Translocation Remodeling in the Primary BALB/c Plasmacytoma TEPC 3610

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Myc-activating chromosomal 12;15 translocations, the hallmark mutations of inflammation-induced BALB/c plasmacytomas, have recently been shown to undergo remodeling by isotype switch-like genetic recombinations that remove ~ 180 kb of immunoglobulin heavy-chain sequence in the vicinity of the rearranged, expressed *Myc* gene. Here we combine cytogenetic data on the 12;15 translocation (SKY and FISH) with the molecular analysis of key junction sites (long-range PCR followed by DNA sequencing) to demonstrate that translocation remodeling occurred as an infrequent, stepwise, and disomic tumor progression event in the tetraploid, fully transformed, and transplantable plasmacytoma TEPC 3610. This result was used, in conjunction with previously obtained molecular data on five other primary plasmacytomas, to devise a hypothesis that predicts that the selective pressure to undergo translocation remodeling may be predetermined by the location of the break site in *Myc*. The pressure may be low if the break occurs 5′ of the normal promoter region of *Myc*, but it may be considerably stronger if the break occurs 3′ of the *Myc* promoter.

INTRODUCTION

G-banding of tumor metaphase cells established more than 20 years ago that the great majority (~ 90%) of malignant plasma cell tumors (plasmacytomas) that develop in genetically susceptible BALB/c mice harbor a consistent cytogenetic aberration, the chromosomal 12;15 translocation (Ohno et al., 1979). The T(12;15) generates an enlarged chromosome 12 (hereafter referred to as derivative 12 or der 12) and a reduced chromosome 15 (hereafter referred to as derivative 15 or der 15). It soon became clear that the translocation occurs as a reciprocal genetic exchange that juxtaposes the immunoglobulin heavy-chain gene cluster, *IgH*, on chromosome band 12F1 to the *Myc* proto-oncogene on chromosome band 15D2. Subsequent molecular studies revealed that the translocation results invariably in the deregulated expression of *Myc* (Shen-Ong et al., 1982), a key oncogenic event in BALB/c plasmacytomagenesis (Potter et al., 1992). The most recent methodological breakthrough in the continuing elucidation of the T(12;15) is marked by the development of PCR methods for the detection of 12;15-typical junction fragments between *IgH* and *Myc*. Since its inception, PCR analysis has been used to demonstrate that the translocation is a very early, if not initiating, oncogenic step during plasmacytoma development (Janz et al., 1993), that the fine structure of translocation differs between fully transformed plasmacytomas and their premalignant precursors (Müller et al., 1994), that translocated cell clones are capable of migrating into different tissues (Müller et al., 1997), and that the propensity to undergo translocation is not correlated with the genetic susceptibility to plasmacytoma development (Müller et al., 1996; Roschke et al., 1997) . These findings have contributed significant new insights into the T(12;15) and have supported the proposal that this translocation should be considered a uniquely valuable model system for the better understanding of the multitude of oncogene-activating chromosomal translocations that occur in leukemias, malignant lymphomas, and multiple myelomas in humans.

Molecular analysis of the fine structure of the T(12;15) by the most reliable PCR method available today, long-range PCR (Kovalchuk et al., 2000), led to the realization that plasmacytomas that are typically characterized by genetic recombinations between *Myc* and the most distal locus of the *IgH* gene cluster, *Cα*, can in fact be derived from precursors that are distinguished by recombinations between *Myc* and the most proximal locus

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of the *IgH* gene cluster, *Cμ*. The precursor-product relationship between *Cμ/Myc*-recombined precursors and *Cα/Myc*-recombined progenitors could be unambiguously established in molecular terms when it was shown that the *Cμ/Myc* to *Cα/Myc* transition was caused by aberrant isotype switching on der 12; however, the nature of the cell in which the original *Cμ/Myc* exchange resided, and the sequence of the progression steps that characterized the cytogenetic evolution of this cell, remained largely unknown (Janz et al., 1997; Kovalchuk et al., 1997). Since most plasmacytomas at the time of discovery are (sub)tetraploid and contain two copies of der 12, it is possible that the switch from *Cμ/Myc* to *Cα/Myc* occurs on just one der 12 or, alternatively, on both of them. Moreover, if both derivatives 12 were involved, the switch could take place simultaneously or separated in time. Here we report on the first BALB/c plasmacytoma, TEPC 3610, that revealed some insights on the cytogenetic evolution of der 12. By combining FISH analysis of all 12;15 translocated chromosomes with the detailed molecular elucidation of key junction sites, we were able to demonstrate that the critical transition to the *Cα/Myc* rearrangement occurred as a relatively infrequent, stepwise, and disomic (bichromosomal) tumor progression event at the stage of a fully transformed, tetraploid, and transplantable tumor cell.

