Cell Cycle-dependent Usage of Transcriptional Start Sites

A NOVEL MECHANISM FOR REGULATION OF CYCLIN B1*

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Cyclin B1 mRNA is expressed temporally throughout the cell cycle with peak expression in G2 and M phase. Both transcriptional and posttranscriptional controls are important for this cell cycle-dependent regulation of cyclin B1 mRNA. In this study, we observed that cyclin B1 has two major transcripts: (a) a constitutively expressed transcript, and (b) a cell cycle-regulated transcript expressed predominantly during G2-M phase. These different transcripts are due to alternative start sites. The constitutively expressed transcript starts 65 bases upstream from the cell cycle-regulated message. Changes in mRNA stability did not appear to control the expression of the cell cycle-specific transcript, but we were able to identify a 24-base pair region of the cyclin B1 promoter spanning the start site of the cell cycle-regulated transcript that was critical for its cell cycle-regulated promoter activity. This suggests that transcriptional regulation is responsible for controlling the presence of each message. The 24-base pair sequence required for cell cycle regulation was notable for containing the nucleotides GGCT repeated three times. The possibility that these two transcripts might be physiologically distinct was raised when the cell cycle-specific transcript was found to be translated more efficiently in vitro than the constitutively expressed transcript. These results characterize a novel mechanism for the regulation of cyclin B1 throughout the cell cycle that is dependent upon the use of different transcriptional start sites.

The cell cycle-dependent expression of cyclin B1, the regulatory subunit of mitosis promoting factor (MPF), is critical for the proper timing of a cell’s entry into mitosis. The cyclin B1 protein accumulates during interphase and peaks at the G2-M phase transition. This pattern of expression is seen not only at the protein level, but also at the mRNA level in somatic cells (1–3). The levels of cyclin B1 mRNA are regulated at both the level of transcription and the level of message stability. Previously reported data from our laboratory have shown that cyclin B1 mRNA is highly stable in G2 and mitosis but is markedly less stable in G1 (4). Although these variations in message stability contribute to the cell cycle regulation of the cyclin B1 gene, transcriptional regulation also influences the expression of cyclin B1 mRNA.

Several studies have identified the transcriptional start sites for cyclin B1 (5–7). However, the locations of these start sites differ from each other. Using primer extension and RNase protection assays, Piaggio et al. (6) identified a start site that is located 63 bases upstream of the start site identified by Cogswell et al. (7) using RNase protection assays. In addition, Pines and Hunter (5) defined the cyclin B1 transcriptional start site at an intermediate position. In this report, we describe the experiments that we performed to reconcile these seemingly disparate results.

Studies of the human cyclin B1 promoter show that transcriptional variation also plays a role in the regulation of the cyclin B1 gene (6–8). The upstream region of the human cyclin B1 gene has been isolated and shown to have cell cycle-regulated promoter activity. Piaggio et al. (6) demonstrated that the activity of the cyclin B1 promoter was minimal in quiescent cells and gradually increased after serum stimulation. Our laboratory has shown that the cyclin B1 promoter has cell cycle regulation; its activity was low in G1 and maximal in G2 and mitosis (8). Deletions from the 5’ end of the cyclin B1 promoter allowed us to identify a region of 90 bp that retained cell cycle-regulated promoter activity. Cogswell et al. (7) have found that the activity of the cyclin B1 promoter was greater in G2-M phase than in S phase using in vitro transcription assays and transfected cells. They suggested that an E-box element within the cyclin B1 promoter contributed to its cell cycle regulation. In their experiments, upstream stimulatory factor (USF) was found to bind to the E-box in a cell cycle-dependent manner. The binding of upstream stimulatory factor to the E-box was shown to be increased in cells blocked in G2-M phase compared with cells blocked in S phase or cycling cells. This suggested that upstream stimulatory factor may play a role in the transcriptional regulation of cyclin B1 during the G2-M phase transition. In a more recent report, Katula et al. (9) found that cell cycle-regulated activity could be conferred by a smaller fragment excluding the E-box. The promoter activity of this fragment was dependent upon the presence of two CCAAT box elements (9).

