Retrovirally Transduced Antisense Sequences Stably Suppress P210<sup>BCR-ABL</sup> Expression and Inhibit the Proliferation of BCR/ABL-Containing Cell Lines

By P. Martiat, P. Lewalle, A.S. Taj, M. Philippe, Y. Larondelle, J.L. Vaerman, C. Wildmann, J.M. Goldman, and J.L. Michaux

There is now strong evidence that the BCR-ABL gene product of the Philadelphia chromosome (P210) plays a crucial role in the pathogenesis of chronic myeloid leukemia (CML). We have previously shown that introduction of antisense oligonucleotides into K562 cells could transiently block the expression of P210 and specifically inhibit cellular growth in culture. In this report, we describe the use of a retroviral vector to introduce selected antisense and sense sequences, first into murine B10 cells, previously rendered interleukin-3 (IL-3) independent by transfection of BCR-ABL sequences, and second into K562 cells. The antisense transcripts generated under the control of MoMLV promoter specifically killed B10 cells in the absence of IL-3 and inhibited P210 expression almost completely. In K562 cells, the antisense sequences led to a dramatic reduction of P210 expression and increased their doubling time by more than twofold. This effect was not reversed by the addition of exogenous IL-3 to the culture medium. Control HeLa or HL60 cells infected with the same constructs did not show any change in proliferation rate, despite abrogation of the normal BCR gene products. Rather unexpectedly, P210 suppression was not lethal in K562 cells, showing that such a cell line does not rely entirely on the expression of P210 for surviving, but depends on it as far as growth properties are concerned. We conclude that this approach can successfully achieve stable suppression of the oncogenic protein P210 and may be used to study further the mechanisms by which P210 is transforming cells. The effect on fresh CML cells in bone marrow cultures remains to be assessed before we can tell whether this technique may be used for selective suppression of leukemic hematopoiesis in vitro.

© 1993 by The American Society of Hematology.

**MATERIALS AND METHODS**

**Constructs**

Vectors generating RNA molecules containing sequences complementary to the 5′ portion of the hybrid BCR-ABL gene were constructed using standard recombinant DNA techniques. A 585-bp SfiI/BamHI fragment (INS1) containing the ATG initiation codon was isolated from a plasmid containing the entire BCR-ABL sequences (KW3; provided by Dr. G. Grosveld, Rotterdam, The Netherlands) and cloned both in a sense (P2-S1) and antisense (P2-AS1) orientation in the retroviral expression vector pZIP/NeoSV(X). The structure of the recombinants was verified by restriction enzyme digestion of isolated plasmid DNA. To generate cell lines producing the recombinant retroviruses, the amphotropic packaging cell line GP12+enAM-12 was transfected with either the sense or antisense constructs using the calcium phosphate transfection method. Individual colonies were isolated in G418 (1.2 mg/mL), expanded, and assayed for the production of recombinant viruses by infection of NIH-3T3 cells. Production of recombinant RNAs was assayed in infected NIH-3T3 cells, after G418 selection, using Northern blot techniques and the (32P)-dCTP-labeled insert as a probe. The second sense and antisense constructs (P2-S2 and P2-AS2), the effects of which are briefly described in the Results section, were made in the same way. The insert INS2 was a 200-bp junctional fragment, produced by PCR, cloned into the same vector and spanning exactly 100 bp on each side of the b3a2 junction (Fig 1) of the BCR-ABL cDNA.