MATERIALS AND METHODS

Spectral Karyotyping and Tumor Propagation

Spectral karyotyping (SKY) was performed as described elsewhere (Liyanage et al., 1996; Coleman et al., 1997). Due to its low mitotic index, the primary plasmacytoma (G0) had to be passaged three times (G1–G3) in pristane-primed BALB/c mice before the 10 to 20 matching karyotypes that were required for a reliable cytogenetic evaluation of all clones and subclones were accumulated. TEPC 3610 was propagated in vivo by injecting 1 ml of plasmacytoma-containing ascites into one pristane-primed BALB/c mouse for each of the first two generations (G1/2) and six pristane-primed BALB/c mice for the third generation (G3).

Long-Range PCR and Southern Analysis

Clonotypic chimeric *IgH/Myc* junctions, the molecular indicators of the T(12;15), were detected by PCR and Southern blotting. High-molecular-weight genomic DNA prepared from plasmacytoma nodules was analyzed by long-range PCR methods whose sensitivity and specificity have been described elsewhere (Kovalchuk et al., 2000). Although not strictly quantitative, long-range PCR has been shown to detect abundant and infrequent translocation-indicator fragments by single-round amplifications with one primer pair and two-round amplifications with nested primer pairs, respectively. The indicator fragments were DNA-sequenced to determine the breakpoint in *Myc* (on both products of translocation), identify the heavy-chain constant gene (C_H) *Myc* had been juxtaposed to (on der 12), and elucidate the structure of the unitary or composite Ig switch regions that resided in the intervening sequence between *Myc* and C_H on der 12, or between *Myc* and D_HJ_H on der 15. For Southern analysis, a total of 10 μg of tumor DNA was digested to completion with *Bsm*BI, fractionated by electrophoresis, blotted, and probed with a genomic 2.2-kb SpeI/NheI clone that included exon 2 of *Myc*. The location of the Southern probe and the *Bsm*BI restriction sites in *Myc* and *Cμ* are indicated in Figure 1D by the striped blue bar and filled triangles that are labeled, respectively.

Fluorescence In Situ Hybridization

Myc, *Cμ*, and *Cα* were visualized by FISH (Esa et al., 2000) with the assistance of mouse BAC probes that were labeled with fluorochromes according to standard nick translation protocols. Two different labeling schemas were used. FISH probes for *Myc*, *Cμ*, and *Cα* were tagged with Cy5 (blue), Spectrum-Orange (red), and FITC (green) to mark the metaphase chromosomes shown in Figure 1B and were tagged with Cy5, FITC, and Spectrum-Orange to mark the interphase chromosomes shown in Figure 2. The maximum projection FISH images depicted in Figure 2 (left) were extracted from 3D confocal data sets. Helper images (right) were prepared to facilitate the visual reconstruction of the FISH signals in the maximum projection images, particularly on der 12. The helper images were generated from the combined gray-scale images of the same cell by overlaying the FISH signals detected in the individual channels with computer-enhanced false-color dots that corresponded to the utilized FISH probe.

RESULTS

Spectral Karyotyping

SKY revealed a cytogenetically diverse tumor that is postulated to have originated with the tetraploid precursor clone P (Fig. 1A). This clone must have contained two copies of der 12, as expected for a tumor in which tetraploidization is

