Expression of v-src induces a myeloproliferative disease in bone-marrow-reconstituted mice

Gordon Keller$^{1,3}$ and Erwin F. Wagner$^{2,3}$

$^1$Basel Institute for Immunology, CH-4005 Basel, Switzerland; $^2$European Molecular Biology Laboratory, D-6900 Heidelberg, FRG; and $^3$Research Institute of Molecular Pathology, A-1030 Vienna, Austria

A recombinant retrovirus, N-TK-src, was used to introduce the v-src oncogene into mouse hematopoietic cells. This vector efficiently expresses both the neo and v-src genes in different hematopoietic lineages in culture as well as in mice reconstituted with infected bone marrow cells. Expression of v-src had no dramatic effect on the proliferative and differentiative capacity of hematopoietic precursors when assayed in methyl cellulose cultures. However, in mice reconstituted with N-TK-src-infected bone marrow cells, expression of v-src leads to the rapid development of a severe myeloproliferative disease, characterized by splenomegaly, anemia, and a shift of hematopoiesis from the bone marrow to the spleen.

[Key Words: v-src; N-TK-src; myeloproliferative disease; hematopoiesis]

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The hematopoietic system consists of clones of developing blood cells that are derived from primitive multipotential stem cells [Wu et al. 1968; Abramson et al. 1977; Dick et al. 1985; Keller et al. 1985; Keller and Wagner 1986; Lemischka et al. 1986]. Development of mature blood cells from these primitive stem cells is thought to occur in a stepwise fashion involving the generation of intermediate-stage precursor cells, whose differentiation potential is more restricted than that of the stem cell. The most mature cells within this precursor pool are those committed to a single blood cell lineage. The events involved in the differentiation of a multipotential cell to one that is committed to a specific lineage are poorly understood. This lack of knowledge, in part, from the fact that the most immature cells within the system are found at an extremely low frequency in normal hematopoietic tissues [Boggs et al. 1982; Metcalf 1984; Harrison et al. 1988]. Methods for purifying stem cells are improving, however they tend to be long and tedious and the number of cells obtained are, as a rule, too low to allow their biochemical characterization [Müller-Sieburg et al. 1986; Spangrude et al. 1988].

An alternative approach toward gaining some insight into the sequence of events involved in this complex developmental program is to disrupt the system through the expression of specific oncogenes. One of the goals of these experiments would be to arrest the development of hematopoietic precursors and thereby expand their number as has been demonstrated to occur in the avian system [Beug et al. 1985; Khazaie et al. 1988]. Expression of various oncogenes could also interfere with the function of the cell populations involved in the regulation of hematopoietic development. This disruption in function could lead to a better understanding of the role played by the regulatory cells in normal hematopoiesis. Recently, a considerable body of evidence has accumulated indicating that the v-src oncogene can alter the development of hematopoietic cells in vivo as well as in vitro. It has been reported that mice infected with a retrovirus that expresses the v-src oncogene develop an erythropoietic disease characterized by increased spleen size and anemia [Anderson and Scolnick 1983]. In culture, expression of this oncogene appears to have a wide variety of effects on mouse hematopoietic cells. These include transformation of erythroid precursors [Anderson et al. 1985], transformation of cells early in the lymphoid lineage [Pierce et al. 1984], immortalization of pluripotential precursors [Spooner et al. 1984, 1986; Wyke et al. 1986], and induction of factor independence in interleukin-3 (IL-3)-dependent cell lines [Overell et al. 1987; Watson et al. 1987]. In the avian system, it has been shown that v-src can transform erythroid precursors in vitro and in vivo [Kahn et al. 1984; Palmieri 1985] and can render growth-factor-dependent myeloblasts independent of exogenous factor [Adkins et al. 1984].

On the basis of these observations, we were interested in determining what effect expression of v-src would have on various hematopoietic precursors, including the most primitive stem cells, when assayed in culture or in irradiated, reconstituted mice. To introduce the v-src oncogene efficiently into different hematopoietic precursors, we constructed a recombinant retrovirus, N-TK-src, which expresses both the neo and the v-src genes. The approach we took to analyze the effects of v-src expression differed in several ways from those described above. In vitro, we used a culture system that...
supports the clonal growth of precursors from all the myeloid lineages. This allowed us easily to detect an effect on many hematopoietic lineages in culture. For the in vivo analysis, we first infected bone marrow cells with N-TK-src in culture and then used these cells to reconstitute irradiated mice. Using this protocol, it has been shown that one can introduce genes into primitive multipotential stem cells and that these genes are expressed in the progeny of these cells for long periods of time (Dick et al. 1985; Keller et al. 1985; Keller and Wagner 1986; Lemischka et al. 1986; Snodgrass and Keller 1987; Dzierzak et al. 1988). In this paper, we demonstrate the efficient expression of v-src in hematopoietic precursors and describe the consequences of this expression in bone-marrow-reconstituted mice.