**Cellular Experiments**

Because we expected P210 inhibition to cause the death of CML cells having integrated the antisense sequences, we chose, as a first target, the B10 lymphoid cell line, which has been rendered interleukin-3 (IL-3) independent by infection with a retroviral vector encoding P210 (b3a2-type junction) sequences. In the event of inhibition of P210 expression, the cells could be rescued by addition of exogenous IL-3, thereby allowing to assay P210 expression on living material. As this cell line was shown to be very resistant to infection with the
amphotropic constructs (data not shown) and as it already contained a neomycin-resistance gene that did not allow for selection of cells infected with our constructs, two types of experiments were designed to assess the efficiency of the constructs: transient expression after electroporation for a rapid first screening of the constructs and stable expression, after cotransfection with a plasmid encoding hygromycin B resistance. The aim of the first experiments was to verify whether, during transient expression, there was a difference in the growth pattern of cells transfected with the sense and antisense constructs, before moving to the next step consisting of making stable transfectants. This first step involved only cell viability studies, at short term (4 days) and a P210 kinase assay. Constructs that seemed to have an effect (in this case, pZip/AS1) were characterized more precisely in the stable expression assay.

**Transient Expression in B10 Cells**

**Transfection.** The B10 cells were electroporated BioRad (UK) Gene Pulser, 960 μF, 250 V in 800 μL RPMI with 20 μg of pZip/Neo-SV(X), pZ/SI, or pZ/AS1. After electroporation, the cells were divided into two and grown in IL-3-supplemented and -deprived culture medium. Viable cells were counted at 24, 48, 72, and 96 hours after electroporation. WEHI-3B cells supernatant was used as a source of IL-3.

**P210 assay.** P210 assay was performed as described previously. Briefly, the cells were washed in phosphate-buffered saline (PBS) and lysed in kinase lysis buffer. The lysates were cleared of insolvable material and immunoprecipitated with the anti-BCR monoclonal antibody, bcr (Ab-2), clone 7C6 (Oncogene Sciences). The immunoprecipitated proteins were labeled in vitro with (32P)-γATP. The proteins were resolved on a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was then dried and autoradiographed. The assay was performed on 4 × 10^6 living cells (Trypan Blue exclusion method), grown with IL-3 to ensure survival of cells in which P210 inhibition could occur, and collected 72, 96, and 108 hours after electroporation. The same type of experiments were performed using pZ/S2 and pZ/AS2.

**Stable Expression in B10 Cells**

**Stable transfectants.** Because the B10 cells are already carrying a neomycin resistance gene, no further selection for the expression of the sense or antisense sequences was possible using the neomycin-resistance gene encoded by pZip/S1 or pZip/AS1. The B10 cells were
therefore coelectroporated, as described above, with two plasmids, pZ/AS1 or pZip/S1, and a plasmid carrying the sequences encoding hygromycin B resistance. After 2 days, the cells were selected using hygromycin B (200 μg/mL). Resistant cells were then cloned, using the limiting dilution methodology. Individual clones were screened for the presence of the constructs S1 and AS1 using polymerase chain reaction (PCR) performed on DNA. The whole procedure was performed in IL-3-supplemented medium.

**Proliferation studies.** Several culture experiments were performed on the selected sense or antisense clones, in the presence or absence of IL-3. After growth in IL-3-supplemented medium, B10/S1 and B10/AS1 were washed with PBS and put back into culture medium with or without IL-3. The cells were then counted every day. Cell death was assessed using Trypan Blue exclusion. This procedure was repeated at least three times on every selected clone.

**P210 measurements.** These experiments were repeatedly performed on 4 × 10⁶ living cells (B10/S1, B10/AS1, and unmanipulated B10 cells) using the procedure described above. HeLa cells extracts were used as a negative control to evaluate the specificity of the assay.

**Southern blot studies.** DNA was extracted from B10/S1 and B10/AS1 cells using standard procedure,14 digested with Xba I, which cuts within the 5' and 3' long terminal repeat (LTR) of the constructs encoding the BCR-ABL sequences18 and the sense/antisense constructs used in the transfection procedure. The digestion products were electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the (32P)-dCTP-labeled17 5' insert (INS1) as a probe. This probe will pick up both the BCR-ABL sequences and the sense/antisense sequences. This, together with the PCR studies described below, were performed to ensure, as much as we could, that clones having lost the expression of the BCR-ABL mRNA after a genomic rearrangement had not been selected by chance during the cloning procedure.

