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Accuracy of in Vivo Aminoacylation Requires Proper Balance of tRNA and Aminoacyl-tRNA Synthetase

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The fidelity of protein biosynthesis in any cell rests on the accuracy of aminoacylation of tRNA. The exquisite specificity of this reaction is critically dependent on the correct recognition of tRNA by aminoacyl-tRNA synthetases. It is shown here that the relative concentrations of a tRNA and its cognate aminoacyl-tRNA synthetase are normally well balanced and crucial for maintenance of accurate aminoacylation. When *Escherichia coli* Gln-tRNA synthetase is overproduced in vivo, it incorrectly acylates the *supF* amber suppressor $tRNA^{Tyr}$ with Gln. This effect is abolished when the intracellular concentration of the cognate $tRNA_2^{Gln}$ is also elevated. These data indicate that the presence of aminoacyl-tRNA synthetase and the cognate tRNAs in complexed form, which requires the proper balance of the two macromolecules, is critical in maintaining the fidelity of protein biosynthesis. Thus, limits exist on the relative levels of tRNAs and aminoacyl-tRNA synthetases within a cell.

AMINOACYL-TRNA SYNTHETASES catalyze the acylation of tRNAs with their respective amino acids, a set of reactions whose specificity is critical for fidelity in the biosynthesis of proteins (*1*). In *Escherichia coli*, each of these enzymes must select its few cognate tRNAs from among approximately 80 tRNA species. Nevertheless, the low frequency of errors found in protein sequences (for example, ovalbumins and globins) indicates that aminoacyl-tRNA synthetases display an extremely high degree of substrate discrimination (*2*). In fact, the term "superspecificity" has been applied to aminoacyl-tRNA synthetases when compared with other enzymes involved in amino acid metabolism, for example, proteases (*3*). Superspecificity can be explained in part by a number of proposed proofreading mechanisms that prevent incorrectly recognized amino acids entering the aminoacyl-tRNA pool of the cell (*4*). The accuracy of tRNA recognition by an aminoacyl-tRNA synthetase is an intrinsic part in achieving the specificity of aminoacylation. However, an additional factor is very important. As we show in this report, competition effects between different tRNA–aminoacyl-tRNA synthetase complexes lead to enhanced binding specificity and are critical for accurate tRNA charging in vivo.

To investigate the accuracy of in vivo aminoacylation, we have made use of a sensitive assay for misrecognition of tRNA by *E. coli* Gln-tRNA synthetase (GlnRS). Certain nonsense mutants are suppressed (that is, a functional protein is made) in the presence of some suppressor tRNAs, but not of others, depending on the amino acids carried by the tRNA. For example, the β-galactosidase amber mutant $lacZ_{1000}$ can be suppressed by *supE* $tRNA^{Gln}$ (which inserts Gln in response to amber codons) but not by *supF* $tRNA^{Tyr}$ (which inserts Tyr), presumably because the mutant enzyme with Tyr at the amber mutation site is inactive. Thus, the *E. coli* strain BT32 ($lacZ_{1000}$, *supF*) is normally phenotypically Lac^-. However, if GlnRS can aminoacylate the *supF* suppressor $tRNA^{Tyr}$ with Gln (forming suppressor Gln-$tRNA^{Tyr}$), active β-galactosidase is made in this strain. With this test system we showed that an altered form of GlnRS can misacylate (mischarge) *supF* $tRNA^{Tyr}$ with Gln (*5, 6*).