Results

Construction and characterization of a retroviral vector expressing v-src

The aim was to derive a replication-defective retrovirus capable of constitutive expression of the v-src gene in all lineages of the hematopoietic system. To this end we have constructed a vector pN-TK-src in which the v-src gene from SR-RSV (Schmidt-Ruppin) strain is transcribed from the thymidine kinase (TK) promoter and the selectable neomycin (neo) gene from the viral 5’ long terminal repeat [LTR] (Fig. 1). The TK promoter was used, as it has been shown to function in a wide variety of cell types (Stewart et al. 1987). Helper-free stocks of replication-defective virus were obtained by transfection of pN-TK-src onto the 62 packaging cell line (Mann et al. 1983) followed by selection in G418. Infection of NIH-3T3 fibroblasts leads to efficient expression of both the neo and v-src genes, resulting in G418 resistance and foci of transformed cells with comparable efficiency [titers of 1.0 x 10^6 to 2 x 10^6 colony-forming units (CFU/ml).

Expression of v-src in hematopoietic precursors in culture

To determine whether v-src was expressed in the different hematopoietic precursors in culture, G418-resistant (G418R colonies, generated from N-TK-src infected bone marrow cells [from 5-fluorouracil [5-FU]-treated mice] were analyzed for the presence of pp60src kinase activity. Colonies grown from precursors infected with N2, a virus that expresses only the neo gene [Keller et al. 1985 and Fig. 1], were used as controls. To obtain enough cells for the kinase assay, colonies containing cells of the same lineages were pooled. Figure 2 demonstrates that colonies consisting of neutrophils and macrophages (GM), erythroid cells (E), and multiple myeloid lineages (MIX) all contain readily detectable levels of pp60src-specific kinase activity. Colonies from precursors infected with the control N2 virus contained no kinase activity. This finding indicates that v-src is expressed in the different myeloid lineages in culture. Because G418R colonies were used in the kinase assay, this experiment also shows that both the neo gene and the v-src oncogene can be expressed efficiently with the same retroviral vector in hematopoietic cells in culture.

To determine what possible effect the expression of v-src might have on the proliferative and differentiation potential of the various precursors, colonies derived from them were picked and analyzed for aberrant cell types as well as for enhanced replating potential. In addition, precursors expressing the v-src gene were plated in methyl cellulose cultures that contained no growth factors [minus IL-3, erythropoietin, and human bladder carcinoma cell line 5637 supernatant] to determine whether expression of v-src alone could render them factor independent. In three separate experiments, we analyzed a total of 400 colonies and were unable to demonstrate any difference in the growth requirements of precursors infected with N-TK-src compared with those infected with N2. In addition, colonies derived from precursors infected with this virus did not differ with respect to size, replating potential, or the state of differentiation of the cells they contained when compared with colonies derived from precursors infected with the control virus [data not shown].

Figure 1. Schematic presentation of the retroviral vectors N2 and N-TK-src.

Figure 2. In vitro IgH phosphorylation assay of pp60 v-src tyrosine kinase activity in cells from erythroid (E), mixed (MIX), and granulocyte/macrophage (GM) colonies, derived from bone marrow cells infected with either N-TK-src or the control N2 virus. [3T3] Fibroblasts infected with either N-TK-src or N2.
It has been reported that in vitro infection of spleen and bone marrow cells from phenylhydrazine (PHZ)-treated mice with a retrovirus that expresses the v-src gene leads to an enhanced growth of erythroid precursors when these cells are plated in the methyl cellulose colony assay (Anderson et al. 1985). Because we were unable to demonstrate any effect of v-src on erythroid precursors from 5-FU-treated mice, we were interested in determining whether infection with N-TK-src would affect the development of these cells from PHZ-treated mice. Two points are worth mentioning regarding this experiment. First, large numbers of erythroid colonies developed in cultures of spleen or bone marrow cells in the presence of erythropoietin (Ep), regardless of whether they were infected with N-TK-src or N2. This made it difficult to demonstrate any enhanced growth of erythroid colonies related to the expression of v-src. Second, G418® erythroid colonies that did develop in the N-TK-src-infected cultures were not different from those found in the N2 cultures, with respect to size, replating potential, or the state of differentiation of the cells they contained. From these experiments, we conclude that v-src, when expressed within the N-TK-src virus, is unable to alter the growth and differentiation of erythroid precursors in short-term methyl cellulose cultures.

**Expression of v-src in reconstituted mice**

The previous series of experiments indicated that v-src was expressed in the different hematopoietic lineages in culture. However, we were unable to demonstrate any effect of this expression on the growth and differentiation of the various precursor cells. In the next experiments, we infected bone marrow cells with the N-TK-src and then used this marrow to reconstitute irradiated mice. Control mice were reconstituted with N2-infected bone marrow cells. This protocol allows one to determine the effect of v-src expression on a broad spectrum of cells, including the most primitive multipotent stem cells, which are not detected by the in vitro colony assay.