**PCR studies.** Clones of B10 cells stably transfected with either the sense (B10/S1) or the antisense (B10/AS1) construct were investigated for expression of BCR-ABL mRNA and antisense sequences as follows. Total RNA was extracted from B10/AS1 and B10/S1 cells according to the method described by Chomczynski and Sacchi.20 RNA was first subjected to DNase I treatment (to avoid amplification of the integrated BCR-ABL or antisense DNA), re-extracted, and reverse transcribed using either a primer complementary to the antisense RNA:INS (Fig 2) or to the BCR-ABL exon a3 RNA:A3. As a control, the same procedure was performed without adding reverse transcriptase. The samples were amplified using a couple of primers spanning the BCR-ABL junction (B1/A3) during 25, 30, and 35 cycles of amplification, as previously described21 or a couple of amplimers (LTR/INS) located on the antisense construct (Fig 2) during 30 cycles of amplification. The 3' primer, INS, was complementary to the antisense RNA:INS (Fig 2) or to the BCR-ABL exon a3 RNA:A3. As a control, the same procedure was performed without adding reverse transcriptase. The samples were amplified using a couple of primers spanning the BCR-ABL junction (B1/A3) during 25, 30, and 35 cycles of amplification, as previously described21 or a couple of amplimers (LTR/INS) located on the antisense construct (Fig 2) during 30 cycles of amplification. The 3' primer, INS, was complementary to the antisense RNA only. If a BCR-ABL message is present, the B1/A3 primers pair will produce a 395-bp fragment for a b3a2 junction and the LTR/INS pair will amplify a 551-bp fragment for a RNA in the

---

**Fig 3. Transient expression of the constructs in B10 cells.** (A) Proliferation of the B10 cells transfected with the sense (S1) or antisense (AS1) constructs in the absence (above) or presence of IL-3. The indicated numbers are the total numbers of living cells, as assessed by Trypan Blue exclusion. Time is in hours, starting from electroporation. (B) Comparison of the amount of P210 in 4 × 10⁶ living cells collected 72 hours after electroporation. The lanes pZ/S2 and pZ/AS2 show the results obtained with the second antisense construct (INS2) that was not efficient in inhibiting P210 expression.
Fig 4. Stable expression in B10 cells: proliferation studies and P210 assay. (A) Comparison of the proliferation rate of B10 cells stably expressing the antisense sequences (AS1) in the presence (+IL-3) or absence (−IL-3) of IL-3. The proliferation rate of B10 cells expressing the sense sequences (−IL-3) is given for comparison. (B) P210 kinase assay comparing the protein expression in 4 × 10^6 B10 cells stably transfected with the antisense construct to its expression in the same number of unmanipulated B10 cells (B10) and in the cells expressing the sense construct (B10/S1). The negative control consists of HeLa cells.

antisense orientation (AS1). The sequences of the amplimer sequences are as follows: A3, 5'-GTGATTATAAGCCTAAGACCCGAGC-3'; B1, 5'-GAAGAAGTGGTCTTGAAGCTTCCTCC-3'; LTR, 5'-TGTGGTCGTGCTGTCC-3'; INS, 5'-GAACGGGACGACCGGGGA-3'.

Northern blot studies. Total RNA was extracted according to the same protocol as described for RT-PCR studies. Twenty micrograms of total RNA was loaded onto a denaturing gel (agarose 0.7%, formaldehyde 3%), electrophoresed for 20 hours at 40 V in a MOPPS buffer, transferred to a nylon membrane using 10× SSPE, and hybridized with the (32P)dCTP-labeled junctional insert (INS2) as a probe to pick up only BCR-ABL mRNA. The hybridization conditions were as follows: 48°C in 50% formamide with 5× SSPE.