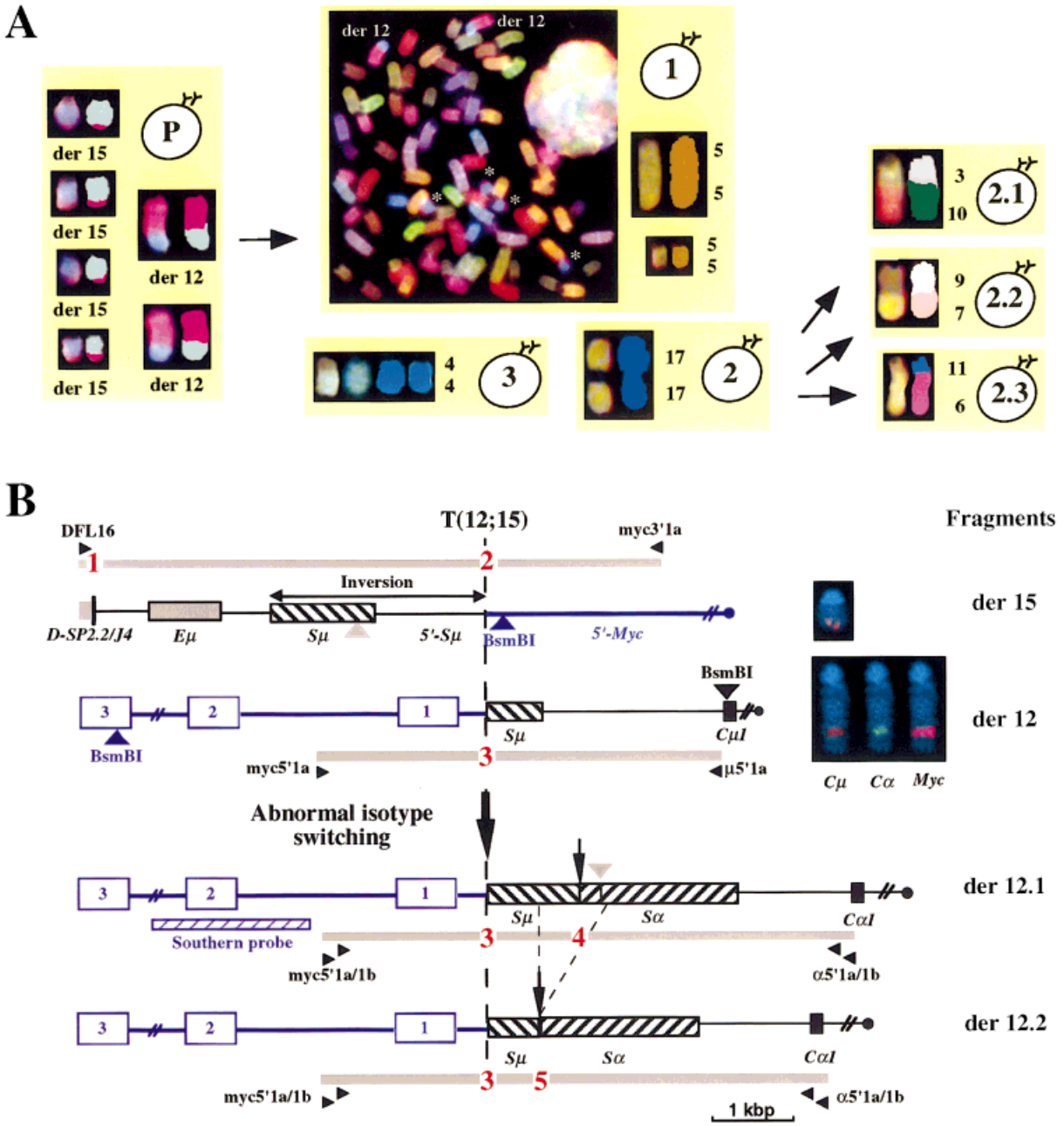

Figure 1. Spectral karyotyping and molecular analysis of the T(12;15). Illustrated are the cytogenetic makeup of TEPC 3610 (**A**), the fine structure of the 12;15 translocation (**B**), the persistence of the *Cμ/Myc* rearrangement during tumor propagation in vivo (**C**), and the abundance of the *Cμ/Myc* rearrangement in relation to the normal *Myc* gene (**D**).