To determine whether v-src was expressed in the reconstituted recipients, we first analyzed the hematopoietic tissues from some of the animals for the presence of v-src-specific mRNA. Figure 3 shows that the spleens from mice src-1 and src-3 and the bone marrow and spleen from mouse src-4 all contain two transcripts that hybridize to a v-src-specific probe. The larger one of 6.1 kb is the predicted size of a transcript initiated at the 5′ LTR, whereas the smaller one of 2.8 kb is the predicted size of a transcript initiated from the internal TK promoter. This finding would indicate that both the LTR and the TK promoter function in hematopoietic cells in vivo.

Having detected v-src-specific mRNA in these recipients, we were next interested in determining whether or not the protein, pp60v-src kinase, could also be detected in the various tissues as well as in isolated cell populations derived from reconstituted mice. Figure 4 shows that kinase activity was present in the spleen, bone marrow, spleen-derived T cells, and bone-marrow-derived macrophages of all recipients analyzed (src-16, src-18, src-19, src-20, src-23). Interestingly, the thymus from two of the recipients (src-16 and src-18) showed very low levels of activity. The reason for this is presently unclear because their hematopoietic tissues contained levels of kinase activity comparable to those from the other recipients. From mouse src-23, peripheral blood was analyzed for kinase activity at week 10 [PB1] and week 13 [PB2] following reconstitution. Activity could not be detected at week 10, but was present at week 13. Kinase activity could also be detected in the spleen of this mouse at weeks 10 and 13, as well as in spleen cells (adherent and nonadherent) that were cultured for 10 days. These findings demonstrate that v-src is expressed efficiently in different tissues and cell populations from mice reconstituted with N-TK-src-infected bone marrow cells.

**Is v-src present in multipotent stem cells?**

To determine whether multipotent stem cells had been infected with the N-TK-src virus, DNA from the various tissues and cell populations from the different mice was analyzed for unique viral integration sites. Macrophages (MAC) and T cells (T) grown in culture were used to represent the myeloid and lymphoid lineages, respectively. In all recipients analyzed, it is possible to identify common viral integrations in both the myeloid and lymphoid lineages, indicating that a multipotential cell had been infected [Fig. 5]. For example, in

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**Figure 3.** Northern analysis of mRNA from hematopoietic tissues of mice reconstituted with N-TK-src-infected bone marrow cells. (Lanes src1, src3, and src4) 3 µg of poly[A]+ RNA was loaded per lane. (SPL) Spleen; (BM) bone marrow; (MC) IL-3-dependent mast cells derived from infected BM cells cultured in vitro. Mice src-1 and src-3 were analyzed at week 6 and mouse src-4 at week 10 following reconstitution.
mouse src-16, the same band is found in DNA from the spleen, macrophages, and thymus, and in mouse src-19, at least two common bands are present in the DNA from the spleen, thymus, macrophages, and T cells. The lane on the right side of the tissues from mice src-16, src-18, and src-19 (labeled MAC, MAC, and SPL, respectively), represents DNA digested with SacI, an enzyme that cleaves once in each of the LTRs. The band at 6.1 kb is the predicted size of the N-TK-src provirus, indicating that there have been no significant rearrangements within the retroviral vector in the reconstituted animals. DNA from the spleens of mice src-16, src-19, src-20, and src-23 and from the thymus of mice src-19 and src-20 (male mice reconstituted with female bone

Figure 5. Lineage analysis of five long-term reconstituted mice (src16, src18, src19, src22, and src23). High-molecular-weight DNA was isolated from various tissues and purified cell populations and digested with BamHI, which does not cut the proviral genome. Southern blots were hybridized with a neo-specific probe. Abbreviations of tissues as in Fig. 4. Lanes marked 6.1 are DNAs digested with SacI showing the intactness of the proviral genome.
marrow cells for 7–13 weeks) also was hybridized with a Y-chromosome-specific probe to estimate the relative proportion of host- and donor-derived cells. On the basis of the intensity of the hybridizing bands relative to male control DNA, we calculated the proportion of donor cells in the spleens of these recipients to be as follows: src-16, 95%; src-19, 98%; src-20, 90%; and src-23, 98% [data not shown]. The cells within the thymus of src-19 and src-20 were also found to be predominantly of donor origin (>95%).

