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The *cdc*22 gene of *Schizosaccharomyces pombe* encodes a cell cycle-regulated transcript

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Two independent DNA sequences, of 5.6 and 2.4 kb in size, were isolated from *Schizosaccharomyces pombe* gene libraries on the basis of their ability to rescue the temperature-conditional lethality conferred by a *cdc*22 mutation. Integration of these sequences into the *S.pombe* genome by homologous recombination, followed by genetic mapping, demonstrated that the site of integration of the 5.6 kb fragment is tightly linked to the *cdc*22 locus, while that of the 2.4 kb fragment is unlinked. This shows that the 5.6 kb fragment carries the authentic *cdc*22+ gene while the 2.4 kb fragment carries an extragenic suppressor sequence. The *cdc*22 transcript was identified by Northern blot analysis and shown to be 3.3 kb in size. The level of the transcript during the cell cycle was investigated in synchronous cultures prepared by elutriation. The *cdc*22 transcript is cell cycle regulated, reaching a maximum level during late G1/S phase, at least 12-fold higher than the minimum level observed in mid G2.

Key words: cell cycle/cell cycle mutant/periodicity/*S.pombe*/transcript

Introduction

The division cycle of a eukaryotic cell is made up of a temporal series of discrete events: the most obvious are the replication of DNA during the S phase, the separation of chromosomes at mitosis, and cell division. Although the molecular basis for the timing of these events is unknown, one possibility is that they rely on the periodic synthesis of specific macromolecules. Thus, ever since methods were first developed to obtain synchronously dividing populations of cells, the search for cell cycle-regulated proteins, and more recently specific mRNA species, has been an important area of investigation.

Early studies on both budding and fission yeast suggested that a large number of enzymes showed periodic 'steps' in activity at specific points in the cell cycle (reviewed in Mitchison, 1971). If enzyme activity was proportional to the amount of enzyme product, it was argued, this would suggest that many proteins were synthesized in a periodic manner during the cell cycle. However, more recent work argues against this hypothesis.

First, a number of independent reports indicated that some of the methods previously used to generate synchronous cultures could cause perturbations in enzyme activity (Mitchison, 1977; Walker *et al.*, 1980; Ludwig *et al.*, 1980). Furthermore, when the activity of some previously identified 'step' enzymes was measured in synchronous cultures prepared by procedures that minimized these perturbations, no periodic changes in enzyme activity were observed (Creanor *et al.*, 1983). A second line of evidence came from measuring the synthesis of individual polypeptides separated by 2-dimensional gel electrophoresis. Such studies in *Saccharomyces cerevisiae* have demonstrated that most proteins are synthesized continuously, though a small number have been identified whose synthesis appears to be cell cycle modulated (Elliott and McLaughlin, 1978; Lorincz *et al.*, 1982).

The development of recombinant DNA techniques and efficient methods of transformation for both *S.cerevisiae* and *Schizosaccaromyces pombe* has enabled a number of genes to be cloned from both organisms. Using these cloned genes as hybridization probes for specific transcripts has offered a new approach to the investigation of cell cycle-regulated gene expression. In *S.cerevisiae*, the steady state levels of very few transcripts appear to vary in a systematic way during the cell cycle. These include the transcripts encoded by the histone *H2A* and *H2B* genes (Hereford *et al.*, 1981), the *CDC*21 gene which codes for thymidylate synthetase (Storms *et al.*, 1984), the *CDC*9 gene which codes for DNA ligase (Peterson *et al.*, 1985) and the *HO* gene which is thought to encode an endonuclease required for mating type interconvention (Nasmyth, 1983). In *S.pombe*, only the histone H2A and H2B transcripts have been demonstrated to be cell cycle regulated (Aves *et al.*, 1985). Thus it appears that while most genes are expressed continuously the expression of a few seems to vary in a periodic manner through the cell cycle.

