP reporter and SV40 polyA sequence. The primers are 5' -cgggggtaccaccaggcacaagaatgatg and 5' -tgaagattgattggcccttg for detection of the 5' -end, 5' -agggcttcacacagcagat and 5' -actccaggacggagtcagtg for the 3' -end, 5' -ccggaattcgccaccatgatggctctgggtagagct and 5' -aaacgcgtcgacttactgtacagctcgtcca for EGFP cDNA, and 5' -ctttcgacgcgtagatacattgatgagtttg and 5' -ctttcgacgcgctcgactctagatcataatc for poly A. For determination of copy number, genomic DNA was purified from tail tip biopsies of each transgenic mouse founder and digested with EcoRI. The BAC-EGFP transgene plasmids with known copy numbers was processed similarly to set up a copy number standard curve. Southern blotting was performed as previously described (Xue et al., 2004) with a portion of the EGFP cDNA as a probe.

Cell separation, in vitro T cell activation, and flow cytometry

Single cell suspensions from bone marrow cells, thymocytes, lymph nodes, and spleen were prepared as described previously (Yu et al., 2010). For activation of T cells, plate-bound anti-CD3 (5 μ g/ml), soluble anti-CD28 (1 μ g/ml) and IL-2 (100 units/ml) were used as previously described (Xue et al., 2002). The cells were surface stained using fluorochrome-conjugated or biotinylated antibodies (eBioscience or BD Biosciences). The antibodies are CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), NK1.1 (PK136), TCR $\gamma\delta$ (GL3), CD25 (PC61.5), CD44 (IM7), CD3 (145-2C11), Mac-1 (M1/70), CD11c (N418), Gr.1 (RB6-8C5), Ter119 (TER-119), IL-7R α (A7R34), and isotype-matched controls. The stained cells were

analyzed on FACS Calibur or Becton Dickinson LSR II flow cytometers (BD Biosciences). The acquired data analyzed using FlowJo software (Tree Star, Inc.).

Infection with LM-Ova and tracking of antigen-specific T cells

BAC-EGFP reporter transgenic mice and non-transgenic littermates were infected with 5×10^6 attenuated LM-Ova as previously described (Zhao et al., 2010). Ova-specific T cells at effector (day 7 after infection) and memory (>40 days after infection) stages were identified by staining with MHC-I SIINFEKL tetramer (Zhao et al., 2010). In some experiments, the Ova-specific CD8⁺ effector T cells were sorted into IL-7R α ⁻, IL-7R α ⁺GFP⁺, and IL-7R α ⁺GFP⁻ subsets. Total RNA from sorted cells was extracted, reverse-transcribed, and used for quantitative measurements of the *Il7r* transcript following our previously described procedures (Yu et al., 2010). The primers for the *Il7r* transcript are 5'-aaagcatgatgtgcctacc and 5'-gactccactcgtccagaag.

Results and discussion

Characterization of reporter systems

In order to systematically identify critical *cis* regulatory elements in the *Il7r* locus that contribute to its dynamic regulation, we set out for generation of BAC-based reporter transgenic mice. This approach has the great advantage of accurately reporting gene expression *in vivo* in different T cell developmental stages and different phases of the T cell response, and has been successfully used to elucidate gene regulation of Rag proteins (Monroe et al., 1999; Yannoutsos et al., 2004; Yu et al., 1999a; Yu et al., 1999b), Th2 cytokine loci (Lee et al., 2003; Loots et al., 2000), and the interferon (IFN) γ locus (Hatton et al., 2006). Advances in recombination-mediated genetic engineering (recombineering) greatly facilitated manipulation of large genomic DNA segments (usually > 200 kb) cloned in a BAC vector (Copeland et al., 2001; Liu et al., 2003). This approach can achieve efficient site-directed homologous recombination using targeting arms containing as short as 50 bp homologous sequences. We initially intended to insert an enhanced green fluorescent protein (EGFP) reporter immediately after the translation start codon in the first exon of the *Il7r* gene. However, the 50 bp DNA sequence upstream of the *Il7r* "ATG" contains more than 10 copies of "TC" repeats, making it unsuitable for sequence-specific homologous recombination. We therefore used the DNA sequence encoding the first 16 amino acids of IL-7R α as the 5' targeting arm, and 50 bp DNA sequence that is immediate downstream of the 5' arm as the 3' targeting arm. We then tested two reporters: IL-7R α -EGFP fusion reporter that encoded a fusion protein of the first 16 amino acids of IL-7R α and EGFP, and IL-7R α -Stop-IRES-EGFP reporter in which EGFP was translated independently of the IL-7R α sequence via separation by a stop codon and an internal ribosome entry site (IRES) (Fig. 1A). Both EGFP reporters were expressed in transiently transfected 293T cells (Fig. 1B). In contrast, the fusion reporter was not detected in either retrovirally infected bone marrow cells or pre-activated T cells, suggesting that the first 16 amino acids of IL-7R α function as a signal peptide which led to secretion of the reporter protein. As shown in bottom panels in Fig. 1B, the independently translated EGFP reporter was retained in both cell types.