### Biological effects of v-src expression in vivo

Unlike the effects in culture, expression of v-src in reconstituted mice had a dramatic effect. Within 6–10 weeks of reconstitution some recipients began to show signs of weight loss and had a ruffled appearance. From this stage, they deteriorated rapidly and died within a few days. Within a matter of several weeks, virtually all recipients followed a similar clinical course and most were dead within 15 weeks of reconstitution. Upon analysis it was found that almost all recipients had enlarged spleens (2- to 10-fold larger than controls) and all were anemic. Many recipients had no detectable thymus and only small lymph nodes. The bone marrow of these animals appeared very white, compared with that of control mice, and in some instances, the marrow plugs were very difficult to remove and dissociate. Morphological and histochemical analysis revealed that the spleens of these recipients consisted predominantly of hematopoietic cells, the majority being from the erythroid (usually >50% of the population) and neutrophil lineages (15–50%). All stages of development, including primitive blasts, could be identified, indicating that the spleens of these recipients had become a major site of hematopoiesis [Fig. 6]. Very few cells with a lymphoid morphology could be identified. The makeup of the cell population within the bone marrow was, in general, not altered as dramatically as that of the spleen. However, there was some variation in the frequency of cells from the erythroid lineage in the marrow of some of the animals and in two there appeared to be a preponderance of cells from the monocyte/macrophage lineage. The peripheral blood of these mice also showed abnormalities and basically reflected the situation found in the spleen, namely an increase in the frequency of immature cells of the erythroid and neutrophil lineages [Fig. 6]. Hematopoietic cells were also found in the lymph nodes and livers, indicating that there was significant extramedullary hematopoiesis in these animals.

### In vitro analysis of precursors from reconstituted mice

When cells from the spleens of these recipients were cultured in the methyl cellulose colony assay it was found that the frequency of all myeloid precursors was increased, compared with control animals (Table 1). The frequency of total precursors in the spleens of mice reconstituted with N-TK-src-infected bone marrow cells ranged from 110 to 2000 per 10^6 cells plated compared

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**Figure 6.** Photomicrograph of cells from: (A) src-16 spleen; (B) src-16 peripheral blood; (C) C-8 spleen; (D) C-8 peripheral blood. Note the presence of blast cells and developing erythroid cells in the spleen and peripheral blood of mouse src-16. In contrast, the majority of cells found in the spleen of mouse C-8 had a lymphoid morphology. (Magnification, ×1000; stain, May-Grunwald-Giemsa).
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Table 1. Frequency of colony-forming cells in the spleens of reconstituted mice

| Mouse | Time of reconstitution (weeks) | Colony-forming cells per 10^6 spleen cells |
|-------|-------------------------------|--------------------------------------------|
|       | CFU-E | E | MIX | GM/MAST | Total | CFU-B |
| src-4 | 10    | 2.3 × 10^8 | ND  | 115     | 80    | 825   | 1020 (33) | ND      |
| src-16| 7     | 10 × 10^9  | 4400| 17       | 20    | 73    | 110 (60)  | 960     |
| src-18| 9     | 4.0 × 10^9  | 3100| 290      | 100   | 1010  | 1400 (60) | 16000   |
| src-19| 10    | 8.6 × 10^9  | 1600| 150      | 50    | 680   | 880 (70)  | 2050    |
| src-20| 10    | 4.6 × 10^9  | 1400| 110      | 90    | 800   | 1000 (70) | 3500    |
| src-25| 6     | 2.0 × 10^9  | 1700| 100      | 10    | 240   | 350 (53)  | ND      |
| src-27| 10    | 3.5 × 10^9  | 4240| 345      | 5     | 650   | 1000 (43) | ND      |
| src-30| 12    | 1.0 × 10^9  | 930 | 870      | 30    | 1130  | 2030 (12) | ND      |
| src-31| 15    | 3.2 × 10^9  | ND  | 140      | 20    | 250   | 410 (9)   | ND      |
| src-4 | 7     | 1.0 × 10^9  | 900 | 60       | 20    | 310   | 390 (14)  | 11300 (6) |
| src-19| 10    | 1.4 × 10^9  | 260 | 26       | 8     | 81    | 115 (66)  | 46000 (8) |
| src-10| 10    | 1.5 × 10^9  | 20  | 7        | 3     | 28    | 38 (44)   | 9200 (17) |
| src-12| 12    | 5.6 × 10^7  | ND  | 180      | 20    | 155   | 355 (30)  | ND      |
| src-31| 12    | 5.0 × 10^7  | 400 | 87       | 15    | 203   | 305 (51)  | ND      |

* Total number of nucleated cells per spleen.

b E refers to those colonies that contain only erythroid cells. They differ from CFU-E-derived colonies in that they are much larger and arise much later (7–9 days) in culture.

c MIX are colonies consisting of erythroid cells plus cells from at least two other lineages.

d GM/MAST includes pure neutrophil, pure macrophage, mixed neutrophil and macrophage, and pure mast-cell colonies.

e Total does not include CFU-E nor CUF-B.

f Colonies consisting of B lymphocytes and ranging in size from 50 to 500 cells.

g Numbers in parentheses represent the percentage of total precursors that were G418³.