A temperature-sensitive mutant in the *cdc*22 gene of *S.pombe* was isolated among a group of mutants whose major defect was in DNA replication (Nasmyth and Nurse, 1981). At the restrictive temperature this mutant, *cdc*22.M45, is blocked early in the nuclear division cycle while growth continues, giving rise to the characteristic elongated cell phenotype of *S.pombe cdc* mutants. Further experiments suggested that the *cdc*22+ gene product was required for the initiation of S phase (Nasmyth and Nurse, 1981). A biochemical investigation of a *cdc*22 mutant strain suggested that the *cdc*22+ gene codes for the DNA precursor enzyme nucleoside diphosphokinase (Dickinson, 1981).

Here we report the cloning of the *cdc*22+ gene and the identification of the *cdc*22 transcript, and demonstrate that the level of the *cdc*22 transcript, unlike that of most genes, is cell cycle regulated.

Results

Isolation of cdc22-M45 complementing clones

The *S.pombe* strain *cdc*22.M45 *leu*1.32 h^- was transformed with the two gene libraries described in Materials and methods. Transformants were isolated by their ability to grow in the absence of leucine at 25°C. These were replica plated to 35°C, and colonies able to grow were picked. Four such *cdc*+ transformants were obtained from the *Hin*dIII library, and two from the *Sau*3A library. The phenotypes of the transformants from the two libraries were very different. At 35°C, all the transformants from the *Hin*dIII library showed wild-type cell size at division. Their growth rate, estimated from the time to form colonies from single cells, was similar to the wild-type. In contrast, the transformants from the *Sau*3A library divided at two to three times the

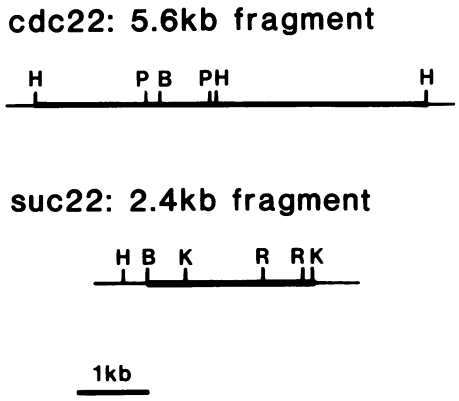

Fig. 1. Restriction maps of the cloned fragments carrying *cdc*22 and *suc*22. Thick lines indicate *S.pombe* inserts; thin lines flanking vector sequences. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*1; R, *Eco*RI. There are two (unmapped) *Eco*RI sites in the 5.6 kb fragment, and no sites for *Kpn*I; there are no *Pst*I sites in the 2.4 kb fragment. The left hand end of the 2.4 kb insert is a reconstituted *Bam*HI site, while the right hand end is very close to the *Kpn*I site.

wild-type cell size, and it took at least twice as long for colonies to form at 35°C as wild-type cells. For all six transformants, the *cdc*+ and *leu*+ characters were both unstably transmitted during mitosis, and mitotic loss of the two characters was coincident. This showed that the *cdc*+ phenotype was plasmid mediated in each case. Plasmid DNA was isolated from each transformant and recovered in *Escherichia coli*. Each plasmid was transformed back into *S.pombe cdc*22.M45 *leu*1.32 and also into *cdc*22.C11 *leu*1.32, and in every case the *cdc* defect was rescued by the presence of plasmid.

Each plasmid was analysed by restriction digestion. The same 5.6 kb *Hin*dIII fragment containing an internal *Hin*dIII site (Figure 1) was found to be present in all four of the plasmids from the *Hin*dIII library. A different insert fragment, 2.4 kb in size, was present in the two plasmids from the *Sau*3A library. The restriction maps of the two types of plasmid (Figure 1) show that the two cloned sequences able to rescue the *cdc*22 mutations were quite different. Southern blot analysis of genomic DNA using the two cloned sequences as probes showed that each sequence was co-linear with the chromosome, and that each was present as a single copy (data not shown).