Selection of a BAC clone and design of the BAC reporter transgene construct

For the BAC clone, we chose to use RP23-365P6, which encompasses the entire *Il7r* locus (23.7 kb) and contains 82 kb 5' sequences and 101 kb 3' sequences which harbor a provisional gene locus *Capsl* (Fig. 2A). It is of note that the murine intergenic sequences between the *Il7r* and its upstream gene *Spef2* loci are much longer than those in human. The RP23-365P6 clone does not contain the entire intergenic sequences between the murine *Spef2* and *Il7r* loci, but contains all the conserved sequences corresponding to the human

intergenic region between these two genes. By recombineering, we inserted the IL-7R α -Stop-IRES-EGFP reporter characterized above into exon1 of the *Il7r* gene (Fig. 2B). To avoid chromosomal position effects which may silence transgene expression, we used insulators from the chicken β -globin locus to shield the transgene from nearby regulatory elements (Bell and Felsenfeld, 1999) (Fig. 2B).

Identification of BAC-EGFP reporter transgene founders

The final BAC-EGFP transgene construct after various modifications was approximately 210 kb in length, was released from the BAC vector, purified by size exclusion chromatography, and microinjected into fertilized mouse eggs. PCR genotyping for the EGFP coding sequence identified seven transgenic founder mice. To evaluate the integrity of the transgene in the founders, we genotyped for both 5' and 3' ends of the BAC-EGFP transgene by PCR and found that three out of the seven founders had all the components in the transgene (Fig. 3A). Southern blotting showed that transgene copy numbers in the founders ranged from one to more than eight copies (Fig. 3B). We next analyzed EGFP expression in T cells from peripheral blood leukocytes (PBLs). Only those founders with 4 or more copies of the transgene (#10, #30, and #54) showed substantial EGFP expression in CD4⁺ T cells (Fig. 3C). Among these 3 founders, #10 and #30 had the complete transgene, whereas #54 lacked a portion of the 5' end of the transgene. It is of note that founder #27 had a single copy of the complete transgene, but failed to express EGFP. Possible explanations might include that the single copy of transgene was fragmented when integrated into the mouse genome, or that EGFP expression from a single copy transgene is below the threshold of detection since detection of EGFP does not use fluorescent antibody to amplify the signal, as is the case for IL-7R α . In the following studies, we used #10 and #30 founder-derived progeny and obtained similar results in progeny of both founders.

Fidelity of the BAC-EGFP transgene in reporting endogenous IL-7R α expression

To determine the specificity of BAC-EGFP reporter transgene expression, we measured the expression of both IL-7R α and EGFP reporter in developing thymocytes. Consistent with previous studies (Sudo et al., 1993), IL-7R α was expressed in DN and SP thymocytes but silenced in DP thymocytes in wild-type control mice (Fig. 4A upper panels). The BAC-EGFP reporter transgene did not perturb normal thymocyte development and cellularity (Fig. 4A and 4B far-left panels). In the BAC-EGFP transgenic mice, the EGFP reporter and IL-7R α showed synchronized downregulation in DP and increased expression in both CD4⁺ and CD8⁺ SP thymocytes (Fig. 4A). However, in DN thymocytes, only a portion of IL-7R α ⁺ cells expressed EGFP (Fig. 4A). The DN cells can be further divided into 4 sequential developmental stages marked by distinct expression of CD25 and CD44. IL-7R α is highly expressed in DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), and DN3 (CD44⁻CD25⁺) subsets, and starts to decline in DN4 (CD44⁻CD25⁻) thymocytes (Fig. 4B upper panels). In the BAC-EGFP transgenic mice, however, EGFP reporter exhibited synchronized expression with IL-7R α in DN1 subset only, with only a small portion of IL-7R α ⁺ DN2–4 thymocytes expressing the reporter (Fig. 4B lower panels). There might be several possible explanations, for example, the transgene might be silenced in DN thymocytes due to gene variegation, or DN thymocytes might have limited protein factors to support transcription from multiple copies of transgenes, or alternatively positive regulatory cis-elements for IL-7R α expression in DN thymocytes might be absent from the BAC-EGFP transgene. Nevertheless, the 210 kb BAC-EGFP transgene faithfully reported IL-7R α regulation in DP and SP thymocytes.