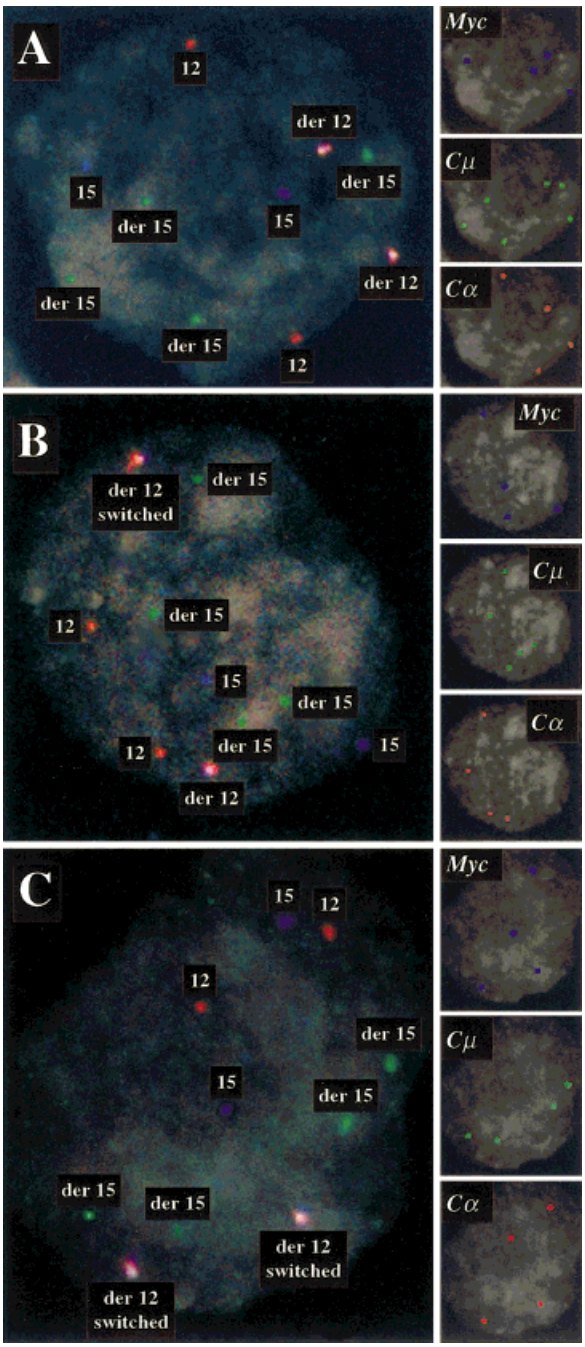

Figure 2. Detection of aberrant isotype switching on der 12 by interphase FISH. Shown are maximum projection images of tumor cells that contained two *Cμ/Myc* rearrangements (**A**), one *Cμ/Myc* and one *Cα/Myc* rearrangement (**B**), and two *Cα/Myc* rearrangements (**C**).

generally preceded by 12;15 translocation (Wiener, 1984). The derivatives 12 are illustrated in the inset that depicts the chimeric chromosomes in SKY display colors to the left and SKY classification colors to the right. The precursor, P, which also contained as many as four copies of der 15, must have evolved into three distinct clones, designated 1, 2, and 3. Clone 1 was distinguished by the reciprocal 5F;5B. Clone 2 was marked by the Rb(17A1,17A1) and its further diversification into three subclones, 2.1, 2.2, and 2.3, which were distinguished by the unique translocations T(3;10)(A4/B;F), T(9;7)(E4;D), and T(11;6)(A;B), respectively. Clone 3 harbored a distinctive T(4;Del4[A2-E])(A;A1). Figure 1A includes a representative metaphase of clone 1 that is shown in SKY display colors. The two copies of der 12 are labeled and the four copies of der 15 are indicated by asterisks. Of significance for the main point of this study is that all tumor cells, regardless of their clonal or subclonal affiliation, contained two copies of der 12. This chromosome harbors the deregulated *Myc* gene, which is thought to be important for the development of plasmacytomas and, presumably, the maintenance of the malignant phenotype.

Molecular Structure of the t(12;15)

The results of the fine structural analysis of the T(12;15) by PCR are summarized in Figure 1B. Presented at the top is a schema of the *Myc* rearrangement on der 15. The genetic exchange recombined the near 5′ flank of *Myc* (junction 2; junction sites are labeled by red numbers) with an inverted (3′ to 5′) segment of DNA that consisted of the truncated *Sμ* region and an approximately 1-kb-long upstream flank of *Sμ*, designated *5′-Sμ*. The extent of the *Sμ/5′-Sμ* inversion is indicated by a horizontal double arrow labeled "Inversion." *Sμ* contained a large internal deletion depicted by a triangle pointing up. The D_HJ_H rearrangement (junction 1; joining *D-SP2.2* with *J4*) that was found upstream of the intronic heavy-chain enhancer, *Eμ*, is also depicted. On der 12, *Myc* was juxtaposed, just 5′ of exon 1, to *Sμ* in the characteristic head-to-head (5′ to 5′) fashion (junction 3). Recombination in *Myc* was perfectly reciprocal, i.e., no nucleotides were found to be lost or added when the *Myc* sequence on der 12 was compared with the corresponding *Myc* sequence on der 15. Junctional fragments, der 12 and der 15, were detected with the primer pairs myc5′1a/μ5′1a and DFL16/myc3′1a, respectively. The primers are designated by small triangles at the ends of the PCR indicator fragments shown as gray horizontal bars; for primer sequences, see Kovalchuk et al. (1997, 2000). FISH labeling of metaphase chromosomes demonstrated the expected three-color signal for der 12 (colocalization of the *Cμ*, *Cα*, and *Myc* probes) and the expected single-color (*Cμ*) signal