To determine which, if any, of the cloned sequences contained the authentic *cdc*22+ gene, both were integrated into the *S.pombe* genome by homologous recombination and the site of integration mapped. Both fragments were first subcloned into the integrative plasmid pESP1. The 5.6 kb *Hin*dIII fragment was transferred into the *Hin*dIII site of pESP1, generating pESP1(5.6). The 2.4 kb fragment was excised using the *Hin*dIII and *Sal*I sites flanking the insertion site, and ligated into the corresponding sites within the *Tet* gene of pESP1, generating pESP1(2.4). These plasmids were used to transform *cdc*22.M45 *leu*1.32 to leucine prototrophy, and transformants showing mitotically stable transmission of this character were taken as putative integrants.

Five stable *leu*+ transformants were obtained from the strain transformed with pESP1(5.6). Four of these showed full complementation of the *cdc*22.M45 defect, while the fifth was *cdc*− in phenotype. Presumably the *cdc*− transformant had arisen by gene conversion from the chromosomal copy during transformation or integration (cf. Falco *et al.*, 1983). One *cdc*+ transformant and the *cdc*− transformant were crossed to a *cdc*+ *leu*1.32 strain, and the meiotic progeny subjected to tetrad analysis (crosses 1 and 2 in Table I). In both crosses leucine prototrophy segregated 2:2 indicating that the plasmid had integrated into the

Table I. Test crosses of integrant strains

Cross	Parental genotypes (*cdc* phenotype)	Tetrad types[a] PD	NPD	TT
1	*cdc*22 *leu*1 [pESP1(5.6)] × *cdc*+ *leu*1 (*cdc*+)	Not applicable: progeny all *cdc*+		
2	*cdc*22 *leu*1 [pESP1(5.6)] × *cdc*+ *leu*1 (*cdc*−)	19	0	0
3–6	*cdc*22 *leu*1 [pESP1(2.4)] × *cdc*+ *leu*1	12	7	23

[a]PD, parental; NPD, non-parental ditype; TT, tetratype.
[b]Data pooled from crosses of four equivalent integrant strains.

S.pombe genome. In cross 1, involving the *cdc*+ transformant, no *cdc*− progeny were observed in the 17 tetrads dissected. This shows close linkage of the site of integration of the *leu*+ marker to the chromosomal *cdc*22 locus. Similarly, no recombinant progeny were observed among 19 tetrads analysed in cross 2, again indicating close linkage. These experiments therefore indicate that the 5.6 kb cloned fragment contains the authentic *cdc*22+ gene.

Similar experiments were carried out using pESP1(2.4) for transformation of a *cdc*22 *leu*1.32 *h*− strain. Four integrants were obtained which were phenotypically *cdc*−, indicating that the presence of an extra copy of the cloned 2.4 kb fragment in the genome was unable to rescue the *cdc*22.M45 defect. It seems likely that the cloned fragment contains a sequence that can rescue or suppress a *cdc*22 mutation only when present in multiple copies on an autonomously replicating plasmid.

Each of the four transformants obtained was crossed to a *leu*1.32 *h*+ strain and again the site of integration was tested by tetrad analysis (crosses 3–6 in Table I). In all four crosses leucine prototrophy segregated 2 *leu*+:2 *leu*− as expected for an integrated plasmid, and the *cdc*22 character also segregated 2:2. The high proportion of tetrads showing tetratype and non-parental ditype segregation for the two characters indicates that the site of integration of the plasmid and the *cdc*22 locus are not linked. Other crosses (data not shown) show that the *leu*+ character of the strains derives from a genetic site unlinked to the *leu*1 gene and is therefore not due to reversion at this locus. Finally, Southern blot analysis of two of the four integrants showed that the pESP1(2.4) plasmid had integrated by homologous recombination (data not shown).