[ND] Not determined.

with a frequency of 38–390 for the same number of cells plated from spleens of control mice. Many of the precursors from the spleen were G418³, indicating that the neo³ gene is being expressed.

In contrast to cells of the myeloid lineages, the frequency of cells from the lymphoid lineages detected as clonable B cells (CFU-B) and surface Ig- and Thy-1-positive cells, was reduced dramatically. In fact, in most recipients there were almost no detectable B or T cells in the spleen, as determined by fluorescent staining (data not shown).

The bone marrow from many of these mice was hypocellular and contained relatively few precursor cells, indicating a general shift of hematopoietic activity from the marrow to the spleen (Table 2). The shift also included an increase in the frequency of precursors found in the peripheral blood, as shown in Table 3. Although the frequency of precursors in the different tissues was altered dramatically by v-src expression, the size of the total precursor population was not changed significantly (data not shown). The myeloid precursors found in the spleen, bone marrow, and peripheral blood of these mice appeared normal. The myeloid precursors found in the spleen, bone marrow, and peripheral blood of these mice contained normal differentiated blood cells and showed no increased replating activity compared with controls.

Spleen cells from both experimental and control mice

Table 2. Frequency of colony-forming cells in the bone marrow of reconstituted mice

| Mouse | CFU-E | E | MIX | GM/MAST | Total |
|-------|-------|---|-----|---------|-------|
| src-4 | ND    | 20| 30  | 440     | 490 (20) |
| src-16| 1750  | 90| 10  | 660     | 770 (14) |
| src-18| 1880  | 330| 100 | 1920    | 2350 (41) |
| src-19| 790   | 120| 50  | 730     | 900 (60) |
| src-20| 2300  | 450| 220 | 2630    | 3300 (50) |
| src-25| ND    | 160| 15  | 435     | 610 (44) |
| src-31| ND    | 80 | 25  | 190     | 295 (7) |
| src-4 | 950   | 460| 170 | 2570    | 3200 (17) |
| src-16| 1600  | 420| 250 | 2430    | 3100 (54) |
| src-18| 790   | 350| 270 | 1880    | 2500 (30) |
| src-19| ND    | 467| 117 | 1549    | 2133 (33) |
| src-20| ND    | 339| 98  | 1178    | 1615 (23) |

See Table 1 footnotes for details.
Numbers are obtained from cultures that contained $10^6$ spleen cells. In contrast, supernatants from spleen cells expressing the v-src gene did not stimulate the growth of only small colonies of macrophages. This would suggest that the stimulus for the growth of these colonies within these cultures was mediated by a locally secreted factor or by a factor that is extremely labile.

### Discussion

In the present study we show that the recombinant retrovirus N-TK-src efficiently expresses both the neo gene and the v-src gene in the different hematopoietic lineages in culture as well as in mice reconstituted with infected bone marrow cells. No dramatic effect of v-src expression could be demonstrated on precursor cells in short-term methyl cellulose cultures. In contrast, expression of the v-src oncogene in reconstituted mice leads to the rapid development (6–10 weeks) of a severe myeloproliferative disorder, characterized by splenomegaly and a shift of hematopoiesis from the bone marrow to the spleen.

A number of other reports have indicated that expression of v-src can alter mouse hematopoietic development, both in vivo as well as in culture. Anderson and Scolnick (1983) first showed that injection of a virus that expresses the v-src oncogene murine Rous sarcoma virus (MRSV) into mice leads to splenomegaly and anemia. On the basis of the morphology of the cells within the spleen, they concluded that expression of v-src induces an erythroproliferative disease. In a following study, Anderson et al. (1985) reported that spleens from mice infected with MRSV contained elevated numbers of erythroid precursors, a significant fraction of which grew and gave rise to erythroid colonies in the absence of erythropoietin. Because precursor cell numbers from other lineages were not reported, it is not possible to judge whether they were elevated also. In none of the mice reconstituted with N-TK-src-infected bone marrow cells did we observe the development of erythroid precursors that could grow in the absence of erythropoietin. Thus, although their numbers were increased by expression of v-src, the growth-factor responsiveness of the erythroid precursors from these animals appeared normal. The difference between our findings and those of Anderson et al. (1985) may be due to the differences in the expression levels of the v-src gene in the different mouse strains used.
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al. (1985) may reflect a difference in the viruses or a difference in the culture conditions used to assay the precursors.

In the same report these authors found that infection of fetal liver cells or spleen or bone marrow cells from PHZ-treated mice leads to the growth of increased numbers of erythroid colonies in semi-solid cultures. When we used spleen or bone marrow cells from PHZ-treated mice as a source of precursors, we observed the development of large numbers of erythroid colonies in the presence of erythropoietin in cultures infected with N-TK-src as well as in the control cultures infected with N2. The growth of these erythroid colonies was cell-density dependent, indicating that factors produced within the culture dish were involved in their development. This observation, together with the fact that the erythroid colonies described by Anderson et al. (1985) could not be replated or expanded in liquid culture, makes it difficult to determine whether the effect they described resulted from expression of v-src in the erythroid precursors or in another population, which in turn stimulated the growth of the erythroid cells.