In addition to $\alpha\beta$ T cells, the thymocytes contain natural killer T (NK-T) and $\gamma\delta$ T cells, and both subsets express CD3 on the cell surface. NK-T cells express semi-invariant TCR recognizing glycolipid antigens presented by the non-polymorphic MHC class I-related protein, CD1d (Matsuda et al., 2008). Consistent with a previous report (Lee et al., 2010),

most of the NK-T cell expressed high levels of IL-7R α , and the EGFP reporter showed similarly high expression in these cells in EGFP-BAC transgenic mice (Fig. 4C). $\gamma\delta$ T cells have limited repertoires of $\gamma\delta$ TCRs, which recognize both MHC-related and -unrelated molecules (Bonneville et al., 2010). In spite of critical dependence of $\gamma\delta$ T cell development on IL-7-derived signals, IL-7R α is only expressed in a subset of $\gamma\delta$ T cells (Fujihashi et al., 1996) (Fig. 4D). In the EGFP-BAC transgenic mice, the EGFP reporter was detected in a substantial portion of IL-7R α ⁺ $\gamma\delta$ T cells (Fig. 4D). These results indicate that the BAC-based reporter transgene mirrored IL-7R α expression in non-conventional thymic subsets as well.

Similar to thymic CD4⁺ and CD8⁺ single positive thymocytes, the mature CD4⁺ and CD8⁺ T lymphocytes in the spleen and lymph nodes expressed high levels of the EGFP reporter, exhibiting synchronized expression with endogenous IL-7R α (Fig. 5A and 5B). A subset of natural killer (NK) cells expressed IL-7R α , and the same subset exhibited EGFP expression in the BAC transgenic mice (Fig. 5A). For the B-lineage cells, IL-7R α is expressed in the most immature pro- and pre-B cells in the bone marrow, but switched off in immature B cells in the bone marrow as well as mature B cells in the spleen. Analogous to the situation in DN2–4 thymocytes, the EGFP reporter was detected in only a small portion of IL-7R α ⁺ pro- and pre-B cells (data not shown), which may be explained by similar reasons discussed above. The EGFP reporter was not detected in splenic mature B cells either (Fig. 5A), indicating that the BAC transgene did not result in ectopic expression of IL-7R α in inappropriate lineages. These data collectively indicate that the expression of the BAC-EGFP reporter transgene coincides with endogenous IL-7R α expression in mature lymphocytes in periphery.

IL-7R α is also dynamically regulated in T cells during cellular immune responses. Upon activation in vitro by TCR crosslinking, both CD4⁺ and CD8⁺ cells rapidly downregulate IL-7R α expression (Xue et al., 2002). To test the fidelity of EGFP reporter in this process, we activated T cells isolated from lymph nodes with plate-bound anti-CD3 in the presence of soluble anti-CD28 and IL-2. Within 24 hrs after stimulation, IL-7R α expression was down-regulated in most of CD4⁺ and CD8⁺ cells and remained low during prolonged culture (Fig. 5B). However, the downregulation of the EGFP reporter was only more evident at 48 hrs after activation. Similar results were obtained when splenic T cells were tested or when phorbol 12-myristate 13-acetate and ionomycin were used in lieu of anti-CD3/CD28 to activate the T cells (data not shown). The delayed downregulation of EGFP is likely due to the reported slow turnover of GFP protein (Corish and Tyler-Smith, 1999), which has been observed in other studies using GFP as a reporter (Muroi et al., 2008). This observation highlights the necessity in utilizing destabilized reporters with shorter half-life to monitor dynamically regulated proteins such as IL-7R α .