Identification of the cdc22 transcript

Northern blot analysis of total RNA prepared from a wild-type strain showed only a single transcript of 3.3 kb that hybridized to the 5.6 kb DNA fragment (data not shown). This transcript is shown to be encoded by the *cdc*22 gene from the following argument. By subcloning of the 5.6 kb *Hin*dIII fragment into two smaller *Hin*dIII fragments we showed that neither of the smaller fragments so generated were able to complement a *cdc*22 mutation. The central *Hin*dIII site therefore lies within the *cdc*22 gene. When the subcloned 2.6 and 3.0 kb fragments were used as hybridization probes in Northern blot analysis, each hybridized to the same transcript as the 5.6 kb fragment (data not shown). This shows that the transcribed region crosses the central *Hin*dIII site and therefore the transcript is encoded by the *cdc*22 gene.

Level of the cdc22 transcript during the cell cycle

In order to investigate the level of the *cdc*22 mRNA through the cell cycle, RNA samples were prepared from a synchronous culture of strain 972 maintained at 35°C for two generations after synchronization. The increase in cell number (Figure 2C) shows the high degree of synchrony obtained.

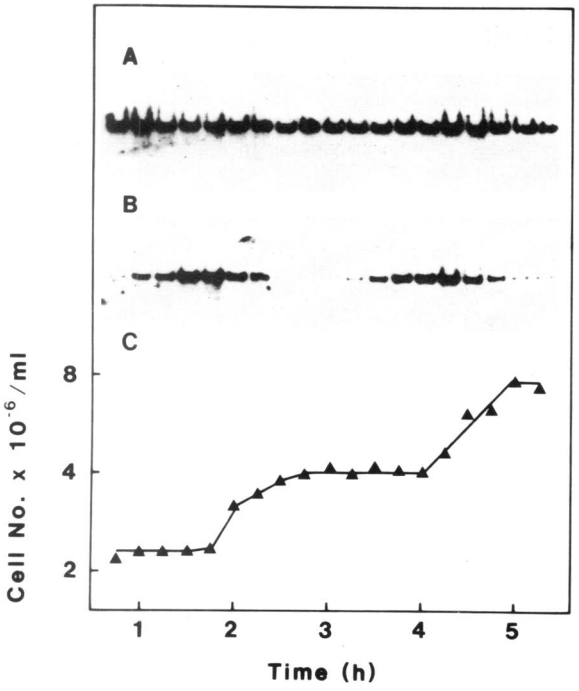

Fig. 2. Cell cycle periodicity in the level of *cdc*22 transcript. A synchronous culture of strain 972 prepared by elutriation was sampled for cell number determination (**C**) and for RNA preparation at 15-min intervals. The RNA samples were separated by gel electrophoresis, blotted and probed with the 5.6 kb cloned fragment containing the *cdc*22 gene (**B**). The probe was stripped off the blot, which was then re-probed with the *S.pombe* ADH gene (**A**).

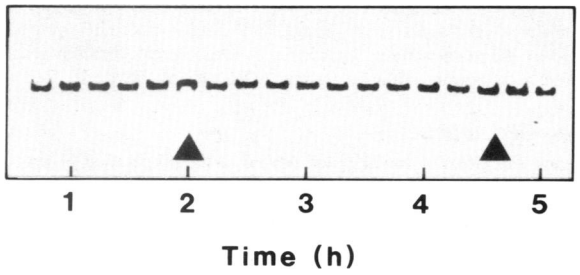

Fig. 3. Absence of periodicity of the *suc*22 transcript. Conditions were as for Figure 2: the probe used was the cloned 2.4 kb fragment carrying the *suc*22 sequence. The arrows indicate the mid-point rise in cell number in this synchronous culture.