In another series of experiments, Boettiger et al. (1984) reported that infection of long-term bone marrow cultures with MRSV leads to an increase in the frequency as well as in the total number of precursor cells (GM-CFC and CFU-S) in these cultures. In subsequent studies, it was shown that cells from these infected cultures could be grown indefinitely in the presence of interleukin-3 (IL-3) (Spooner et al. 1984, 1986; Wyke et al. 1986). These IL-3-dependent cell lines do not appear to be blocked in their developmental program because they contain some precursor cells that are able to generate multilineage colonies when plated in methyl cellulose cultures (Spooner et al. 1986). One unexpected finding was that most of these cell lines contain only the helper virus and not MRSV (Wyke et al. 1986). This would indicate that expression of the v-src oncogene is not required for maintenance of these cell lines. However, the fact that one cannot generate these lines from cultures infected with only the helper virus would suggest that v-src is required for their establishment. The effect of the v-src oncogene then appears to be indirect and could possibly be mediated through a regulatory cell population within these cultures.

Expression of v-src in a regulatory cell population may, in part, be responsible for the myeloproliferative disease that we observe in mice reconstituted with N-TK-src-infected bone marrow cells. Because the total bone marrow population is exposed to the virus, it is quite possible that, in addition to the hematopoietic cells, the nonhematopoietic stromal cells will be infected and express the v-src gene as well. Expression of v-src in these stromal cells could lead to the alteration that we have observed. The fact that the precursor frequency from many lineages is altered, but the precursor cells themselves appear to be normal, would support this notion. If expression of v-src in a regulatory cell population is indeed responsible for the myeloproliferation, the primary effect could be in the bone marrow where a change in these cells leads to an environment that is unable to support hematopoesis. As a consequence, the spleen would become a hematopoietic organ, to compensate for the lack of hematopoesis in the bone marrow. Alternatively, expression of v-src in a regulatory cell population in the spleen could lead to increased proliferation of hematopoietic precursors, with the resulting splenomegaly. The observation that spleen cells from mice that express the v-src gene, when plated at high cell densities, can support the growth of erythroid, neutrophil/macrophage, and multilineage colonies in the absence of added growth factors (Table 4) would indicate that factors are being produced within the culture. The inability to demonstrate any significant amounts of colony-stimulating activity in medium conditioned by these spleen cells would suggest that the growth of these colonies is not mediated by a long-range secreted factor. The nature of this colony-stimulating activity and its relationship to the expression of the v-src oncogene remain to be determined. That expression of v-src can lead to growth-factor production has been demonstrated in the avian system by Adkins et al. (1984). They found that expression of v-src in factor-dependent myb-transformed chicken myeloblasts induced these cells to produce their own growth factor.

Alternatively, expression of v-src in stem cells and the various precursor populations could also be responsible for the myeloproliferative disease in the reconstituted mice. Expression of v-src in these cells could subtly change their growth characteristics and/or their ability to “home” to the appropriate tissues when introduced into the recipient mouse. A number of studies indicate that v-src can transform or at least alter the growth of cells of hematopoietic origin. Pierce et al. (1984) reported that infection of bone marrow cells with MRSV leads to the development of colonies of blast cells in soft agar. By propagating cells from these colonies on bone-marrow-adherent cells for several months, it was possible to establish continuously growing lines that lacked all known differentiation markers. The fact that several months were required for their establishment indicates that events other than the expression of v-src were involved.

In another series of experiments, Overell et al. (1987) found that expression of v-src in an IL-3-dependent cell line leads to the generation of factor-independent cells. However, only a relatively small fraction of infected cells becomes factor independent, indicating that, as in the previous study, expression of v-src alone is not sufficient for this change. In the avian system, the v-src oncogene is capable of transforming erythroid precursors in culture as well as in vivo in a relatively short period of time (Kahn et al. 1984, Palmieri 1985). Cell populations derived from these transformed precursors can be grown in cultures in the absence of Ep. However, after 20–30 population doublings they appear to senesce.

When we infected bone marrow cells in culture with N-TK-src we did not detect any alteration in the growth and differentiation of precursors within the 10 to 14-day culture period. It is possible that the avian v-src onco-
gene is not as efficient in mouse cells as in chicken cells, and thus, we would have to analyze more infected cells for a longer period of time to detect a similar transformation event. In a recent series of experiments, we passaged spleen and bone marrow cells from primary reconstituted animals to secondary and tertiary recipients. In these recipients we have detected precursors with a transformed phenotype that grow continuously in culture in the presence of specific growth factors [G. Keller, unpubl.]. Preliminary evidence indicates that at least some of these cells are committed to the erythroid lineage. These findings are consistent with the notion that expression of v-src can lead to the transformation of precursors, albeit at a low frequency. The fact that infected cells had to be passaged through several recipients would suggest that, again, other events are required for the establishment of a transformed phenotype.