We found that wild-type GlnRS also possesses a certain affinity for some noncognate *E. coli* tRNAs and can mischarge these species in vitro (*6*), and thus we investigated the conditions under which such reactions might take place in vivo. The most likely factor to influence the extent of mischarging was the concentration of GlnRS present in the cell, so we varied in vivo GlnRS levels by constructing strains with different copy numbers of *glnS*, the structural gene for GlnRS. The mischarging indicator strain BT32 (described above) contains only the chromosomal copy of *glnS*. The λ-lysogen BT32(λ*glnS*), which carries a single extra copy of *glnS* as part of the prophage, contains two copies of the gene. In addition, a multicopy plasmid clone of *glnS* was introduced into BT32 to form BT32/pBR322*glnS*. Liquid cultures of these strains were grown to mid-log phase, and the levels of $tRNA^{Gln}$, GlnRS, and β-galactosidase (arising from suppression of $lacZ_{1000}$) were determined (Table 1). We found that BT32 makes significant levels of active β-galactosidase only when it markedly overproduces GlnRS because of the presence of *glnS* on the multicopy plasmid. Little enzyme activity was observed in strains BT32 and BT32(λ*glnS*), and also in the control strain BT32/pBR322. Since a $lacZ_{1000}$ mutant synthesizes active β-galactosidase when Gln, but not Tyr, is inserted in response to the $lacZ_{1000}$ amber codon, it appears that the *supF* $tRNA^{Tyr}$ suppressor in BT32 can be charged with Gln by wild-type GlnRS, given sufficiently high intracellular GlnRS concentrations. The transformed strain BT32/pBR322*glnS* yielded dark red colonies on lactose MacConkey agar plates. GlnRS charged *supF* tRNA less efficiently than its cognate *supE* suppressor tRNA, as can be seen by comparing the amounts of β-galactosidase activity found in BT32 and in the BT32(λ*supE*) control. In another strain (BT32/pBR322*supF*), the *supF* amber sup-

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pressor $tRNA^{Tyr}$ was overproduced. In this case, only a very slight elevation of β-galactosidase activity was observed, showing that GlnRS, when it is present at normal intracellular concentrations, cannot efficiently mischarge this tRNA, as the tyrosine-containing enzyme has little enzyme activity in vivo.

But why should a high in vivo level of GlnRS alter its specificity? This change might be related to the state in which tRNAs and aminoacyl-tRNA synthetases are present in the cell. Most GlnRS and $tRNA^{Gln}$ molecules may normally be complexed with each other in vivo. The K_m of GlnRS for $tRNA^{Gln}$ is about 0.1 μ*M*, whereas both the enzyme and the tRNA are normally present at approximately micromolar concentration in vivo (*7*). Thus there is probably little free GlnRS in the cell. If, however, cellular GlnRS levels become elevated without a concomitant increase in $tRNA^{Gln}$ levels, then uncomplexed synthetase molecules are free to interact with noncognate tRNA species, which would then lead to mischarging. If this explanation were correct, then simultaneous overproduction of GlnRS and of $tRNA^{Gln}$ should abolish the misacylation seen when only the synthetase is overproduced.

To test this hypothesis, we decided to overexpress in the same cell both the *glnS* gene and genes encoding $tRNA^{Gln}$ (see Fig. 1). To do this, we made use of a plasmid in which the production of $tRNA^{Gln}$ was placed under the control of a strong inducible promoter. The plasmid, pRS3, carries two tandemly linked *E. coli* $tRNA_2^{Gln}$ genes behind the λp_L-promoter. Production of tRNA directed by this plasmid is completely repressed at 28°C if it is maintained in a strain that synthesizes a thermolabile λ repressor. Transcription from the λp_L-promoter can be activated by raising the temperature to 42°C, and after prolonged induction up to 60% of unfractionated tRNA isolated from such cells is $tRNA^{Gln}$ (*8*). In order to perform such a temperature-shift experiment in our tester strain, a chromosomal copy of the λ*c*I857 gene (which codes for a temperature-sensitive λ repressor) was introduced into strain BT32 to give strain RS108 (*9*). For simultaneous GlnRS and $tRNA^{Gln}$ overproduction, we recloned the *glnS* gene into pACYC184 (*10*), a plasmid compatible with pRS3 and other pBR322-derived plasmids. RS108 was then transformed with both the new *glnS*-containing plasmid (pRS11) and pRS3 to make a BT32-derived strain that could overproduce both GlnRS and, by induction, $tRNA_2^{Gln}$ (Fig. 1). A control strain was also made by transforming RS108 with pRS11 and the expression vector pPLc28 containing no insert. This strain (RS108/pRS11/pPLc28) constitutively overproduces GlnRS, but it cannot be induced to overproduce $tRNA^{Gln}$.