Total RNA was prepared from each sample, and was subjected to gel electrophoresis and Northern blot analysis using the 5.6 kb cloned fragment as hybridization probe. The autoradiograph obtained (Figure 2B) clearly demonstrates that the level of *cdc*22 transcript exhibited dramatic changes during the cell cycle. The difference between maximum and minimum values was estimated by densitometry measurements on the autoradiograph to be at least 12-fold. The time between the appearance of the two peaks in *cdc*22 transcript level was close to 2.5 h. This corresponds to the generation time of wild-type cells at 35°C (Figure 2C). The time in the cell cycle at which the level of *cdc*22 transcript is maximal can be estimated from the cell number curve (Figure 2C) at ~20 min before the midpoint of cell number rise. The S phase under the conditions used is short and its midpoint occurs almost simultaneously with cell division (Mitchison and Creanor, 1971). Thus the level of *cdc*22 transcript is maximal during late G1 or S phase.

As a control, the filter was stripped of probe and re-hybridized with the cloned alcohol dehydrogenase (ADH) gene from *S.pombe*, whose transcript is reported to be present at essentially the same level throughout the cell cycle (Aves *et al.*, 1985). In contrast to the *cdc*22 transcript, the ADH transcript did not vary significantly in level in the RNA prepared from the synchronous culture (Figure 2A).

A single transcript of 1.5 kb was found to hybridize to the cloned 2.4 kb fragment carrying extragenic suppressor activity. The level of this transcript during the cell cycle was estimated by probing a filter carrying RNA from cells at different cycle stages with the cloned 2.4 kb fragment. The resulting autoradiograph (Figure 3) shows that the level of 1.5 kb transcript remained essentially constant throughout.

Discussion

In this paper we report the cloning of the *S.pombe cdc*22+ gene and the identification of its transcript. We also demonstrate that the level of this transcript varies dramatically during the cell cycle. When followed for two cell cycles, the level of the *cdc*22 transcript was found to peak at the same time in each cycle, during late G1 or early S phase. We believe this pattern to be representative of the normal cell cycle since synchronous cultures were prepared by elutriation, a procedure shown to minimize metabolic perturbation (Creanor and Mitchison, 1979). As a further control, the ADH transcript, previously reported to be aperiodic (Aves *et al.*, 1985) was observed to be present at a constant level throughout the experiment. With the single exception of the histone *H2A* transcript (Aves *et al.*, 1985), the *cdc*22 transcript is the only reported case of cell cycle regulation of a specific transcript in *S.pombe*. The observed periodicity might be caused in one of two ways: the *cdc*22 gene might be periodically transcribed, or there might be cell cycle-specific changes in the stability of the *cdc*22 transcript. Alternatively, both mechanisms might operate, as has been demonstrated for the histone transcript in *S.cerevisiae* (Hereford *et al.*, 1981). The observations presented here cannot distinguish between the possibilities, and further experiments will be needed to resolve them.

The *S.pombe cdc*22+ gene product is required for progression into or through S phase. In *S.cerevisiae* there are several examples of genes required for S phase showing cell cycle regulation. The histone genes, *CDC*9 (encoding DNA ligase), *CDC*8 (thymidylate kinase) and *CDC*21 (thymidylate synthase) are all cell cycle regulated, the latter three co-ordinately (L.H.Johnston, personal communication). Perhaps these are examples of a general phenomenon in which the expression of a number of genes required for S phase is subject to cell cycle regulation.

The *cdc*22+ gene has been reported (Dickinson, 1981) to encode the DNA precursor enzyme nucleoside diphosphokinase (NDPK), but we feel that more recent observations call for a re-appraisal of this conclusion.

First, observations in this laboratory (J.Creanor, personal communication) have failed to show any difference in NDPK activity between either of the *cdc*22 mutants available and the wild-type, using a more direct assay than that used by Dickinson (1981). Since the maximum difference in activity quoted in that report was only 2-fold, the overall evidence for temperature sensitivity of NDPK activity in *cdc*22 mutants is not compelling. Furthermore, introduction of the cloned *cdc*22+ gene into *S.pombe* cells did not lead to an increase in NDPK activity (J.Creanor, personal communication).