To further demonstrate the fidelity of EGFP reporter during immune responses in vivo, we immunized BAC-EGFP transgenic mice and littermate controls with *Listeria monocytogenes* expressing chicken ovalbumin (LM-Ova) and tracked Ova-specific CD8⁺ T cells with the MHC-I SIINFEKL tetramer. Whereas >90% naïve CD8⁺ T cells expressed IL-7R α (Fig. 4), most of the antigen-specific effector T cells (7 days after infection) downregulated both IL-7R α and EGFP expression (Fig. 6A). The tetramer-negative CD8⁺ T cells contained bystander naïve CD8⁺ T cells as well as effector T cells responding to other LM epitopes, and the latter likely downregulated IL-7R α expression. Interestingly, most of the IL-7R α ⁺ tetramer-negative CD8⁺ T cells expressed the EGFP reporter (Fig. 6A). It is of note that among the tetramer-positive IL-7R α ⁺ effector CD8⁺ T cells, only a portion of them remained positive for EGFP. To determine if the reporter positivity resulted from difference in the *Il7r* gene transcription, we sorted the tetramer positive CD8⁺ effector T cells into IL-7R α ⁺, IL-7R α ⁺GFP⁺, and IL-7R α ⁺GFP[−] subsets and measured *Il7r* transcript levels by

quantitative RT-PCR. Consistent with the cell surface IL-7R α expression levels, the *Il7r* transcript in IL-7R α ⁻ effector T cells was measured to be $0.7 \pm 0.5\%$ of a housekeeping gene *Hprt1*. In contrast, the *Il7r* transcript in IL-7R α ⁺GFP⁺ and IL-7R α ⁺GFP⁻ effector T cells was $116 \pm 33\%$ and $87 \pm 32\%$ of the *Hprt1* transcript, respectively. Although the GFP⁺ cells contained higher *Il7r* transcripts than the GFP⁻ cells, their difference was not statistically significant ($p = 0.25$, $n = 3$). Thus, the EGFP reporter appeared to be more sensitive to TCR-mediated downregulation than the endogenous IL-7R α at the effector phase, which was probably explained by that the GFP fluorescent intensity is influenced by the inflammatory cytokine milieu at the height of cellular immune responses.

Following the acute phase of infection, a small portion of effector T cells that survive contraction become memory T cells, which upregulated IL-7R α expression. After >40 days of infection, Ova-specific CD8⁺ T cells were detected at a lower frequency compared with the effector phase (Fig. 6B). Most of the tetramer-positive memory T cells restored IL-7R α expression, and in the BAC transgenic mice, these cells also upregulated EGFP reporter expression (Fig. 6B). These results indicate that the BAC-EGFP transgene reports IL-7R α expression with high fidelity during T cell responses.

Summary and implications

In this study, we describe a strategy to generate a BAC-EGFP reporter transgenic mouse strain to monitor dynamic regulation of IL-7R α in vivo. The BAC transgene exhibited high fidelity in reporting IL-7R α expression in DP and SP thymocytes and during mature T cell responses. These observations indicate that all the critical *cis* regulatory elements responsible for dynamic IL-7R α regulation in these biological processes are embedded within the 210 kb sequence flanking the *Il7r* locus. Our study thus constitutes a critical initial step for systematic identification of the *cis* elements and their interacting protein factors. It would be important to determine which of these elements/factors are utilized specifically in developing thymocytes and antigen-responding mature T cells, and which are common regulatory modules in these cells under different developmental/activation stages. Design of future BAC transgenic constructs can be based on cross-species sequence conservation and genome-wide mapping of DNase I hypersensitivity sites in T lineage cells. It is of note that within the 210 kb BAC sequence used in this study, a total of 20 DNase I hypersensitivity sites were identified in human T cells, and 8 of them exhibited perfect overlap with highly conserved sequences (Christopher Wilson, personal communication). Cross-species comparison of conserved non-coding sequences may identify more putative regulatory regions (Boffelli et al., 2004). Incorporation of additional genome-wide information such as histone modification state (Wei et al., 2009) and transcription factor binding (ENCyclopedia of DNA Elements/ENCODE project) (ENCODE Project Consortium, 2004) should also assist the construct design for expeditious mapping of functionally important *cis* regulatory elements.