The β-galactosidase activity in these strains was followed as an indication of misacylation in vivo. When a culture of the control strain RS108/pRS11/pPLc28 (which constitutively overproduces GlnRS, but not $tRNA^{Gln}$) was subjected to a temperature shift to 42°C, the level of β-galactosidase activity increased in parallel with cell density after an initial decrease (Fig. 2A). The initial decrease may be due to suppression of $lacZ_{1000}$ by Gln insertion being slightly temperature sensitive or adaptation of cells to fresh media. Thus misacylation occurs as expected from the earlier experiment (Table 1). However, when the experimental strain RS108/pRS11/pRS3 was shifted to 42°C, the amount of β-galacto-

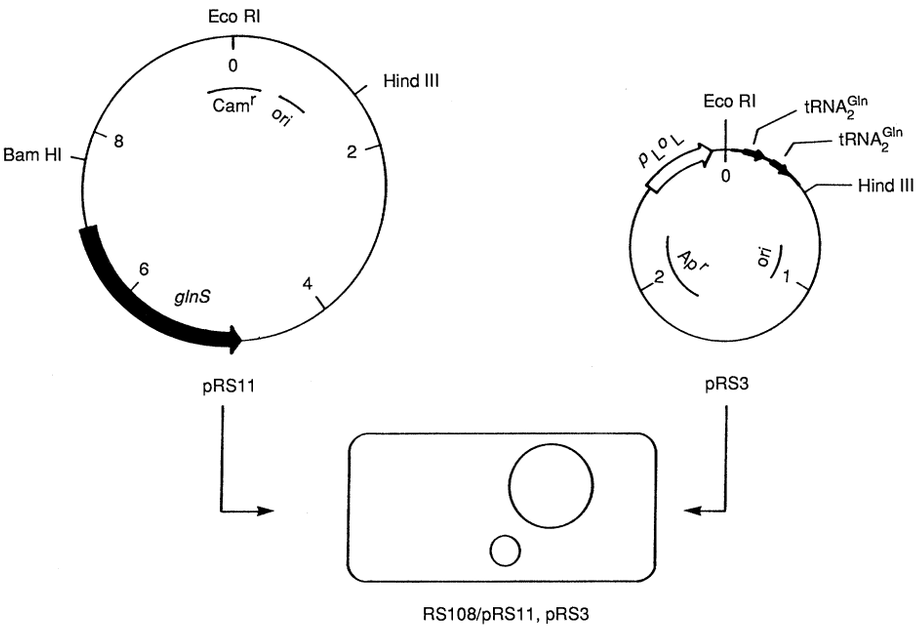

Fig. 1. Plasmids used for the simultaneous overproduction of GlnRS and of $tRNA^{Gln}$. The plasmid pRS11 consists of a 5.8-kb *glnS*-containing Pvu II/Hind III DNA fragment cloned between the Eco RV and the Hind III sites of pACYC184. Plasmid pRS3 is a derivative of the expression vector pPLc28 (*22*) that contains two $tRNA_2^{Gln}$ genes from the phage λ*psu*°2 (*23*) downstream of the inducible λ p_L-promoter and operator (p_Lo_L). Both strains were used to transform the indicator strain RS108. Heavily shaded regions of the plasmids indicate cloned *E. coli* chromosomal DNA. Lengths are given in kilobases.

Table 1. Steady-state mischarging by wild-type GlnRS. Derivatives of *E. coli* strain BT32 ($F^-lacZ_{1000}met_{am3}trp_{am}str-$ $tsx_{am}bfc_{am}supF$) were made that harbored either transducing λ prophages or multicopy plasmids. The plasmid designated pBR322*glnS* is the same as pYY105, which is a 6.5-kb *glnS*-containing Eco RI/Hind III DNA fragment cloned into pBR322 (*16*). Phage λ*glnS* is the same 6.5-kb fragment cloned into the vector λNM540. pBR322*supF* is plasmid pSSU101, a ColE1 derivative carrying a synthetic *supF* supressor $tRNA^{Tyr}$ gene (*17*), and λ*supE* is an in vivo–derived transducing phage carrying the *supE* suppressor gene (*18*). Individual precultures of each construct were grown in triplicate in minimal A medium supplemented with 0.2% glucose, 0.2% vitamin assay casamino acids (Difco), 1 m*M* isopropyl-β-D-thiogalactopyranoside and, as necessary, with ampicillin (100 μg/ml). The precultures were then used to inoculate fresh medium of the same kind. Cultures were grown at 37°C until the A_{600} was approximately 0.5. Then, duplicate 0.5-ml samples were taken for β-galactosidase assays (*19*). Samples were also taken for determination of GlnRS activity (10 ml) and for Tyr and Gln acceptor activity assays (100 ml) of crude tRNA preparations, as described (*20, 21*). Relative levels were normalized; 1.0 then represents 4170 cpm per A_{260} absorbance units for tRNA levels, 441 cpm per microgram of protein for GlnRS levels, and 0.3 Miller units for β-galacotosidase activity.