Second, mutants of *S.cerevisiae* defective in DNA precursor

metabolism such as *cdc*21 and *cdc*8 (Game, 1976; Sclafani and Fangman, 1984) behave as chain elongation mutants in reciprocal shift experiments to test dependency relationships (Hartwell, 1976). In *S.pombe*, the *cdc*22 block occurs prior to the chain elongation steps, as defined by hydroxyurea sensitivity (Nasmyth and Nurse, 1981).

Third, the *cdc*22 transcript is 3.3 kb in size, sufficient to code for a polypeptide of ~100–120 kd. The enzyme from *S.cerevisiae* has a monomer size of 17 kd (Palmeri *et al.*, 1973). Although by no means certain, it seems improbable that the corresponding enzymes from the two yeasts are so different in size. With the cloning of the *cdc*22$^+$ gene reported here, it should be possible finally to settle the question of whether the gene encodes NDPK.

In addition to the *cdc*22$^+$ gene, we report the isolation of a 2.4 kb DNA fragment that is able to rescue the temperature lethal effect of both known *cdc*22 alleles. Rescue only occurred when the cloned fragment was carried on an autonomously replicating vector, and such transformant strains grew more slowly than the wild-type, with a substantially increased cell size at division. No rescue of *cdc*22 mutations was observed when the sequence was integrated into the genome. This suggests that for rescue to occur, the 2.4 kb fragment must be present in multiple copies, probably because overproduction of a product encoded by the 2.4 kb fragment is required for suppression of *cdc*22 mutant phenotype. Rescue of mutant phenotype by cloned extragenic fragments carried on multicopy plasmids has been reported by a number of workers (Hicks *et al.*, 1979; Calderon *et al.*, 1983; Weiss and Friedberg, 1985). In some cases (e.g. Pringle *et al.*, 1984) the extragenic sequences have provided valuable clues about possible interactions between different genes. We have tested the 2.4 kb fragment that suppresses *cdc*22 mutants for its ability to complement or suppress other *cdc* mutations defective in S phase. In no case was any effect of the introduced fragment observed.

Materials and methods

Strains and media

E.coli strains. Strain JA221 (*rec*A1 *leu*B6 *trp*E5 *Hsd*R$^-$ *Hsd*M$^+$ *lac*Y C600) was used routinely for transformations, and for the growth and maintenance of plasmids. Strain BJ5183 (*r*$^-$ *rec*BC *sbc*B *endo*I *gal meth Str*^{r} *thi biot hsd*) was used for the recovery of plasmids from yeast (Beach et al., 1982a). Growth medium for *E.coli* was LB, supplemented with 1.5% agar for plates.

S.pombe strains. The strains used were 972*h*$^-$ (wild-type); *leu*1.32 *h*$^+$; *cdc*22.M45 *leu*1.32 *h*$^-$ (Nasmyth and Nurse, 1981); *cdc*22.C11 *leu*1.32 *h*$^-$ (J.Creanor and C.Gordon, unpublished data). *S.pombe* was grown in YEPD (0.5% yeast extract/0.5% peptone (Difco)/3% glucose), or in minimal medium as described by Creanor and Mitchison (1982) but with 2% glucose, supplemented where necessary with 75 mg/l L-leucine. For plates, 2% agar was added. Standard genetic procedures were as described by Gutz *et al.*, (1974); the growth and handling of liquid cultures was as described by Mitchison (1970).