Farina et al. (10) also proposed a role for the E-box in cyclin B1 expression during the G2–G1-phase transition. They found that the Max protein binds to the E-box consensus element in quiescent cells, suggesting that the E-box has an inhibitory role during G0. Because the 90-bp region of the cyclin B1 promoter that we have identified that confers cell cycle regulation does not include the E-box element, it appears to be unnecessary for cell cycle control. These contrasting results imply that the E-box is not the only element that plays a role in the regulation of cyclin B1 expression throughout the cell cycle. It is most likely that multiple elements are important for the cell cycle-dependent regulation of cyclin B1 mRNA expression.

In this report, we describe the identification of two cyclin B1

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‡ The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction.
transcripts: (a) a constitutive transcript that appears in several phases of the cell cycle, and (b) a cell cycle-regulated transcript that appears only during G2-M phase. In addition, these two cyclin B1 transcripts may also be regulated at the translational level.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cell Synchronization, and RNA Extraction—**HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. Cells were blocked at different phases of the cell cycle by treatment with one of the following: (a) aphidicolin (1 μg/ml) for 16 h, (b) mimosine (400 μM) for 24 h, or (c) nocodazole (0.04 μg/ml) for 16 h. Total RNA was extracted from HeLa cells using TRIzol reagent (Life Technologies, Inc.), which is an adaptation of the guanidine isothiocyanate method described by Chomczynski and Sacchi (11).

**Synthetic Oligonucleotides—**Oligonucleotides were synthesized by the University of Pennsylvania Cancer Center (Philadelphia, PA). The sequences of these oligonucleotides are shown in Table I.

**Primer Extension Analysis—**An oligonucleotide consisting of 20 bp of the 5′ sequence from the cDNA of the human cyclin B1 gene (from positions 10 to 30) was labeled at the 5′ end with [γ-32P]ATP and T4 polynucleotide kinase. 5 × 10^5 cpm of the labeled primer were hybridized with 90 μg of RNA in 1× aqueous hybridization buffer (1 mM NaCl, 167 mM HEPES, pH 7.5, and 335 μM EDTA, pH 8.0) overnight at room temperature. The primer extension reaction was carried out as described previously (12). The extended products were then separated and analyzed by electrophoresis on a 6% denaturing polyacrylamide gel.

**Reverse Transcription-PCR—**An oligonucleotide corresponding to the human cyclin B1 cDNA sequence from positions 250 to 270 (referred to as cycB-250 RT) was used as the primer for cDNA synthesis in the reverse transcription reaction. 5 μl of primer was annealed to 4 μg of RNA. Reverse transcription was carried out at 42 °C for 90 min. The cDNA synthesized from each reverse transcription reaction was used for two separate PCR amplifications. The cycB-250 RT oligonucleotide was used for the downstream primer in both PCR amplifications. For one set of reactions, the oligonucleotide ccreg.start.887 was used for the upstream primer. For the other set of reactions, the oligonucleotide con.start.848 was used for the upstream primer. The sequences of both of these oligonucleotides lie within the sequence of the human cyclin B1 promoter at positions 887–913 and 848–873, respectively. The products of each PCR amplification were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

**In Vitro Transcription and Translation—**The DNA templates used for in vitro transcription reactions were PCR-amplified from a plasmid containing the human cyclin B1 promoter upstream of a luciferase reporter gene (pGL2-upB) (upstream region of cyclin B1)) using the following oligonucleotides for primers: (a) T7.ccreg.start for the upstream primer for the cell cycle-regulated template, (b) T7.const.start for the upstream primer for the constitutive template, and (c) lucid.down.stop for the downstream primer for both templates. The T7.const.start primer contains the T7 promoter for RNA synthesis and spans the region from positions 885 to 907, which includes the cell cycle-regulated start site. The T7.const.start primer contains the T7 promoter and spans the region from positions 823 to 845, which includes the constitutive start site. The lucid.down.stop primer is located within the luciferase coding region (positions 802–826) and includes a stop codon. Capped RNA transcripts were synthesized from these PCR-amplified templates using the RiboMAX System (Promega). 200 ng of each RNA transcript (along with 100 ng of a luciferase control transcript) were used in an in vitro translation reaction containing rabbit reticulocyte lysate (Promega), [35S]methionine, and an amino acid mixture. The labeled proteins were then denatured and separated by 10% SDS-polyacrylamide gel electrophoresis. The levels of labeled protein were quantitated using optical densitometry.