Construct	Relative $tRNA^{Gln}$ levels	Relative GlnRS levels	Relative β-galactosidase activity
BT32	1.0	1.0	1.0
BT32(λ*glnS*)	1.1	2.1	3.4
BT32/pBR322	1.0	1.0	1.5
BT32/pBR322*glnS*	1.1	24.0	56.0
BT32/pBR322*supF*	0.7	0.9	3.3
BT32(λ*supE*)	0.9	1.0	241.0

sidase activity progressively diminished (Fig. 2B). These results were exactly as one would expect if $tRNA^{Gln}$ overproduction were to cause misacylation to cease. Thus, the balance of $tRNA^{Gln}$ complexed to GlnRS is important for the accuracy of glutaminylation, not the absolute amounts of either macromolecule. How much $tRNA^{Gln}$ is needed in the cells to stop misacylation? After 60 min of induction of the strain RS108/pRS11/pRS3, at which time active β-galactosidase production was clearly repressed (Fig. 2B), 40% of the unfractionated tRNA consisted of Gln-accepting tRNAs (that is, 626 pmol of Gln-accepting activity per A_{260} absorbance unit). This corresponds to an approximately 22-fold elevation in $tRNA^{Gln}$ levels and sets an upper limit to the amount of $tRNA^{Gln}$ required to abolish misacylation caused by a 30-fold elevation of GlnRS levels. GlnRS levels were also determined after 60 min of induction and were about 30 times normal in both the experimental and control strains. Therefore, elevation in the level of $tRNA^{Gln}$ following induction does not effect mischarging indirectly by repressing *glnS* overexpression.

The above results indicate that GlnRS misacylates the *supF* suppressor $tRNA^{Tyr}$ when the enzyme is present at elevated levels in vivo. Since there are normally approximately the same number of GlnRS and $tRNA^{Gln}$ molecules in the cell (7), GlnRS apparently mischarges the noncognate $tRNA^{Tyr}$ suppressor when GlnRS molecules are present in excess over cognate $tRNA^{Gln}$ molecules. Thus, the balance of in vivo complex formation between tRNA and its cognate aminoacyl-tRNA synthetase contributes significantly to the fidelity of aminoacylation. Mischarging may be the product of as many as three steps: the dissociation of two different cognate aminoacyl-tRNA synthetase–tRNA complexes and the subsequent matching of the incorrect pair. On the basis of in vitro data, which showed that the presence of cognate tRNAs inhibited the mischarging of noncognate tRNAs, it was postulated earlier that the existence of parallel synthetase-tRNA systems might contribute to the specificity of aminoacylation in the cell (*11*). Our data show that this is indeed the case in vivo and that the contribution of parallel systems to the accuracy of tRNA charging is significant.

What is the tRNA specificity of GlnRS in the mischarging reaction? This glutaminylation reaction is probably not limited to *supF*. In vitro studies with pure synthetase and tRNA species (*6*) have demonstrated that several noncognate wild-type *E. coli* tRNAs can be charged with Gln at measurable rates. Presumably, there is a set of tRNA species that have some of the required characteristics (discriminants) present on $tRNA^{Gln}$ needed for efficient recognition by *E. coli* GlnRS (*12*). The affinity of GlnRS for these tRNA species is lower than that for the cognate $tRNA^{Gln}$ and may explain why elevated *supF* $tRNA^{Tyr}$ levels did not cause substantial mischarging at normal intracellular GlnRS concentrations.

Are other synthetases also capable of mischarging? Although GlnRS may have a "more relaxed" specificity than some other aminoacyl-tRNA synthetases, the phenomenon is likely to be more general. The recent in vitro generation of several new tRNA suppressors has led to demonstrations that *E. coli* Lys-tRNA synthetase is also capable of misacylating noncognate tRNAs (*13*).