Plasmids, gene banks and cloning procedures

The yeast/*E.coli* shuttle vectors pDB248 (Beach *et al.*, 1982a) and pDB262 (Beach *et al.*, 1982b) have been described previously. Both contain, in addition to bacterial plasmid sequences, part of the 2 μm circle and the *LEU*2 gene from *S.cerevisiae*. These plasmids can replicate autonomously in *S.pombe* and confer the *leu*$^+$ phenotype on *leu*1.32 strains. The integrative vector pESP1 which carries the *S.cerevisiae LEU*2 gene but is unable to replicate in *S.pombe* was constructed from pDAM6 (Beach *et al.*, 1982b) by deleting the *Sal*I site in the *S.cerevisiae* region, leaving a single *Sal*I site in the *Tet* gene available for cloning. pADH (Aves *et al.*, 1985) contains the *S.pombe* ADH gene.

Plasmid isolation and transformation of E.coli

These procedures were carried out as described by Maniatis *et al.* (1982). The preparation of *S.pombe* genomic DNA, recovery of plasmids from *S.pombe*, and transformation of *S.pombe* were as described by Beach *et al.* (1982a). Two gene libraries were used: one consisted of partial *Hin*dIII fragments of total genomic DNA cloned into the *Hin*dIII site of pDB262 and was the gift of Dr P.Nurse; the other was constructed in this laboratory by ligating partial *Sau*3A fragments into the *Bam*HI site of pDB248.

Preparation of synchronous cultures of S.pombe

Synchronous cultures of strain 972 were prepared using an elutriator rotor (Beckman JE-6) essentially as described by Creanor and Mitchison (1979). Cultures were grown at 35°C in Edinburgh Minimal Medium to a density of 5×10^6 cells/ml. Cells were pumped at 25 ml/min into the pre-warmed (35°C) rotor preset at 4000 r.p.m. Cells were loaded for ~20 min. To harvest the small cells for growing on synchronously, the pump speed was increased by 10–15% while leaving the rotor speed constant. A sample was examined under the microscope to estimate the homogeneity of cell size in the separated fraction, a good indicator of the likely course of synchrony. A total of 150 ml of culture at up to 3×10^6 cells/ml was routinely harvested. During growth of the culture at 35°C, samples were taken at intervals for cell density estimation and for preparing RNA. Cell number was estimated using a Coulter electronic counter (Mitchison, 1970).

Preparation of total RNA from S.pombe cells

For samples from synchronous cultures, aliquots containing ~2×10^7 cells were filtered onto Oxoid membrane filters (25 mm diameter; pore size 0.45 μm). The cells were washed on the filter twice with ice-cold NaCl (150 mM) and the filter immediately frozen and stored at −20°C. Larger samples were harvested by centrifugation. Cells were re-suspended from the filters at 0°C into 0.5 ml RNA extraction buffer (Tris.HCl, 50 mM, pH 7.5; EDTA 10 mM; 2% TNS (Tri-iso-propylnaphthalenesulfonic acid sodium salt, Eastman Kodak)). After removal of the filter, an equal volume of acid-washed glass beads (0.4–0.5 mm), and an equal volume of phenol were added. The mixture was vortexed vigorously for 40 s, placed on ice for 60 s, and then vortexed for a further 40 s. The cell lysate was transferred to a 1.5 ml Eppendorf tube and centrifuged at 4°C for 5 min. The aquenous phase was removed and extracted with phenol/chloroform and then with chloroform. RNA was then precipitated with 2.5 volumes of ethanol at −20°C and re-dissolved in TE buffer (Tris-HCl, 10 mM, pH 7.5; EDTA, 1 mM). The concentration of RNA was determined from its absorbance at 260 nm, taking 1 absorbance unit to be equivalent to 40 μg/ml).

Gel electrophoresis at RNA and Northern blotting

Denaturing 1.2% agarose gels containing formaldehyde were used for electrophoresis (Goldberg, 1980). 5 μg of each RNA preparation was loaded, the gel run, and the separated RNA transferred to GeneScreen (New England Nuclear). Transfer, pre-hybridization, hybridization and washing procedures were as recommended by the manufacturer. Radioactivity labelled hybridization probes were prepared by nick-translation of plasmid DNA (Rigby *et al.*, 1977).

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