**Plasmids and Transient Transfection Assays—**The pGL2-upB plasmid contains the full-length promoter of human cyclin B1 upstream of the luciferase reporter gene (8). Plasmids containing cyclin B1 promoter sequences downstream of either the constitutive start site or the cell cycle-regulated start site regulated by the SV40 promoter were constructed by using PCR. The appropriate fragments were amplified and subcloned into pGL2-PROMOTER (Promega), which contains the SV40 promoter upstream of the luciferase reporter gene. The plasmid containing the SV40 promoter-controlled constitutive start site sequences is referred to as SV.const.start. The plasmid containing the SV40 promoter-cell cycle-regulated start site sequences is referred to as SV.ccreg.start. A luciferase plasmid containing the cyclin B1 promoter up to position 842, which includes the constitutive start site, was generated by PCR amplification of the appropriate fragment followed by subcloning into pGL2-BASIC (Promega). This plasmid is referred to as upB.const.842. A plasmid containing the cyclin B1 promoter up to position 896, which includes the cell cycle-regulated start site, was generated by digesting a plasmid containing the cyclin B1 promoter that contains a BamHI linker from positions 897 to 908 with BglII to excise the sequences downstream of the cell cycle-regulated start site. This remaining plasmid was re-ligated and is referred to as upB.ccreg.896.

The pGL2-upB373 plasmid contains the full-length human cyclin B1 promoter with a 76-bp deletion at its 3′ end from positions 873 to 949. This was accomplished by removing the cyclin B1 promoter from pGL2-upB by digestion with Kpn1 and Xhol. The cyclin B1 promoter was then digested with DpnI to remove the 76-bp region from the 3′ end before re-ligating it back into the pGL2-BASIC plasmid. HeLa cells were transiently transfected with various plasmids using the calcium-phosphate method as described previously (8, 13). The pSVβ-gal plasmid was co-transfected as an internal control for transfection efficiency. The transfected cells were then treated with aphidicolin to block them in G1-S phase or with nocodazole to block them in G2-M phase. Cells were harvested using reporter lysis buffer (Promega) and assayed for luciferase and β-galactosidase activity as described previously (8).

**RESULTS**

**Variation of the 5′ Ends of the Cyclin B1 mRNA with the Cell Cycle—**To determine whether the transcriptional start sites of the cyclin B1 gene vary throughout the cell cycle, we performed primer extension analysis on total RNA extracted from HeLa cells blocked in different phases of the cell cycle. HeLa cells were treated with one of the following drugs before RNA extraction: (a) aphidicolin to block cells at G1-S phase, (b) mimosine to block cells in late G1, and (c) nocodazole to block cells in mitosis. Using flow cytometry, we have previously found that 70–75% of a population of cells were arrested at the G1-S-phase boundary by aphidicolin, 65–75% of cells were arrested in G1 by mimosine, and 85–90% of cells were blocked in G2-M phase by

| TABLE I | Synthetic oligonucleotides |
|---------|-----------------------------|
| coding-cycB | 5′-AGCCAGAGAGGCTTCGACCCG-3′ |
| cycB-250 RT | 5′-TCTTCAAGGAGGTTGCGCGGATTGT-3′ |
| ccreg.start.887 | 5′-CTTTCTCCTGGGCTGCGCCTGGCGC-3′ |
| con.start.848 | 5′-AGGGAGGAGGCTGCGGGGTTAAT-3′ |
| T7.ccreg.start | 5′-ATGGTTAATACGCTATAGGCGGGTTGCTCGTCCGCGGTTGCGGCG-3′ |
| T7.const.start | 5′-ATGGTTAATACGCTATAGGCGGGTTGCTCGTCCGCGGTTGCGGCG-3′ |
| lucid.down.stop | 5′-TTATCGTAAACACGCTCTTCTCCAAA-3′ |