Several aspects of the myeloproliferative disease found in the mice expressing the v-src oncogene are similar to the symptoms exhibited by patients suffering from the clinical disorder myelofibrosis. These patients are often anemic and have a grossly enlarged spleen, which contains large numbers of hematopoietic cells (Bouroncle and Doan 1962, Ward and Block 1971). Their bone marrow has characteristic areas of fibrosis and in some cases has almost no hematopoietic activity. Elevated levels of hematopoietic precursors have been detected in the circulation of these patients (Chervenick 1973; Douer et al. 1983), and in addition to the spleen, extramedullary hematopoiesis has been found in the liver and lymph nodes as well as in a variety of other tissues (Ward and Block 1971). Whether these similarities reflect a common mechanism remains to be determined; however, they would suggest that the role of c-src in this myeloproliferative disorder needs to be investigated.

In conclusion, our findings demonstrate that the recombinant retrovirus N-TK-src efficiently expresses two genes, neo and v-src, in hematopoietic cells in culture as well as in long-term reconstituted mice. The primary effect of v-src expression appears to be the development of a severe myeloproliferative disorder, in which one observes an increase in the frequency of precursor cells from the various myeloid lineages in the spleen and peripheral blood and a decrease of these cells in the bone marrow. Expression of v-src does not appear to lead to a rapid transformation of these precursors in the primary recipients, as they appear normal with respect to their differentiation potential and growth-factor requirements. Preliminary experiments would indicate that passaging spleen cells from primary to secondary recipients does lead to a transformation event, allowing the establishment of continuously growing cell lines. Current experiments are aimed at identifying the cells within these lines and elucidating the mechanism by which expression of v-src leads to their establishment.

Materials and methods

**Vector construction**

pN-TK-src was constructed by the insertion of a 1.9-kb v-src fragment from the AccI to the NruI sites of the SR-A Rous sarcoma virus [Czernilovsky et al. 1983] into the BglII site of pXT1 (Boulter and Wagner 1987).

Ten micrograms of plasmid DNA was transfected onto 62 cells [Mann et al. 1983] plated at 5 x 10⁶ cells per 100-mm dish 24 hr previously. After 48 hr, cells were selected in the presence of 0.75 mg/ml of G418 and individual colonies were picked after 8–10 days. Supernatants from these clones were then assayed for infectious virus.

**Infection of NIH-3T3 cells to assay viral titers**

NIH-3T3 cells (5 x 10⁴) were plated in 60-mm dishes 1 day prior to infection. For infection, the medium was removed and replaced with virus and 4 μg/ml Polybrene. After 2 hr incubation with occasional shaking, fresh medium was added. Selection in 0.75 mg/ml G418 was started 36 hr later. The medium was changed every 2 days and G418R colonies were counted 10–12 days later. This method was also used to determine whether infectious particles were present in the sera of reconstituted mice. It was found that most animals contained between 100 and 1000 infectious cfu per milliliter of blood, indicating a low level of viral spread. The origin and nature of the virus is presently unknown.

**Virus production**

The 62 cells producing the N-TK-src virus were cultured in large tissue culture flasks (175 cm²) in Iscove’s modified Dulbecco’s medium [IMDM] plus 5% fetal calf serum (FCS). When the culture became confluent, the supernatant was removed, discarded, and replaced with fresh medium (25 ml per flask). Every 6–12 hr following this point, the supernatant was harvested, filtered (0.2 μm), and frozen (–70°C). Supernatant from the N-TK-src-producing cells harvested under these conditions contained 1 x 10⁶ to 2 x 10⁷ CFU/ml when assayed for the induction of G418R colonies on NIH-3T3 cells. N2 (Keller et al. 1985) stocks produced under similar conditions had titers of 5 x 10⁶ to 1 x 10⁷ CFU/ml.

**Protein kinase assay**

v-src tyrosine kinase activity in cell lysates, quantitated photometrically for total protein using Pierce reagent, was assayed essentially as described by Collett and Erikson (1978). For the immunoprecipitation, a tumor-bearing rabbit [TBR] polyclonal serum against v-src was used [obtained from S. Courtneidge, Heidelberg].

**Mice**

Either CBA/N bii or CBA/J [IIFA-CREDO] mice 12 weeks of age or older were used as recipients in the reconstitution experiments. CBA/HT6 T6 bii mice were used as bone marrow donors for the CBA/N recipients, and CBA/J mice were used as donors for the CBA/J recipients. In most experiments the mice used for bone marrow were male and those used for recipients were female. Recipients were irradiated with 950 R prior to reconstitution. In those experiments indicated, mice were treated with phenylhydrazine [0.04 mg/g] 48, 48, and 40 hr prior to sacrifice, as described by Hankins and Scolnick [1981].