Stable Expression in K562 Cells

Infection procedure. We next introduced the constructs pZ/S1 and pZ/AS1, the efficacy of which had been verified in the B10 stable expression assay, into K562 cells by retroviral infection followed by neomycin-resistance selection. K562 cells were cultured in the presence of the retroviral supernatants for 2 days. Four days after the beginning of the procedure, G418 (2.5 mg/mL) was added to the culture medium and resistant cells were selected. Cell growth rate measurements and P210 assays were performed on control K562 and K562 expressing the sense (K562/S1) or antisense sequences (K562/AS1). As a control for the specificity of any effect on growth properties, we used the same procedure to generate clones of HeLa and HL60 cells expressing the sense or antisense constructs.

Western blot studies. Experiments were performed on 4 × 10^6 unmanipulated K562, K562/S1, K562/AS1, or on the same number of HeLa/S1 and HeLa/AS1 using the monoclonal antibody bcr (Ab-2; Oncogene Sciences) according to the manufacturer’s protocol, except that proteins were first immunoprecipitated with the Ab-2 monoclonal antibody, as in the kinase assay described above, before being loaded onto the polyacrylamide gel. This achieved better results in terms of BCR-related protein resolution.

RESULTS

Constructs

The titers of retroviral supernatants generated by the two chosen (GP12/pZ-AS1 and GP12/pZ-S1) clones, determined by resistance of NIH/3T3 fibroblasts to G418, were 4 × 10^4 and 6 × 10^4 colony-forming units (CFU)/mL, respectively. Northern blot studies, performed after G418 selection, on infected NIH/3T3 fibroblasts showed these cells to express a high level of the recombinant RNAs (data not shown).
Fig 5. Stable expression in B10 cells: Southern blot, Northern blot, and PCR studies. (A) DNA extracted from B10/AS1 and B10/AS1 digested with XbaI and hybridized to the labeled insert INS1. Both lanes show the presence of an 11-kb (BCR-ABL construct) and a 4.5-kb (sense or antisense constructs) band integrated into the B10 cell genome. (B) Total RNA extracted from B10/AS1 and B10/AS1 hybridized to the labeled insert INS2 (that picks up only the BCR-ABL mRNA), showing a strong reduction in the level of BCR-ABL message in B10/AS1 cells. (C) RT-PCR studies on B10/S1 and B10/AS1 cells. The three lanes of 25, 30, and 35 cycles (BCR-ABL) refer to the amplification of the BCR-ABL message in the two types of cells, with NC being a negative control without RT (35 cycles). The two lanes AS show the result of RT-PCR performed with the primers pair amplifying the antisense RNA.

B10 Cells

**Transient Expression**

Cell proliferation studies. The 5' antisense transcripts (AS1) were able to specifically inhibit the proliferation of B10 cells in the absence of IL-3, as shown by cell proliferation studies, while not affecting them in IL-3-supplemented culture medium (Fig 3A). This effect on cell growth was maximal between 48 and 72 hours after electroporation. After that period, the growth suppression was released and the B10 cells electroporated with pZip/AS1 started to grow again. The sense construct had no detectable effect on cell kinetics when compared with the original plasmid [pZip/NEO-SV(X)] electroporated using the same experimental protocol.

**P210 assay.** P210 kinase assays showed an inhibition of the protein expression by more than 90% (Fig 3B) in B10/AS1 cells grown in IL-3-supplemented medium collected at 72 hours. P210 expression returned to normal 96 to 108 hours after electroporation and IL-3 independence was restored by that time. B10/S1 cells did not show any reduction in P210 expression. The second construct (pZ/AS2) had no detectable effect, neither on cell growth nor on P210 expression (Fig 3B).

**Stable Transfection**

Proliferation studies. In B10 cells expressing the antisense sequences (B10/AS1), independence from IL-3 was completely abrogated (Fig 4A), whereas the cells infected with the sense construct were unaffected. True cell death was observed in B10/AS1 grown without IL-3. Three days after the cells had been transferred to an IL-3-deprived culture medium, no surviving cell could be observed using Trypan Blue and the addition of IL-3 at that stage did not lead to any cell regrowth.

**P210 assay.** A marked reduction of P210 expression (Fig 3B) was observed in the clones of B10 cells expressing the antisense sequences.