![Image](406x664 to 456x729)
nocodazole (8). An oligonucleotide corresponding to sequences from the 5′ end of the human cyclin B1 cDNA was used as the radiolabeled primer. As shown in Fig. 1, two major transcripts were found: (a) a cell cycle-regulated transcript, and (b) a constitutively expressed transcript. A longer transcript that mapped to 117 bases from the 3′ end of the primer was observed in equivalent amounts in HeLa cells blocked in late G1, G1-S phase, and G2-M phase. A shorter transcript that mapped to 52 bases from the primer was observed only in HeLa cells blocked in mitosis. As expected, we observed a greater amount of total cyclin B1 mRNA in G2-M phase than in late G1 or G1-S phase.

The presence of the longer transcript was confirmed by reverse transcription-PCR. An oligonucleotide composed of a sequence from within the coding sequence of human cyclin B1 (cycB-250 RT) was used as the downstream primer. An oligonucleotide (con.start.848) that can detect only the longer transcript was used as the upstream primer. The predicted PCR product of 351 bp was detected in cells blocked in all phases of the cell cycle, as shown in Fig. 2. An upstream primer (ccreg.start.887) that can detect both transcripts was also used as a positive control. The resulting PCR product amplified from these reactions is 312 bp. A larger amount of this PCR product is observed in cells blocked at G2-M phase because the upstream primer used in these reactions detects both cyclin B1 transcripts that are present at this time during the cell cycle. No products were seen when reverse transcriptase was omitted from the reactions.

These experiments indicate that there are two different cyclin B1 transcripts. One transcript is constitutively expressed and is transcribed from a start site that has been mapped by Piaggio et al. (6). The other, shorter transcript varies markedly throughout the cell cycle. The start site for this transcript corresponds to the start site that was mapped by Cogswell et al. (7). These experiments reconcile the reports of two different transcriptional start sites (shown in Fig. 3). One start site appears to be differentially regulated and cell cycle dependent, whereas the other is constitutively active throughout the cell cycle.

Transcriptional Start Sites of Cyclin B1

CTCTCCAGGTGGCGCTGCAGCTGCGGCCAGAGCAGGCAGGCGAGGAAGAGA 700
CCACGTAGAGGCTGGCAGGCCTGCTGGCTAGGCTGACAGCCGCCGC 750
CCCTCCGAGACGCTCGGCAGTGCAGCTGCCCTGGAACGAGATTCTCTGCGAC 800

constitutive

CGGCAGCCCGCAGATGGAAAGGGAAGTGAGTGCCAGCAACAGGCCAAATAGG 850

Piaggio et al.

cell cycle-regulated

AGGAGACGTGCGGGTGTTAATCTGAGGCTAGGCTGCTCTTCCTCGGC 900

Pines and Hunter

TGGCTCGGGCGGAAACGCGCTTGTTTCTCTGTGGGTAGTCTCTGCTGCCTGG 949
GGCCGCTCCCTGTCTGGTCTTCTGCCTCCTGCCGACTGCTGGTGAG 1000
AGGAAGCATTG

first MET
G1-S phase and G2-M phase. The plasmid containing the SV40 promoter upstream of the constitutive start site, SV.const.start, also had approximately the same amounts of activity in both G1-S phase and G2-M phase. Both of these plasmids behave similarly to pGL2-CONTROL, a plasmid containing the SV40 promoter upstream of a luciferase reporter gene. The pGL2-upcB plasmid, which contains the full-length cyclin B1 promoter, was included in this experiment as a control for cell cycle regulation. The cyclin B1 promoter was found to be approximately 4-fold more active in G2-M phase than in G1-S phase. These results would suggest that the sequences within the 5' untranslated regions of the cyclin B1 transcripts do not confer changes in mRNA stability that could regulate the expression of the two different cyclin B1 transcripts.