**Bone marrow infection and reconstitution**

Bone marrow from mice injected 5 days earlier with 5-fluorouracil [5-FU; 150 mg/kg] was used as a source of cells for infection, as described previously [Keller et al. 1985]. The bone marrow cells were infected by mixing them with a virus-con-
taining supernatant rather than direct co-culture with the virus-producing ±2 cells. The infections were carried out in large [175 cm²] tissue culture flasks for a period of 18–24 hr. The culture consisted of supernatant from the virus-producing cells [80%], Polybrene [5 µg/ml], WEHI-3BD-conditioned medium [10%] as a source of interleukin-3 [IL-3], and supernatant from the human bladder carcinoma cell line 5637 [10%] as a source of interleukin-1 [IL-1] [Machizuki et al. 1987]. Infection was most successful with a multiplicity of infection of at least 3. Following infection, the cells were harvested, centrifuged, and resuspended in a ‘preselection’ culture that contained fresh IMDM [55%], FCS [5%], WEHI-3-conditioned medium [25%], 5637-conditioned medium [15%], and G418 [1.5 mg/ml, powder = 750 µg/ml active substance]. In the initial experiments, the bone marrow cells were preselected for 48 hr, however, in the more recent studies, this was extended to 72 hr. The preselection step selects for cells that were infected with the virus and express the neo gene. Under the conditions described above, between 10 and 20% of all precursors were G418± immediately following infection, and more than 75% were G418± following the preselection step. After the preselection culture, the cells were harvested and injected into irradiated recipients [950 R]. In most experiments, the marrow from femurs and tibias of 5–8 donors was used to reconstitute each recipient. Following 5-FU treatment, infection, and preselection, this represents approximately 1 × 10⁶ to 2 × 10⁶ cells per recipient.

Clonal assays

The colony assay for precursors of the myeloid lineages was carried out in 1-ml cultures [35-mm Petri dishes] that contained 1.0% methyl cellulose in IMDM, 4% FCS, 10 mg defibulated and deionized bovine serum albumin [BSA], 300 µg iron-saturated transferrin, plus a mixture of dipalmitoyl phosphatidylcholine, cholesterol, and oleic acid [22.4, 23.5, and 17.3 µg of each, respectively]. All reagents were prepared as described by Iscove [1984]. DEAE-Sepharose-purified WEHI-3-conditioned medium was used as a source of IL-3, and crude conditioned medium from the human bladder carcinoma line 5637 was used as a source of IL-1. Human erythropoietin was kindly provided by Genetics Institute [Cambridge, Massachusetts]. All reagents were tested to give optimal colony growth. Cultures normally contained 1 unit erythropoietin [international standard units], 10 units IL-3 [1 unit = ½ maximum activity], and 15% 5637-conditioned medium as indicated.

Colonies derived from the more mature erythroid precursors, the CFU-E, were scored on an inverted microscope, following 2 days incubation at 37°C in a humidified environment which consisted of 5% CO₂ in air. Pure erythroid, macrophage, neutrophil, and mixed macrophage–neutrophil colonies were scored at days 7–8 and mast-cell and multilineage colonies were scored at days 10–12 of incubation. Clonable B cells [CFU-B] were grown in 1-ml agar cultures, as described previously [Paige and Skarvall 1982]. These colonies were scored 5 days after incubation.

Cell populations for kinase assay and DNA analysis

To generate populations enriched for T cells, spleen cells were grown for 5–7 days in the presence of concanavalin A [5 µg/ml] and interleukin-2 [IL-2]. Following the culture period, the cells were harvested, centrifuged over a Lymphoprep density gradient (to remove dead cells), and then used for DNA preparation. Greater than 95% of these cells express Thy-1. Populations of macrophages were prepared in a two-step procedure. First, bone marrow cells were cultured in tissue culture flasks [2.0 × 10⁶ cell/ml] in IMDM containing WEHI-3 [IL-3 source] and L-cell-[CSF-1 source] [Stanley and Heard 1977] conditioned medium. Forty-eight hours later the nonadherent cells [containing precursors] were removed and replated in flasks in the presence of IMDM with only L-cell-conditioned medium [35%]. Seven to ten days later, the cells were harvested and used for DNA preparation. At this time, more than 90% of the cells in the population expressed the macrophage marker MAC-1 [Springer et al. 1979].

Preparation of spleen-cell-conditioned medium

Spleen cells were cultured in either tissue culture flasks [25 cm²] or 24-well plates at a concentration of 2.5 × 10⁶ cells/ml in IMDM plus 5% FCS. After 5–7 days, the supernatant was removed, filtered, and stored at −70°C until tested.

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