It may be that regulation of the ratio of synthetase to cognate tRNA is far more important for some synthetases than for others. Aminoacyl-tRNA synthetases are, after all, an extremely heterogeneous class of enzymes with regard to the number of subunits, mechanisms of regulation, and finally, their means of ensuring specific tRNA charging (*1, 4*). Tyr- and Cys-tRNA synthetases, for example, do not have proofreading mechanisms similar to the ones possessed by several other synthetases. These enzymes, however, compensate for this lack of an editing function by being singularly specific in the binding and activation of their cognate amino acids (*14*).

The regulation of the enzyme-to-substrate ratio may play an important role in enhancing the specificity of other enzymes. For example, there is evidence that *E. coli* RNA polymerase is present only at low levels in the cell and is perhaps held bound in complexes at promoters (*15*). High levels of RNA polymerase could conceivably lead to increased levels of transcription from improper start sites by free polymerase molecules. Indeed, mechanisms for enhancing specificity by sequestering enzymes in complexes with substrate molecules may not be uncommon in living systems. It is interesting to consider the evolution of aminoacyl-tRNA synthetases in this light. Not only has it been necessary for the structures of these exquisitely selective enzymes to evolve, but it has also been necessary for the regulation of synthetase and tRNA levels to evolve concomitantly in order to assure sufficiently accurate aminoacylation to sustain the process of protein biosynthesis.

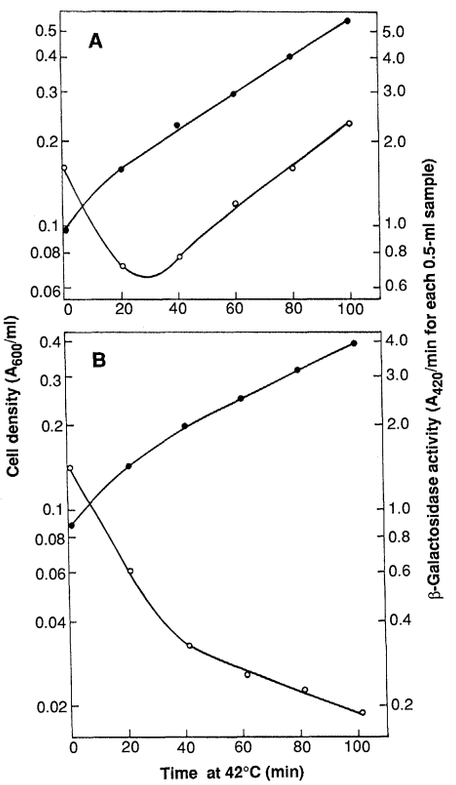

Fig. 2. Overproduction of $tRNA_2^{Gln}$ abolishes overproduction mischarging by GlnRS. Log-phase cultures of RS108 transformed with pRS11 and either pPLc28 (**A**) or pRS3 (**B**) were grown at 30°C in minimal A medium supplemented with 0.2% glucose, 0.2% vitamin assay casamino acids (Difco), biotin (0.1 μg/ml), 1 m*M* isopropyl-β-D-thiogalactopyranoside, chloramphenicol (30 μg/ml), and ampicillin (100 μg/ml). The cultures were diluted 1:5 into the same medium prewarmed to 42°C, and the cell densities (●) and β-galactosidase levels (○) of the cultures were measured from duplicate samples after various times of incubation at 42°C.

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Competitive Inhibition of *hsp70* Gene Expression Causes Thermosensitivity

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A novel method has been developed for modulating the expression of an endogenous chromosomal gene in a higher eukaryote, by competitive inhibition at the level of gene transcription. The gene studied was the *hsp70* gene, which encodes a 72-kilodalton (kD) heat shock protein that is synthesized after thermal stress. The 5′ control region of the *hsp70* gene was inserted on a plasmid containing the eukaryotic gene for dihydrofolate reductase. The hybrid plasmid was then introduced into a Chinese hamster ovary cell line and elevated in copy number approximately 20,000-fold by selection of cells with methotrexate. Heat-inducible expression from the intact *hsp70* gene was reduced by at least 90% in the modified cells when compared with the induction in control cells, and the modified cells also displayed elevated thermosensitivity. The change in heat shock protein synthesis is presumably caused by competition among the increased number of binding sites for the heat-shock transcription factor, leading to altered expression from the native heat shock gene. These results support a role for heat shock protein in the recovery of mammalian cells from acute thermal stress.

