SHORT COMMUNICATION

Differential expression of the splicing regulatory factor genes during two-step chemical transformation in a BALB/3T3-derived cell line, MT-5

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Although the alternative splicing of various genes is a common event in human tumors, the mechanisms behind it have not been characterized. We hypothesized that the expression of splicing regulatory factors would be changed during cellular transformation. Gene expression of three splicing regulatory factors, alternative splicing factor/splicing factor 2 (ASF/SF2), heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) and the 65 kDa subunit of U2 small nuclear ribonucleoprotein particles auxiliary factor (U2AF65), were examined by northern blotting in a two-step chemical transformation model. This in vitro model is composed of BALB/3T3 cells and a BALB/3T3-derived N-methyl-N-nitro-N-nitrosoguanidine (MNNG)-initiated cell line (MT-5). MT-5 cells can be transformed on exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA). ASF/SF2 mRNA levels were decreased 2-fold in both MNNG-initiated cells and TPA-induced transformed cells compared with the normal parental cells, whereas hnRNP A2 mRNA expression did not significantly change between these three types of cells. U2AF65 mRNA levels were markedly increased (~4.7-fold) associated with progression of cellular transformation. Moreover, RT–PCR analysis showed that distinct forms of ASF/SF2 mRNA were present in the MNNG-initiated cells and TPA-induced transformed cells but not in the parental cells. These findings indicate that ASF/SF2 or U2AF65 gene expression is altered during in vitro two-step chemical transformation. The data suggest that the differential expression of splicing regulatory factors is one cause of aberrant expression of alternatively spliced mRNAs encoded by various genes in tumor cells.

The splicing of pre-mRNA is a fundamental mechanism in the expression of most eukaryotic genes encoding functional proteins. In the case of alternative splicing, flexibility in the selection of splice sites is important in increasing the coding capacity of genes and allows for the generation of several protein isoforms from one gene. Alternative pre-mRNA splicing occurring in many genes is controlled in a tissue-specific or developmental-specific manner (1,2). Recently, the expression of alternatively spliced mRNAs encoding a variety of altered forms of proteins, such as growth factors, products of tumor suppressor genes or proto-oncogenes, have been reported in various tumors including pancreatic cancers (3), breast cancers (4), gastrointestinal cancers (5) and head and neck cancers (6). This way of producing altered forms of proteins seems to play an important role in some steps of tumorigenesis and the relaxation of pre-mRNA splicing fidelity may contribute generally to abnormal expression of normal cellular genes in tumors. Although tumor-specific alternative splicing of pre-mRNA appears to be a common phenomenon, the regulatory mechanisms that link it to tumorigenesis have not been defined.

The factors involved in pre-mRNA splicing have been studied extensively in recent years. Alternative splicing factor/splicing factor 2 (ASF/SF2), which is known to be an essential factor for constitutive and alternative splicing of pre-mRNA. It is a member of the SR protein family (9) and contains a RNA-binding domain (RBD) at the N-terminus and a region rich in arginine and serine residues (RS domain) at the C-terminus. The heterogeneous nuclear ribonucleoprotein (hnRNP) protein A2 (10) is similar to A1 and belongs to the basic protein subset of the hnRNP complex in mammalian cells. ASF/SF2 and hnRNP A1 regulate alternative pre-mRNA splicing through antagonistic effects on 5’ splice sites selection in a concentration-dependent manner in vitro and in vivo. An excess of hnRNP A1 favors the use of distal 5’ splice sites, whereas an excess of ASF/SF2 promotes the use of proximal 5’ splice sites (11,12). In addition, hnRNP A2, which has 68% amino acid identity with hnRNP A1, was shown to have stronger 5’ splice site switching activity than hnRNP A1 (13). U2 small nuclear ribonucleoprotein particles auxiliary factor (U2AF) has also been extensively characterized and shown to play an important role in RNA splicing (14). U2AF is composed of two subunits, a 65 kDa protein (U2AF65) and an associated 35 kDa protein. Of these two polypeptides, U2AF65 binds to the pyrimidine tract at the 3’ splice site and is required for in vitro splicing of two model pre-mRNA substrates (15).

These splicing regulatory factors are necessary not only for constitutive splicing, but also for selection of alternative splice sites. The mechanisms of tumor-specific alternative pre-mRNA splicing occurring during tumorigenesis have not been thoroughly investigated. Thus, studies on the regulation of these splicing regulatory factor genes during neoplastic transformation are needed. It was previously reported that MT-5 cells, established as a mouse fibroblast BALB/3T3-derived N-methyl-N’-nitro-N-nitrosoguanidine (MNNG)-initiated cell clone, could be chemically transformed on exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) and form colonies in soft agar medium (16). A series of normal and transformed mouse fibroblasts, such as parental BALB/3T3 cells (17) and MT-5 cells treated with or without TPA, form a unique cellular model for studying the roles of splicing regulatory factors in influencing alternative splicing site selection during two-step chemical transformation. In the present study, we investigated the expression of ASF/SF2, hnRNP A2 and U2AF65 mRNA.
to determine whether differential expression of one or more of these splicing regulatory factors is associated with two-step chemical transformation in vitro.