Molecular dissection of how IL-7R α is dynamically regulated is not only important for in-depth understanding of this critical molecule and related biological processes but also likely provide mechanistic insights into the regulation of other genes showing synchronized expression with IL-7R α . For example, during T cell development, *Deltex1*, a Notch response gene, is expressed in DN, completely extinguished in DP, and re-expressed at high levels on CD4⁺ or CD8⁺ SP thymocytes (Deftos et al., 2000). During T cell responses, the key lymph node-homing receptors CD62L (L-selectin) and CCR7 (Sinclair et al., 2008), Wnt pathway effector transcription factors TCF1 (T cell factor 1) and LEF1 (lymphoid enhancer factor 1) (Zhao et al., 2010) are all expressed in naïve T cells, but are dramatically downregulated in effector T cells, and re-expressed in memory T cells. Our working hypothesis is that at least a subset if not all of the genes showing synchronized expression

changes with IL-7R α may be governed by a shared molecular mechanism to meet functional requirements during T cell development and activation. With massive mouse genome and gene expression array data now available, bioinformatics approaches can aid in the identification of DNA sequences in other gene loci that show high conservation with the *cis* regulatory elements in the *Il7r* locus. Thus, the *Il7r* gene can serve as a “model locus” in search of a unified molecular explanation underlying synchronized expression changes of a group of genes during T cell development and T cell-mediated immune responses.

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Figure 1. Characterization of EGFP reporters

A) Schematic showing the design of the constructs encoding two different reporters. B) Expression of EGFP in different cell types. The reporters were cloned in a retroviral vector where the reporter expression was driven by viral long terminal repeats (LTRs). 293T cells were directly transfected with each reporter, and bone marrow (BM) and pre-activated T cells were infected with retroviruses expressing different reporters. Forty-eight hours after transfection or retroviral infection, EGFP expression was detected by flow cytometry. Shown are the percentages of the EGFP-positive subsets among viable cells (based on the combination of forward and side scatter). Data are representative from two independent experiments.

Figure 2. Design of BAC-EGFP reporter transgene

A) Relative coverage of BAC clones encompassing the *Il7r* locus. The relative locations of *Il7r* and its neighboring gene loci were aligned in the human and mouse genomes. The orientation of gene transcription is shown by arrows, and the length of each arrow is proportional to the size of the corresponding gene locus. The coverage of each BAC clone is shown. B) Design of the BAC-EGFP transgene construct based on the RP23-365P6 BAC clone. The exons in the *Il7r* and *Capsl* loci are shown as vertical lines. The IL-7R α -Stop-IRES-EGFP reporter was inserted into exon 1 of the *Il7r* gene and 2 copies of the chicken β -globin insulator sequence were inserted at the 3'-end of the BAC clone by recombineering. In the initial design of the construct, LoxP, LoxN, and Frt sites (represented by triangles in different colors), were inserted in the indicated locations (marked by vertical arrows). These sites were inserted to facilitate the production of transgenic mouse substrains with internal deletions after mating founders with Cre or FLP recombinase transgenic mice. These studies were not implemented because EGFP expression required more than one transgene copy and the number of possible recombination products after the action of recombinases on multi-copy transgene concatamers would interfere with data interpretation. Lox and Frt sites were targeted to the least conserved regions between human and mice, and the sites did not perturb the fidelity of EGFP expression based on functional analysis of BAC-EGFP transgenic founders and progeny (detailed in following figures).

Figure 3. Characterization of BAC-EGFP reporter transgene founders

A) Integrity of the BAC-EGFP transgene in different founders. PCR primers were designed to detect the presence of 5'- and 3'-ends of the BAC transgene in addition to the EGFP coding and SV40 polyA sequences. The primers for the 5'-end detection used T7 primer derived from the BAC vector backbone, which remained in the 5'-end of transgene construct after NotI digestion, and another primer complementary to the BAC sequence. The primers for the 3'-end detection included one primer complementary to the insulator and the other to the BAC sequence. Copy numbers and EGFP reporter activity are also summarized in this table. B) Copy numbers of the BAC-EGFP reporter transgene. Genomic DNA from each founder was analyzed by Southern blotting with a portion of EGFP cDNA as probe. The

BAC-EGFP transgene plasmid was used to set up a copy number standard curve. C)
Detection of EGFP reporter activity in T cells. PBLs from each founder were isolated,
stained for CD4 and analyzed on a flow cytometer. Percentages of EGFP⁺ in gated CD4⁺
subset are shown.