EUKARYOTIC GENE EXPRESSION IS modulated by interactions among cis-acting control sequences that are topologically contiguous with the regulated genes, and trans-acting regulatory proteins that bind to the control sequences (*1*). It is presumed that changes in activity or abundance of the regulatory proteins are sufficient to explain most aspects of the control of gene expression, but direct tests of these concepts are difficult to construct. In one approach, expression from reporter genes on plasmids transiently introduced into cells can be modulated by cointroduction of another plasmid that contains genes or gene fragments that share control sequences with the first (*2, 3*). When expression from the first plasmid is reduced in such an experiment, it is inferred that the control sequences on the two plasmids are competing for binding to a positive-acting regulatory protein that is present in limiting abundance in the host cell. With excess competing plasmid, fewer regulatory proteins are available for binding to the reporter gene's control region, and reporter gene transcription is correspondingly diminished.

This approach has not been successful, however, in demonstrating modulation of expression from endogenous chromosomal genes. Even in cases where competition among introduced genes is clear, expression from endogenous genes with shared control sequences has remained unaffected (*3*). Several explanations may be offered for this puzzling result. (i) The introduced genes may reside too briefly within cells before degradation to compete effectively with endogenous genes, or they may reside within an intracellular compartment (such as micronuclei) that is isolated from the endogenous genes. (ii) Newly introduced genes may compete effectively with one another during chromatin assembly, whereas the preexisting chromatin configuration of endogenous genes may be stable against such transient disruptions. (iii) Only a small fraction of cells may incorporate transfected DNA (*3*), and the majority of cells may not experience effective competition. (iv) Cells may be transiently damaged during DNA transfection, and this may alter the cell's normal regulatory mechanisms during the course of the assay. (v) Cells may vary the abundance or localization of regulatory proteins in response to transient changes in the number of binding sites for the proteins.

The method of directed gene coamplification provides an opportunity to circumvent these difficulties. To achieve coamplification, genes or gene fragments are transfected into cells together with the gene for dihydrofolate reductase (*4*); the genes may then become stably integrated into the cell's chromosomes at random sites of insertion. By varying the level of methotrexate selection to which transfected cells are subsequently exposed, one can also vary the copy number

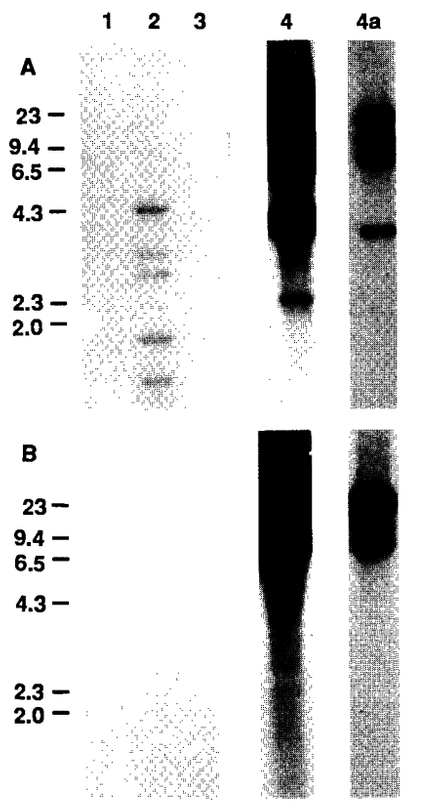

Fig. 1. Coamplification of *dhfr* and *hsp70* control region. DNA was isolated from CHO K_1, *dhfr*$^-$, and CHO DH_f cells, and from an independently derived CHO K_1 line [resistant to 500 n*M* methotrexate and designated B_{11} (*9*)] in which the native hamster *dhfr* gene is amplified 50-fold. The DNA samples were digested with Eco RI, and 5 μg of each sample was separated by electrophoresis in 0.7% agarose, capillary-blotted to nitrocellulose, and probed with radiolabeled (0.5×10^9 cpm/μg) *dhfr* (**A**) or *hsp70* control region (**B**) gene fragments (*9*). Lanes 1, CHO K_1; lanes 2, B_{11}; lanes 3, *dhfr*$^-$; and lanes 4 and 4a, CHO DH_f. Exposure to x-ray film was for 24 hours (lanes 1 to 4) or 45 min (lanes 4a). Molecular size markers are indicated at left in kilobases.

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