Cell Cycle Regulation by the Cyclin B1 Promoter—To identify a region of the human cyclin B1 promoter that is critical for its cell cycle-regulated promoter activity, we constructed a series of plasmids containing various deletions that span the cell cycle-regulated transcriptional start site, as diagrammed in Fig. 5A. The cell cycle-regulated activity of these plasmids was tested by transiently transfecting them into HeLa cells, followed by treatment with cell cycle inhibitors to block the transfected cells at particular points in the cell cycle. The results of these experiments are shown in Fig. 5B. The full-length cyclin B1 promoter, upcB, had approximately 4-fold greater activity in G2-M phase compared with that in G1-S phase. The cyclin B1 promoter with the sequences downstream of the cell cycle-regulated start site would be transcribed and translated in vitro to compare the amounts of protein synthesized from these transcripts. Luciferase sequences were also transcribed and translated in vitro for normalization. The levels of 35S-labeled proteins were quantitated using optical densitometry.
deleted, upcB.creg.896, also had approximately 4-fold greater activity in G2-M phase compared with that in G1-S phase. The cyclin B1 promoter constructs containing deletions that span the cell cycle-regulated start site, upcBA873 and upcB-.const.842, had approximately equivalent levels of activity in both G1-S phase and G2-M phase; i.e. cell cycle regulation was not observed. The sequences that were deleted in both of these plasmids contain the cell cycle-regulated transcriptional start site. Thus, the presence of this transcriptional start site is critical for the increased level of promoter activity during G2-M phase. These results also identify a 24-bp sequence, which is shown in Fig. 5A, that is necessary for cell cycle-regulated promoter activity.

**Translational Efficiency of Each Cyclin B1 Transcript in Vitro**—The presence of two cyclin B1 transcripts raises the possibility of differences in translational efficiency between the constitutively expressed transcript and the cell cycle-regulated transcript that could result in different levels of protein synthesis throughout the cell cycle. To determine if this might be possible, we compared the *in vitro* translational efficiency of each transcript. Template DNA for *in vitro* transcription was PCR-amplified from a cyclin B1 promoter-luciferase plasmid using a primer within the luciferase coding region and either a primer that spans the constitutive start site or a primer that spans the cell cycle-regulated start site. Both of these primers contain promoter sequences for T7 polymerase. After *in vitro* transcription, equal amounts of each mRNA transcript were translated *in vitro* in a rabbit reticulocyte lysate system. As shown in Fig. 6, the level of protein synthesized from the cell cycle-regulated transcript was approximately 3-fold greater than the level of protein synthesized from the constitutive transcript, as determined by optical densitometry. Thus, the increased translational efficiency of the shorter, cell cycle-regulated transcript may contribute to the higher level of cyclin B1 protein during G2-M phase.

**DISCUSSION**

The expression of cyclin B1 mRNA is regulated at both the transcriptional and posttranscriptional levels (4, 5). Several different studies have described the structure of the cyclin B1 promoter along with its cell cycle regulation (6–8). These studies have also reported different transcriptional start sites for the cyclin B1 gene. Our experiments have clarified these discrepancies as well as identified a novel aspect of the regulation of cyclin B1. We have identified two cyclin B1 transcripts that differ by 65 bases in length. The longer transcript is constitutively expressed and appears throughout the cell cycle. The shorter transcript is cell cycle regulated and appears only at G2-M phase. The start sites for these transcripts map to the start sites reported by Piaggio *et al.* (6) and Cogswell *et al.* (7), respectively. The experiments described in these reports used total RNA from unsynchronized cells. This would explain why the shorter transcript was not identified as being cell cycle regulated and the longer transcript was not identified as being cell cycle independent.

Cell cycle-dependent usage of transcriptional start sites has not been described previously. The presence of an additional cyclin B1 transcript at the G2-M-phase boundary may contribute significantly to the increased level of cyclin B1 expression at this point in the cell cycle. This cell cycle-regulated transcript was also found to be translated more efficiently than the constitutively expressed transcript. Approximately three times more protein was synthesized *in vitro* from the M-phase-specific transcript than from the constitutive transcript. Thus, translational control may be an important factor in the regulation of cyclin B1 expression.