Figure 4. Expression of the BAC-EGFP reporter transgene in conventional and non-conventional T cell subsets in the thymus

(A) Detection of the EGFP reporter in developing $\alpha\beta$ T cells. Thymocytes were isolated from the BAC-EGFP reporter transgenic mice and littermate controls and surface stained with CD4, CD8 and IL-7R α . The pattern of CD4 and CD8 staining of thymocytes is shown on the left, and that of IL-7R α and EGFP reporter expression in each thymocyte subset shown on the right. Gating for IL-7R α -positive populations was based on staining with isotype control (not shown). The percentages of different subsets are shown, and data are representative from three independent experiments. Coexpression of IL-7R α and EGFP in CD4 $^{+}$ and CD8 $^{+}$ single positive thymocytes was consistently observed in all BAC transgenic

mice tested, with IL-7R α - and EGFP-double positive cells constituting $63.8 \pm 5\%$ of CD4 $^{+}$ and $67.5 \pm 3.8\%$ in CD8 $^{+}$ T cells ($n = 8$). (B) Detection of the EGFP reporter in DN thymocytes. Total thymocytes were first labeled with biotinylated antibodies against CD3, CD4, CD8, B220, NK1.1, TCR $\gamma\delta$, Mac-1, Gr.1, CD11c, and Ter119 to exclude all known differentiated blood lineages. The cells were then stained with fluorochrome-linked streptavidin, IL-7R α , CD25 and CD44 antibodies. Fractionation of DN1–4 subsets is shown on the left, and the expression of IL-7R α and EGFP reporter in each subset shown on the right. Gating for IL-7R α -positive populations was based on staining with isotype control (not shown). (C) EGFP expression in NK-T cells. (D) EGFP expression in $\gamma\delta$ T cells. Total thymocytes were stained with IL-7R α , CD3, and NK1.1 or TCR $\gamma\delta$ antibodies. The expression of IL-7R α and EGFP reporter was determined in CD3 $^{+}$ NK1.1 $^{+}$ and CD3 $^{+}$ TCR $\gamma\delta$ $^{+}$ subsets, respectively. The percentages of different subsets are marked and representative data are shown ($n = 3$ for B-D). For Figs. 4–6, similar results were obtained from progeny of both #10 and #30 transgenic founders, and representative data from progeny of #30 are shown.

Figure 5. The expression of BAC-EGFP reporter transgene in peripheral lymphocytes
 (A) Lineage-specific expression of the EGFP reporter. Splenocytes were isolated from BAC-EGFP reporter transgenic mice and littermate controls and surface stained with lineage markers and IL-7R α . In each specified lineage, gating for IL-7R α -positive populations was based on staining with isotype control (not shown). The percentages of IL-7R α ⁺, GFP⁺, and IL-7R α ⁺GFP⁺ subsets in each cell lineage are shown. Note that a subset of isotype control-stained cells fell in IL-7R α -positive gate (not shown). Similarly, the small subset of cells that fell in GFP-positive gate in non-transgenic mice should be considered to reflect background expression rather than being true-positive. Representative data from two independent experiments are shown. Coexpression of IL-7R α and EGFP in CD4⁺T, CD8⁺

T, and NK cells was consistently observed in all BAC- transgenic mice tested, with IL-7R α - and EGFP-double positive cells constituting 65.1 ± 9.7 % of CD4 $^{+}$ T, 79 ± 8.5 % in CD8 $^{+}$ T, and 10.4 ± 3.1 % in NK cells (n = 6). (B) The expression of EGFP reporter in T cells primed *in vitro*. CD4 $^{+}$ and CD8 $^{+}$ T cells from lymph nodes were activated with plate-bound anti-CD3 in the presence of anti-CD28 and IL-2. The cells were collected prior to, 24 hrs or 48 hrs after the stimulation, and the expression of EGFP and endogenous IL-7R α was determined. The percentages of different subsets are marked, and representative data are shown (n = 3).

Figure 6. Expression of the BAC-EGFP reporter transgene in antigen-specific CD8⁺ T cells during cellular immune responses
 BAC-EGFP reporter transgenic mice and littermate controls were infected with LM-Ova. Antigen-specific effector (A) and memory (B) CD8⁺ T cells were identified by the combination of CD8 and MHC-I SIINFEKL tetramer staining. The expression of both IL-7Rα and EGFP reporter for tetramer-positive and -negative CD8⁺ subsets was determined. Representative data from three independent experiments are shown. For tetramer-positive CD8⁺ effector T cells in the BAC transgenic mice (A), the frequency of GFP⁺IL-7Rα⁺ was detected at $7.4 \pm 2.6\%$, whereas GFP⁻IL-7Rα⁺ at $29.2 \pm 8\%$ ($n = 7$). For

tetramer-positive CD8⁺ memory T cells in the BAC transgenic mice (B), the frequency of GFP⁺IL-7R α ⁺ was detected at $64.8 \pm 11.5\%$, whereas GFP⁻IL-7R α ⁺ at $24 \pm 6.4\%$ ($n = 6$).