**Southern blot studies.** In B10/S1 and B10/AS1 DNA digested with XbaI (Fig 5A), two bands (approximately 11 and 4.5 kb) are visible, showing that in these cells both the BCR-ABL complete sequences and the sense/antisense sequences are present within the genome.

**Northern blot studies.** In B10/S1 cells, the 8.5-kb BCR-ABL mRNA can be seen after 2 days of exposure (Fig 5B), whereas it cannot be detected in B10/AS1. A longer exposure time (15 days) results in the appearance of a faint band in B10/AS1 RNA, showing that the message is present but in much lower amounts in these cells.

**PCR studies.** After 25 cycles of amplification, the BCR-ABL message is clearly visible, in B10/S1 cells, on the ethidium bromide-stained gel (Fig 5C), and another 5 to 10 cycles do not change significantly the intensity of the band. On the other hand, 30 cycles are needed to clearly identify the mes-
Fig 6. Stable expression in K562 cells: proliferation studies and P210 assay. (A) Comparison of proliferation rate between K562 expressing the antisense RNA (K562/AS1) and the same number of control K562 or K562/S1 growing in the same culture medium. (B) P210 measurement in 4 x 10⁶ K562/S1 cells and K562/AS1 using two different autoradiograph exposure time (6 and 24 hours).

K562 Cells

Proliferation Studies

K562 cells that had integrated the antisense sequences (K562/AS1) showed a reduced growth rate in comparison with control K562 and K562/S1 infected with the sense construct grown in the same culture medium (doubling time: 39.0 ± 2.5, 17.8 ± 2.0, and 18.1 ± 2.3 hours, respectively) (Fig. 6A). This effect on proliferation was not reversed by the addition of IL-3 to the culture medium.

P210 Assay

In K562/AS1, P210 expression was markedly reduced (Fig 6B) when compared with K562/S1 and normal K562 (not shown).

Western Blot Studies

Experiments performed to look at the normal BCR gene products showed a complete disappearance of P160BCR and P130BCR in K562/AS1 (Fig 7) and in HeLa/AS1 cells. No differences were observed in the growth characteristics of HeLa or HL60 cells infected with the antisense construct, showing that the inhibition of the BCR proteins may not be responsible for this change in proliferation rate, inasmuch as conclusions obtained on two cell lines (HL60 and HeLa) can be applied to a third one (K562).