BALB/3T3 cells were obtained from the Cancer Cell Repository (CCR) of Tohoku University (Sendai, Japan) and MT-5 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). All cell lines were cultured in Eagle’s minimum essential medium (Gibco BRL, Grand Island, NY) supplemented with 10% newborn calf serum (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO₂. MT-5 cells were grown in the presence or absence of TPA. MT-5 cells exposed to 100 ng/ml TPA (Sigma, St Louis, MO) for ~2 weeks showed morphological changes and had a cobblestone-like appearance (data not shown). Total RNA was isolated from cells at 80–90% confluency using a RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Northern blot analysis was performed in the parental cells (BALB/3T3) and MNNG-initiated cells (MT-5 cells without TPA) and TPA-induced transformed cells (MT-5 cells with TPA) to test whether gene expression of ASF/SF2, hnRNP A2 and U2AF65 were changed during the process of two-step chemical transformation. Twenty micrograms each of total RNA were denatured using glyoxal and electrophoresed in 1% agarose gels, then transferred to Hybond-N nylon membranes and the TPA-induced transformed cells showed a 4.7-fold increase in U2AF65 expression relative to the parental cells (Figure 2). These results suggest that down-regulation of ASF/SF2 expression caused inactivation of 5' proximal splice site selection in the initiation step of the chemical transformation process. Another splicing factor we examined was U2AF65. U2AF65 is important in the definition of 3' splice sites at the earliest stage of spliceosome assembly (18,19). The level of U2AF65 mRNA was markedly increased associated with the process of two-step chemical transformation. The MNNG-initiated cells showed a 2.6-fold increase in U2AF65 expression relative to the parental cells and the TPA-induced transformed cells showed a 4.7-fold increase relative to the parental cells (Figure 2). These findings suggest that step-up activation of recognition of the 3' splice sites, together with activities of other splicing regulatory factors, affects the alternative splicing system and may contribute to phenotypic changes in the parental cells during tumorigenesis.

Mouse ASF/SF2 pre-mRNA contains a 196 bp alternative intron and a distinct form of mRNA, designated ASF-3 mRNA, was generated by retention of this alternative intron in the process of alternative splicing. If the alternative intron is not removed from the ASF/SF2 pre-mRNA, the protein product would be truncated at a termination codon encountered near the 5'-end of the intron (7). ASF-3 protein, a truncated form of ASF/SF2 protein, thus shares the RBD with ASF/SF2 but
glyceraldehyde 3-phosphate dehydrogenase mRNA and the relative such differential expression was a result of cellular transformation. It is not of conserved domains of hnRNP A1 and other hnRNP A/B proteins.

Northern blot analysis of U2AF 65 gene expression in BALB/3T3, non-treated MT-5 and TPA-treated MT-5 cells. The relative expression levels of U2AF 65 mRNA were determined by comparison with the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA and the relative expression levels are depicted in the figure. Similar results were obtained in two separate experiments and a typical result is shown.

Fig. 2. Northern blot analysis of U2AF 65 gene expression in BALB/3T3, non-treated MT-5 and TPA-treated MT-5 cells. The relative expression levels of U2AF 65 mRNA were determined by comparison with the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA and the relative expression levels are depicted in the figure. Similar results were obtained in two separate experiments and a typical result is shown.


Fig. 3. RT–PCR analysis for ASF-3 and ASF/SF2 mRNA expression using total RNA isolated from BALB/3T3 (lane 1), non-treated MT-5 (lane 2) and TPA-treated MT-5 (lane 3) cells. PCR reactions were electrophoresed in 1% agarose gel and the gel was stained with ethidium bromide. An ASF/SF2-specific band of 485 bp was observed in each lane, while an ASF-3-specific band of 681 bp was detected only in lanes 2 and 3. M, molecular weight marker 6X174/HaeIII.

differs from it in that it lacks the RS domain. Although in vitro studies showed that the RS domain is unnecessary for certain functions performed by ASF/SF2 (20,21), the exact role of the RS domain remains to be elucidated. To examine ASF-3 gene expression, RT–PCR analysis with strand-specific primers for ASF/SF2 was performed. One microgram of total RNA isolated from the cells was digested with RNase-free DNase I (Promega, Madison, WI) and then reverse transcribed in a reaction mixture containing 500 ng of oligo(dT) primers and 200 U of M-MLV reverse transcriptase (Wako Pure Chemicals, Osaka, Japan). One microgram of the RT samples was amplified by PCR in a reaction mixture as previously described (22). The PCR condition was as follows: 30 cycles of denaturation (94°C, 30 s), annealing (54–58°C, 1 min) and extension (72°C, 1 min). Our primers were designed to flank the region containing the alternative intron. Confirmation of the PCR products revealed that the fragment at 485 bp was the original ASF/SF2 and the band at 681 bp was ASF-3. As the band intensity of ASF-3 was weak, it seems that the amount of ASF-3 relative to ASF/SF2 is very small. ASF-3 mRNA was not detected in the parental cells but was in both MNNG-initiated cells and TPA-induced transformed cells (Figure 3). It is interesting that the splicing regulatory factor itself was alternatively spliced during cellular transformation. It is not known whether ASF-3 protein is active or has some functional role, but these results raise the possibility that this isoform is involved in neoplastic transformation. To determine whether ASF-3 protein has some function during neoplastic transformation or not, further studies are needed.

We have shown that altered gene expression of splicing regulatory factors occurs during two-step chemical transformation in our in vitro model. It is not known whether decreases in ASF/SF2 expression or increases in U2AF 65 expression actually alter the alternative splicing pattern of any genes in the cells during our two-step cellular transformation. However, previous in vivo experiments indicated that overexpression of splicing factors, such as U2AF 23 or ASF/SF2 24, influences the selection of alternative splice sites in some genes. Our results support the hypothesis that the alternatively spliced RNAs of various genes commonly expressed in tumor tissues may be caused by differential expression of splicing regulatory factors and give a clue as to the nature of tumor-specific alternative splicing. In addition, our findings suggest that gene expression of ASF-3 participates in tumor progression. It remains unclear whether differential expression of splicing regulatory factors cellular transformation or whether such differential expression was a result of cellular transformation. An understanding of the regulatory mechanism of alternative splicing occurring in neoplastic transformation may help to elucidate the cause of abnormal gene expression in cancer.

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References


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