Several different mechanisms could be responsible for the presence of the additional, shorter M-phase-specific transcript. It could be regulated transcriptionally and be specifically transcribed only at G2 and M phase. It could also be regulated posttranscriptionally; i.e. it could be more stable in G2 and mitosis than it is in G1 and S phase. Changes in message stability have already been shown to be responsible in part for the differences in cyclin B1 mRNA throughout the cell cycle (4). However, these differences in message stability were determined after examination of the total cyclin B1 mRNA and not that of a specific transcript. When the sequences for both cyclin B1 transcripts were placed under the regulation of the SV40 promoter, which is expressed uniformly throughout the cell cycle, we did not observe differences in mRNA stability using a transient transfection assay. Thus, changes in message stability most likely do not regulate the differential expression of the M-phase-specific transcript compared with that of the longer transcript.

To identify a region of the cyclin B1 promoter that is critical for cell cycle regulation, we made a series of deletions from its 3′ end and tested them in HeLa cells in a transient transfection assay. These deletions span the cell cycle-regulated transcriptional start site. From these experiments, we were able to identify a 24-bp sequence that is necessary for cell cycle-regulated activity of the cyclin B1 promoter. This region includes the cell cycle-regulated start site and the sequence GGCT repeats three times. Although this motif or region has not been previously shown to be important for cell cycle-regulated genes that are active at G2-M phase, it may be important for cyclin B1 regulation. The CDE element has been described as an important DNA sequence for the regulation of the cdc25C, cyclin A, and cdc2 genes (14, 15). These genes are active in late S phase and G2 and are not expressed in G0. The CDE element was shown to be necessary for the repression of these genes in G0. The cyclin B1 promoter contains a CDE element, but it does not appear to be critical for its regulation in G2-M phase, because the deletion of this region does not alter the cell cycle-regulated promoter activity. Katula *et al.* (9) have recently identified two CCAAT elements that appear to be important for cyclin B1 regulation. Both of these CCAAT elements were shown to be necessary for the induction of the cyclin B1 gene during S phase. The NP-Y transcription factor was shown to bind to these CCAAT elements. Both of the CCAAT elements and the GGCT repeats may contribute to the regulation of transcription of cyclin B1 during G2-M phase. The GGCT repeats are located in the same region as the cell cycle-regulated transcriptional start site that we have shown to be important for the regulation of the cyclin B1 gene.

**REFERENCES**

1. Grana, X., and Reddy, E. P. (1995) *Oncogene* **11**, 211–219.
2. MacLachlan, T. K., Sang, N., and Giordano, A. (1995) *Cyt. Rev. Eukaryotic Gene Expression* **5**, 127–156.
3. Morgan, D. O. (1995) *Nature* **374**, 131–134.
4. Maita, A., McKenna, W. G., and Muschel, R. J. (1995) *EMBO J.* **14**, 603–609.
5. Pines, J., and Hunter, T. (1989) *Cell* **58**, 833–846.
6. Piaggio, G., Farina, A., Puglisi, D., Manni, L., Fuschi, P., Saschei, A., and Gaetano, C. (1995) *Exp. Cell Res.* **216**, 396–402.
7. Cogswell, J. P., Godlewski, M. M., Bonham, M., Bist, J., and Babiss, I. (1995) *Mol. Cell. Biol.* **15**, 2762–2770.
8. Hwang, A., Maita, A., McKenna, W. G., and Muschel, R. J. (1995) *J. Biol. Chem.* **270**, 28419–28424.
9. Katula, K. S., Wright, K. L., Paul, H., Surman, D. R., Nuckolls, F. J., Smith, J. W., Ting, J. P.-Y., Yates, J., and Cogswell, J. P. (1997) *Cell Growth Different.* **8**, 811–820.
10. Farina, A., Gaetano, C., Crescenzi, M., Pucetini, F., Manni, I., Saschei, A., and Piaggio, G. (1996) *Oncogene* **13**, 1287–1296.
11. Chomczyński, P., and Saschei, N. (1987) *Anal. Biochem.* **162**, 156–159.
12. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1989) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
14. Luchtello, F. C., Truss, M., Zwickler, J., Ehler, F., Beato, M., and Muller, R. (1995) *EMBO J.* **14**, 132–142.
15. Zwickler, J., Luchtello, F. C., Wolfram, L. A., Gross, C., Truss, M., England, K., and Muller, R. (1995) *EMBO J.* **14**, 4514–4522.