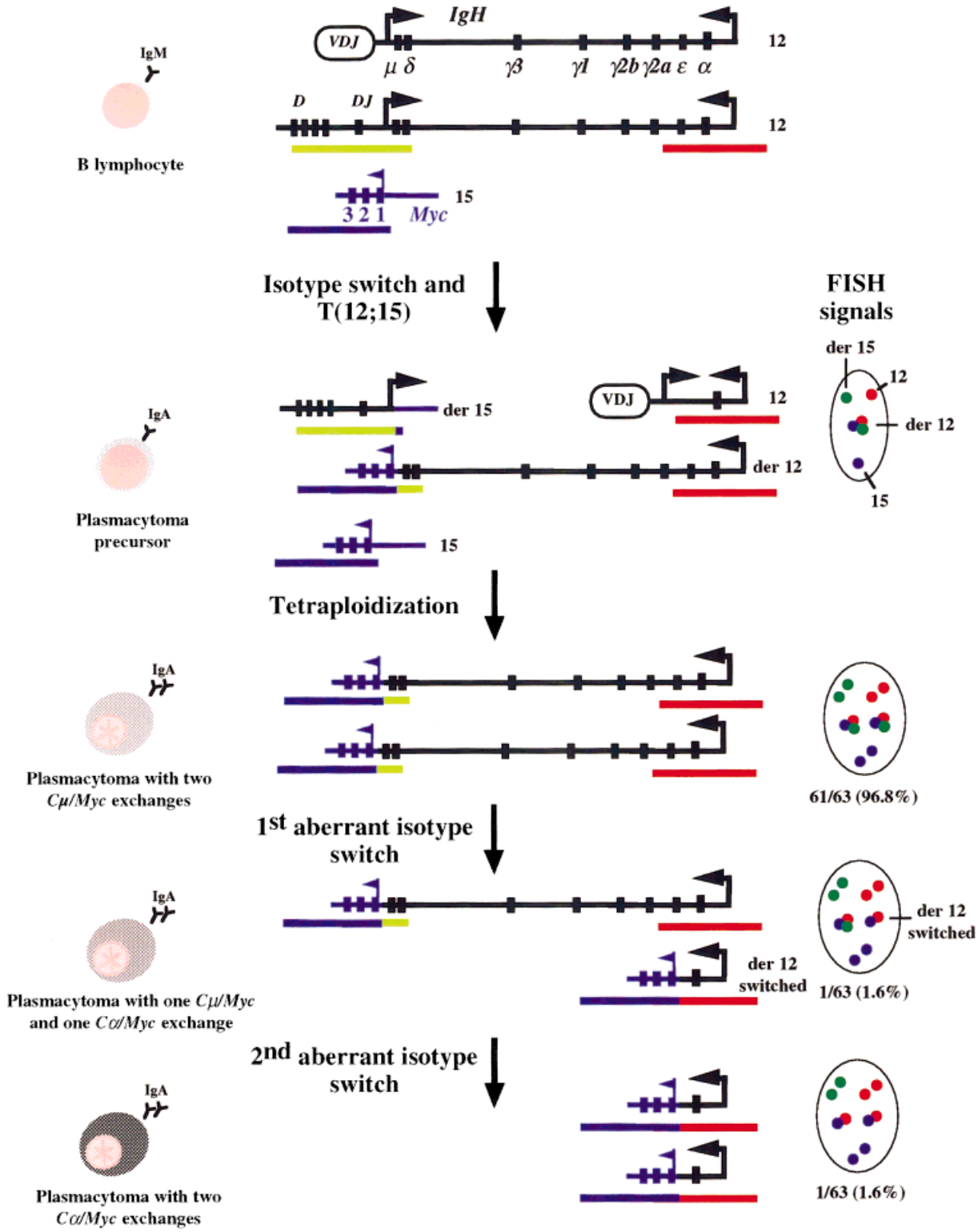

Figure 3. Cellular and cytogenetic evolution of the plasmacytoma TEPC 3610.

for der 15. The recombination partner of *Cμ* on der 15, *Myc*, was not detected because the *Myc* probe covered too small a portion of the gene's upstream flank to be detected by FISH (cf. Fig. 2 and the schema on the approximate boundaries of FISH probes in Fig. 3).

Illustrated at the bottom of Figure 1B are schemas of two additional translocation-indicator fragments, der 12.1 and der 12.2. These fragments must have been derived from the *Cμ/Myc*-recombined precursor, der 12, by an abnormal isotype switch to *Cα* that took place in the immediate vicinity of the breakpoint in *Myc* (junctions 4 and 5). Sites of aberrant switching are indicated by small black arrows pointing down. The descent of der 12.1 and 12.2 from the precursor, der 12, was evidenced by the clonotypic junction between *Myc* and *Sμ* (junction 3); it was common to all three fragments. Two complicating details on the molecular relationship between the three different *Myc* rearrangements will be acknowledged here. First, it is unclear whether der 12.2 was derived from der 12.1 (by an internal deletion, indicated by dashed lines, that eliminated the *Sμ/Sα* junction in the composite switch region of der 12.1), or derived from der 12 (by a *Sμ/Sα* recombination similar to the one that produced the *Sμ/Sα* junction on der 12.1). Second, the precursor, der 12, is thought to have suffered deletions in *Sμ* after giving rise to its progenitor, der 12.1 (because the *Sμ* region in der 12.1 is longer than in der 12). However, these considerations are peripheral and should not distract from the main point that isotype switch-like deletions removed ~ 180 kb of *IgH* sequence (the intervening sequence between *Sμ* and *Sα*) from the *Cμ/Myc*-rearranged precursor, der 12. Junctional sequences 2 and 3 have been published (Kovalchuk et al., 1997), and junctions 1, 4, and 5 have been determined in this study but are not shown. FISH detection of der 12.1 and der 12.2 in metaphase cells was also attempted, but it was not successful because dividing cells containing these rearrangements were not found.

Of importance is that the indicator fragments, der 12 and der 15, were detected with great ease, i.e., only one round of PCR was required, all tumor samples were positive, and fragments were consistently found in repeated PCR reactions. In contrast, the indicator fragments, der 12.1 and der 12.2, were detected with difficulty: two rounds of PCR with nested primer pairs were required, not all tumor samples were positive, and fragments were found sporadically when the PCR was performed in multiple repeats. These results indicated that tumor cells that contained the precursor-type chromosomes der 12 and der 15 were highly abundant, whereas tumor cells that harbored derivative chromosomes with secondary *Cα/Myc* junctions were infrequent, if not rare.

FISH

Three-color FISH was employed to detect the *Cμ/Myc* to *Cα/Myc* transition at the cytogenetic level, and to decide if it occurred as a mono- or disomic recombination event. Figure 2 illustrates how the cohybridization of three FISH probes (*Cμ*, green; *Cα*, red; *Myc*, blue) was used to distinguish the following 10 chromosomes. Chromosomes 15 and 12 (two copies each) were visualized by blue and red signals, respectively. The red-only signal of chromosome 12 was consistent with our finding that the IgA/κ secreting tumor had performed isotype switching to *Cα* and had thereby deleted *Cμ* from the productively rearranged chromosome (data not shown). The sequence of the corresponding productive VDJ recombination (*VHQ52-DFL16.1-JH4*) and the junction with *Cα* (exon 1) was determined by RT-PCR and DNA sequencing (data not shown). The four copies of der 15 were indicated by green-only (*Cμ*) signals because *Myc* was not detectable on this chromosome, as mentioned above. Der 12 (two copies) was visualized by a three-color signal when it contained the *Cμ/Myc* rearrangement but a two-color signal (without green) when it contained the *Cα/Myc* rearrangement. A total of 63 tumor cells had to be analyzed by confocal microscopy (Esa et al., 2000) before at least one clear representative of the following three possible cell types was observed: the first harbored two *Cμ/Myc* rearrangements, the second harbored two *Cα/Myc* rearrangements, and the third coharbored one *Cμ/Myc* and one *Cα/Myc* rearrangement. The overwhelming majority of cells (61 of 63, 96.8%) contained *Cμ/Myc* recombinations on both derivatives 12 (Fig. 2A), and only 1 of 63 (1.6%) cells each exhibited a mixed phenotype (Fig. 2B) or harbored two *Cα/Myc* recombinations (Fig. 2C).

The apparent cytogenetic preponderance of the *Cμ/Myc* rearrangement not only confirmed the PCR result on the predominance of this rearrangement in the genomic DNA, but it was also consistent with the remarkable persistence of the *Cμ/Myc* exchange over three consecutive in vivo passages. Figure 1C illustrates (indicated by the + sign in the center line) that PCR junction fragments representative of the *Cμ/Myc* rearrangement on der 12 and der 15 were consistently detected throughout three consecutive in vivo passages (G1–3) after a single round of PCR. One G3 recipient, G3/6, was negative because the tumor did not take in this mouse (indicated by the − sign). In contrast, PCR indicator fragments typical for the derivative *Cα/Myc* rearrangements on der 12.1 and der 12.2 were

found only after two rounds of PCR, and only sporadically, i.e., in the G0, G3/2, and G3/4 mice (der 12.1) or the G3/1 and G3/5 mice (der 12.2; indicated in the bottom line). FISH data also agreed with Southern analysis of tumor DNA (Fig. 1D), which readily demonstrated the clonal *Cμ/Myc* rearrangement but failed to produce any indication for a clonal *Cα/Myc* rearrangement. Figure 1D depicts the 4.5-kb-long germline fragment of *Myc* (labeled "*Myc*") after digestion at *Bsm*BI sites in exon 3 and the 5′ flank of the gene (~ 200 bp upstream of the translocation breakpoint), respectively. It further shows the 6.2-kb-long, der 12–typical *Cμ/Myc* rearrangement (labeled "*Cμ/Myc*") after restriction with *Bsm*BI in exon 3 of *Myc* and exon 1 of *Cμ*. This fragment was detected in the G2 recipient and all tested G3 recipients (except G3/6; the G0 mouse and the G1 and G3/3 recipients were not included in the analysis). Restriction of the *Cα/Myc* rearrangements, der 12.1 or der 12.2, at the same *Bsm*BI site in *Myc* and the nearest *Bsm*BI site in *Cα* (located in the intervening region between exons 3 and 4) would have resulted in > 9 kb fragments, yet these were not detected. The equal intensity of the normal *Myc* fragment and the *Cμ/Myc* fragment indicated that der 12 (two copies per cell) and chromosome 15 (also two copies per cell) occurred with equal abundance in the tumor. Again, this result was entirely consistent with FISH and PCR.

Natural History of TEPC 3610

The results of the above-described studies permitted us to develop a schema on the molecular and cytogenetic development of TEPC 3610. Figure 3 (top) presents a normal, mature, diploid B-lymphocyte prior to isotype switching. This cell is postulated to have given rise to the plasmacytoma precursor depicted below. It contains two copies of chromosomes 12 and 15. Chromosome 12 contains the *IgH* gene cluster that consists of eight heavy-chain loci, C_H. The intronic heavy-chain enhancer, *Eμ*, and the enhancer complex located 3′ of *Cα* are symbolized by black horizontal arrows pointing right and left, respectively. One chromosome 12 is thought to have undergone productive VDJ rearrangement (indicated by an open oval labeled "VDJ"). It encodes the μ heavy chain, which is expressed, in conjunction with a light chain, as IgM on the surface of the B-cell. The second chromosome 12 is postulated to have aborted VDJ rearrangement after DJ recombination; this is not unusual for B-lymphocytes. Chromosome 15 harbors the *Myc* gene, whose three exons and normal promoter region at the beginning of exon 1 (P1 and P2) are indicated by blue rectangles and a left-pointing arrowhead, respectively. The FISH probes utilized for visualizing *Myc*, *Cμ*, and *Cα* are symbolized by horizontal lines that are colored in blue, green, and red, respectively. The approximate 5′ and 3′ boundaries of the probes (based on PCR mapping; data not shown) are also indicated, even though they were not precisely established by DNA sequencing. Illustrated in the center of Figure 3 is the putative plasmacytoma precursor that must have performed isotype switching on the VDJ-rearranged chromosome 12. Isotype switch recombination joined Sμ with Sα and thereby effected the change from μ heavy-chain expression to α heavy-chain expression. The DJ-rearranged chromosome 12 must have undergone transchromosomal exchange with *Myc*. This resulted in the balanced T(12;15) and juxtaposition of *Myc* to *Cμ*. It is possible that chromosomal translocation was triggered by an attempted isotype switch on the DJ-recombined chromosome, but this has not been demonstrated. Indicated in the lower half of Figure 3 are tetraploidization, a frequently observed tumor progression event in BALB/c plasmacytomas, and the development of the fully transformed, transplantable plasmacytoma that harbored the *Cμ/Myc* exchange on both copies of der 12. The complete set of FISH signals that was diagnostic for this predominant cell type is shown to the right in the symbolic cell nucleus (cf. Fig. 2A). Translocation remodeling effected the *Cμ/Myc*-to-*Cα/Myc* conversion in a minority of tumor cells, either on one copy of der 12 (cf. Fig. 2B) or on both copies of der 12 (cf. Fig. 2C). Note that remodeling was accompanied by the transition of one or two three-color FISH signals (including the green component) to two-color FISH signals (lacking the green component). Note also that the utilized *Myc* probe, which barely reached into the 5′ flank of the gene, was not suitable for detecting der 15.

DISCUSSION

This study has documented that the remodeling of the *Myc*-activating product of the 12;15 translocation (der 12) occurred as a late, infrequent, stepwise, and disomic tumor progression event in the tetraploid, fully transformed, and transplantable plasmacytoma TEPC 3610. When translocation remodeling was first described in mouse plasmacytomas, it was hypothesized that its biological significance may be coupled to the upregulation of *Myc* expression that is thought to be effected by the

180-kb approximation of *Myc* to the powerful 3′-*Cα* enhancers (Kovalchuk et al., 1997). The hypothesis implied that *Myc* expression may be insufficient for plasmacytomagenesis when the gene is recombined with *Cμ* (~ 200 kb away from the 3′-*Cα* enhancers), but rendered adequate for plasmacytomagenesis when the gene is drawn near *Cα* (in proximity to the 3′-*Cα* enhancers). However, the finding that TEPC 3610 was capable of completing plasmacytomagenesis in spite of retaining the primary *Cμ*/*Myc* rearrangement on der 12, combined with the observations that the *Cμ*/*Myc* exchange persisted in three consecutive in vivo passages and that *Myc's* switch to *Cα* occurred only occasionally and relatively late during tumor progression, strongly suggested that *Myc* expression was perfectly sufficient in this case to complete tumor development.

How can this result be reconciled with the above-mentioned postulate on the functional consequence of translocation remodeling? We note that TEPC 3610 is similar to three other plasmacytomas that are characterized by translocation remodeling—TEPC 1194 (Kovalchuk et al., 1996), TEPC 3609, and 4127 (Kovalchuk et al., 1997)—in that the tumor harbors a class II translocation of *Myc* (Cory, 1986). All four class II–translocated tumors showed the same pattern in long-range PCR analysis: the original *Cμ*/*Myc* fragment was abundant, while the derivative *Cα*/*Myc* fragments were rare. In contrast, two additional plasmacytomas—4132 and 5974 (Kovalchuk et al., 1997, 2000)—that were also distinguished by translocation remodeling but contained a class I translocation of *Myc* (Cory, 1986) demonstrated the opposite pattern in PCR analysis: the *Cμ*/*Myc* fragment was rare, while the *Cα*/*Myc* fragments were abundant. This inverse relationship raises the intriguing possibility that *Myc* expression levels, and pressure to perform aberrant isotype switching to *Cα*, may be tightly linked with the translocation breakpoint in *Myc*. *Myc* expression may be sufficient for plasmacytomagenesis after rearrangement with *Cμ* if the breakpoint in *Myc* occurs upstream of its physiological promoters, P1 and P2 (i.e., class II translocation). The pressure to undergo aberrant isotype switching may be modest in these cases, as illustrated here for TEPC 3610, and aberrant switching may reflect no more than an epiphenomenon with little if any impact on tumor progression in vivo. Conversely, *Myc* expression levels may be insufficient for plasmacytomagenesis after genetic exchange with *Cμ* if the breakpoint in *Myc* decapitates the gene by allocating the P1/P2 promoters to the reciprocal product of translocation (i.e., class I translocation). The selective pressure to perform aberrant isotype switching to *Cα* may be significantly stronger in these cases, and abnormal switching may play an important (essential?) role in tumor progression in vivo.

At present, all six plasmacytomas that have been characterized in terms of translocation remodeling adhere to this theory, which is based on significant differences in the efficiency of the 3′-*Cα* enhancers to establish long-range interaction with and effect activation of *Myc*, either in the presence or absence of its normal promoter region. Additional studies are required to evaluate this testable hypothesis. Moreover, additional plasmacytomas will have to be assessed by the combined methodological approach employed here before it can be decided whether TEPC 3610 is an outlier or, as favored by us, an authentic example for a potentially interesting link between the molecular distribution of translocation breakpoints in an oncogene and the built-in tendency of these translocations to undergo secondary, targeted, structural alterations.

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