DISCUSSION

We have shown in this report that almost complete inhibition of P210 expression was achievable, in a stable manner, using retrovirally transduced antisense sequences targeted against the 5’ portion of the hybrid BCR-ABL mRNA. This effect could be obtained in cells expressing the BCR-ABL mRNA at a much higher level than what can be expected in fresh CML cells. If successfully achieved in CML-derived cells, this inhibition might have caused the cells having integrated the antisense sequences to die, thus preventing us to verify our hypothesis. This is why we chose as a first target the B10 lymphoid cell line (previously rendered IL-3 independent upon expression of P210-coding sequences). In the event of inhibition of P210 expression, which would lead to cell death in a culture medium deprived of IL-3, the cells can be rescued by addition of exogenous IL-3, thereby allowing us to assay P210 expression on living material. Their relative resistance to retroviral infection led us to first set up a transient
expression assay that ultimately proved a very useful tool for evaluating the effect of a construct without having to go through each time the long procedure of making packaging cells, cloning them, and testing the supernatants. Our experience with antisense oligonucleotides\textsuperscript{12} led us to first use the sequences complementary to the 5' end of BCR/ABL mRNA before designing junctional antisense constructs that would have the theoretical advantage of leaving unaffected the normal BCR gene product. The 5' antisense transcripts, generated under the control of MoMLV LTR promoter sequences and stably expressed in B10 cells, were able to specifically kill them in the absence of IL-3, as shown by cell proliferation studies, and to deeply inhibit P210. Northern blot studies showed that one mechanism for inhibition of P210 expression is a degradation of the BCR-ABL mRNA. However, PCR experiments showed persistence of some BCR-ABL message, but because inhibition can also occur at the translational level, a further block can account for almost complete blockade of P210 synthesis. The P210 assay performed on K562 expressing the antisense sequences showed a dramatic inhibition of P210 expression; this occurred despite known amplification of the BCR-ABL gene in K562 cells. It is worth mentioning that the K562/AS1 cells that were investigated at different moments showed slight variations in the level of inhibition, ranging from no P210 detectable to a small amount of it being picked up by the assay (the latter case being shown in Fig 6). The fact that these cells are not clonal may account for variation in the expression of the antisense transcripts according to the integration site within the genome. We chose to show an experiment that represents one of the weakest inhibitions we found to not overinterpret the data, but all the P210 assays were at least as good in terms of inhibition, with some of them showing no detectable protein. We may thus speculate that a construct capable of inhibiting P210 to such an extent should be very active in fresh CML cells. We think that the change of the proliferation rate of the K562/AS1 cell is related to P210 inhibition rather than to inhibition of the normal BCR products, because HeLa and HLM60 cells, in which inhibition of the BCR gene has occurred, have the same growth characteristics as the cells expressing the sense sequences. However, inhibition of the normal BCR gene may have different effects in different cell lines, and only specific inhibition of P210 with a junctional antisense construct will allow us to answer that question definitively. Our first attempt to design a junctional antisense construct failed to inhibit P210 expression. We have no data allowing us to understand why this junctional construct had no effect. It was also efficiently transcribed in 3T3 fibroblasts and the lack of effect cannot therefore be attributed to a lower transcription efficiency. It is possible that secondary structures of the hybrid BCR-ABL and/or the antisense RNAs make these junctional sequences less likely to hybridize. However, antisense strategies are still, to a large extent, empirical and further experiments with different types of junctional constructs are under way. These results also show that survival of a cell line derived from a CML blast crisis (K562) is possible despite reduction of P210 expression. We cannot tell from our experiments whether a low level of P210 expression is sufficient to keep the cells alive, or whether other mechanisms play an essential role when P210 is inhibited. The fact that the changes induced in K562 growth characteristics was not reversed by the addition of IL-3 to the medium indicates that P210 must exert its action in CML cell lines via an IL-3-unrelated pathway. Although apparently less dramatic than results obtained on CML cells in bone marrow culture using antisense oligonucleotides\textsuperscript{13} in which inhibition of the Philadelphia-positive compartment occurred upon inhibition of BCR-ABL mRNA, our results may be explained by the fact that a leukemic cell line may have acquired secondary characteristics, making it less dependent on a single genetic event that fresh CML cells do not possess. Further experiments on fresh CML cells in culture will be needed to see whether infection of these cells with antisense constructs, followed by antibiotic selection, will lead to eradication of Philadelphia-positive cells.
or simply to the persistence of these cells, despite abolition of P210 expression, with possible modifications of their leukemic phenotype (Philadelphia-positive, P210-negative cells).

In conclusion, we feel that the use of retroviral antisense sequences may prove useful for research and therapeutic use. K562 cells with stable inhibition of P210 expression may be used to study further the mechanisms by which the effects of the inhibition could be reversed. Furthermore, our results suggest an approach to in vitro gene therapy in CML; Philadelphia-negative or even Philadelphia-positive, P210-negative hematopoiesis might be restored by autografting patients with bone marrow cells that had been incubated with the antisense supernatant, followed by a selection procedure to ensure that all reinfused stem cells had integrated antisense sequences.

ACKNOWLEDGMENT

The authors thank Prof G. Burtonboy for allowing them to use the facilities of his laboratory of virology.

REFERENCES

Retrovirally transduced antisense sequences stably suppress P210BCR-ABL expression and inhibit the proliferation of BCR/ABL-containing cell lines

P Martiat, P Lewalle, AS Taj, M Philippe, Y Larondelle, JL Vaerman, C Wildmann, JM Goldman and JL Michaux

Updated information and services can be found at:
http://www.bloodjournal.org/content/81/2/502.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml