A sequence dimorphism in a conserved domain of human 28S rRNA. Uneven distribution of variant genes among individuals. Differential expression in HeLa cells

Liang-Hu Qu+, Monique Nicoloso and Jean-Pierre Bachellerie*
Centre de Recherche de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique, Université Paul-Sabatier, 118 route de Narbonne, 31062 Toulouse Cédex, France

Received December 31, 1990; Accepted February 4, 1991 EMBL accession nos X56542, X56543

ABSTRACT

In humans, cellular 28S rRNA displays a sequence dimorphism within an evolutionarily conserved motif, with the presence, at position +60, of either a A (like the metazoan consensus) or a G. The relative abundance of the two forms of variant genes in the genome exhibit large differences among individuals. The two variant forms are generally represented in cellular 28S rRNA in proportion of their relative abundance in the genome, at least for leucocytes. However, in some cases, one form of variant may be markedly underexpressed as compared to the other. Thus, in HeLa cells, A-form genes contribute to only 1% of the cellular content in mature 28S rRNA although amounting to 15% of the ribosomal genes. The differential expression seems to result from different transcriptional activities rather than from differences in pre-rRNA processing efficiency or in stabilities of mature rRNAs. G-form ribosomal genes were not detected in other mammals, including chimpanzee, which suggests that the fixation of this variant type is a rather recent event in primate evolution.

INTRODUCTION

With regard to its mode of structural variation during evolution, a ribosomal gene exhibits a complex mosaic structure, with an interspersion of domains evolving at widely different rates. While some sequences motifs are universally conserved in pro- and eukaryotes, other portions of the gene undergo dramatic structural changes, with extensive sequence divergence and length variations, even between closely related species, such as mouse and rat (1–5). Like other multigene families, eukaryotic ribosomal genes undergo a concerted evolution (6–8) and the few hundred gene copies present in the genomes of higher organisms exhibit a very high degree of structural homogeneity within a species. However the sequence homogeneity is not perfect and variant forms have been identified in several eukaryotic species (9–13). In humans, the 28S rRNA sequence shows several sequence polymorphisms (14–16), essentially located within the most rapidly evolving domains of the molecule. These domains exhibit a high level of sequence simplicity in higher eukaryotes (17) and the reported cases of polymorphism mostly correspond to differences in the copy numbers of short tandemly repeated oligonucleotides or in the lengths of homopolymeric sequence tracts (14–16). Such variations are likely to originate from unequal homologous exchanges among the tandemly repeated gene copies or from slippage-like mechanisms taking place during replication (18). However we have recently detected in human 28S rRNA a different sort of sequence variation, i.e., a dimorphism at a nucleotide position of the universally conserved core of the large subunit rRNA molecule (19,20). This nucleotide position (position +60 from the 5'end of eukaryotic 28S rRNA) is part of a single-strand motif which has been perfectly conserved in all the other metazoans examined so far (21). Accordingly this particular nucleotide variation seems unlikely to represent a neutral change in terms of rRNA functioning and its further analysis may prove helpful for better assessing the relative contribution of genetic drift, natural selection and internal dynamics of the genome in the concerted evolution of the multigene family. In this study, we report that the proportions of these two forms of ribosomal genes may largely differ among individuals and also show that the two variant classes may be differentially expressed in some human cell lines.

MATERIAL AND METHODS

RNA purification and analysis

All analyses involving the transformed human cell lines were carried out on exponentially growing cells. Bulk cellular RNA was extracted from cell pellets or frozen tissues in 3 M LiCl, 6M urea according to Auffray and Rougeon (22), with the modifications introduced by Le Meur et al. (23). RNA was analyzed by electrophoresis on 0.8% agarose gels in denaturing conditions (24), in the presence of 2.2 M formaldehyde. The running buffer was 5 mM Na acetate, 1 mM EDTA, 20 mM

* To whom correspondence should be addressed

+ Permanent address: Biotechnology Research Center, Zhongshan University, Guangzhou 510275, China

MOPS (pH 7.0). Before loading, RNA samples were dissolved in 50% formamide, 2.2 M formaldehyde, 5% glycerol in the running buffer and heated for 5 min at 65°C. Separation was achieved in 45 min (6 V/cm, room temperature). Blotting was carried out by passive transfer (25) and nitrocellulose filters were baked in a vacuum oven at 80°C for 2 h before utilization for hybridization.

DNA purification and analysis

Bulk cellular DNA was extracted from cell pellets (for leucocytes and transformed cell lines), or frozen tissues, according to Blin and Stafford (26). After a pancreatic RNAse treatment and extractions with phenol-chloroform, the DNA solution was dialyzed and DNA recovered by precipitation in 0.4 M LiCl. After redissolution in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, DNA concentrations were determined from UV absorption spectra. DNA samples were either spotted directly onto nitrocellulose A85 filters (100 μg DNA per spot) or analyzed on 0.8% agarose gels prior to transfer onto the membranes by Southern blotting (27).

Hybridization

Probes: each synthetic oligodeoxyribonucleotide (22-mers) was 5'end-labeled by (γ-^{32}P) ATP (3000 Ci/mmole) in the presence of T4 polynucleotide kinase. After several steps of ethanol precipitation and redissolution, the probes (specific activity=about 10^6 cpm/pmole) were generally directly used for hybridization. Titration of each form of variant sequence was performed according to a previously reported procedure (20), involving the parallel processing of two identical sets of samples, each one hybridized with one of the two labeled probes, in the presence of the other (non-labeled) oligonucleotide. When the hybridization is performed near the melting temperature of the perfectly matched duplexes, the competition between the cognate (labeled) and the non-cognate (unlabeled) oligonucleotides drastically reduces the signals contributed by one-mismatch labeled hybrids, thus providing the basis for a selective and quantitative assay (20). Since the background signal contributed by non-cognate RNA or DNA sequences was found negligible in these conditions, the titration was routinely carried out by dot-blot hybridizations on total cellular RNA or DNA samples. For each sample, three aliquots were spotted separately on each hybridization membrane, and the average value for the three spots was used for calculating the relative abundance of the two variant forms. For results expressed as percentages of each variant in cellular rRNA or in genomic rDNA, the standard error for the less abundant variant form was ± 1% for percentage values inferior to 10%, or ± 2% otherwise. In order to provide internal references for the quantitation, a set of control samples was also included on each membrane: for DNA titrations, known amounts of plasmid recombinant DNAs coding for each form of human 28S rRNA variant (20) were spotted in parallel; for RNA titrations, purified 28S rRNAs from mouse liver cells and from HeLa cells served as references for the A-form and G-form respectively, either spotted separately or as a 1:1 mixture. After hybridization and washing, the radioactivity retained on each spot was measured and corrected for background level of non-specific binding as reported elsewhere (20). Hybridization signals obtained with the probe recognizing the major variant form amounted usually to $1-2\times10^4$ cpm (Cerenkov) per spot. Prehybridization was carried out in 6×SSC (1×SSC=0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 10×Denhardt's solution (10×Denhardt's solution = 0.2% bovine serum albumin, 0.2% polyvinyl-pyrrolidone, 0.2% Ficoll), 0.5% SDS and 100 μg/ml *E. coli* tRNA for 0.5−3 h at the same temperature than hybridization. Hybridization was performed in the same mixture in the presence of 0.8−1 pmole/ml of labeled probe and 4 pmoles/ml of unlabeled oligonucleotide complementary of the other form. For DNA blots, hybridization was carried out for 18 h at 60°C. For RNA blots, the hybridization mixture was only 5× in Denhardt's solution and incubation was performed for 4 h, either at 60°C (with the 'A' labeled probe) or at 62°C (with the 'G' labeled probe). After hybridization, filters were washed 3 times with 6×SSC at 20°C, 3 times with 1×SSC at 45°C, then 3 times in 0.1×SSC at 45°C, and processed for autoradiography or radioactivity counting.

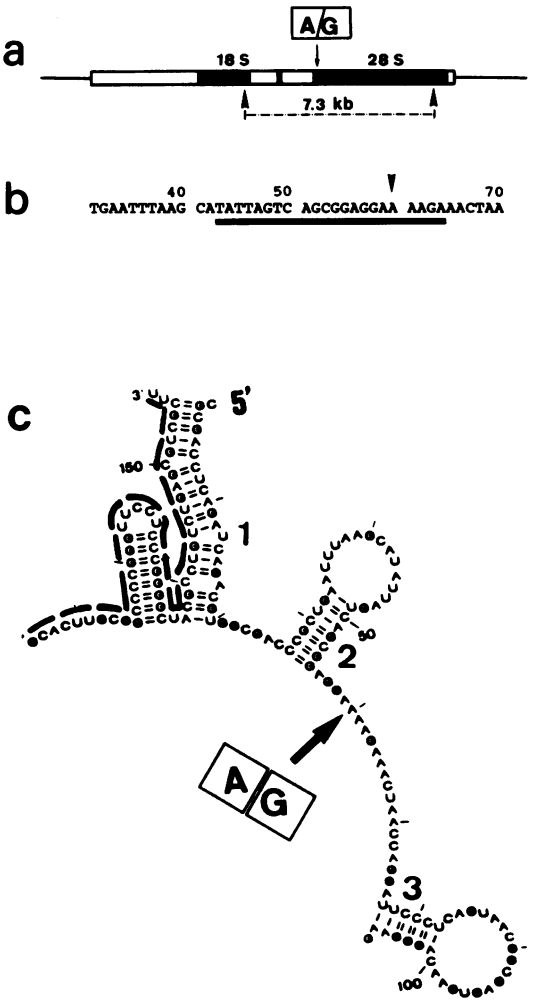

Fig. 1. Location of the A/G sequence variation in human 28S rRNA. a) Schematic representation of the human rRNA transcription unit (transcribed spacers are denoted by open boxes) with indication (arrows) of the two phylogenetically conserved Eco RI sites located in mature rRNA coding region used in Southern blot analyses of genomic DNA (as shown in Fig.2). b) Sequence of the RNA-like strand around the site of variation (nucleotide positions are numbered from the 5' end of mature 28S rRNA). The arrow-head shows the position occupied by a G in a fraction of human ribosomal genes. The pair of synthetic 22-mer oligonucleotides used for selective titration of the variants is complementary to each version of the underlined sequence. c) Secondary structure of the 5' terminal domain of human large subunit rRNA. The base-pairing between the 3'end of 5.8S RNA (broken overline) and the 5'terminal nucleotides of 28S rRNA is represented (stem 1). The site of nucleotide dimorphism, between universally conserved (42) stems 2 and 3, is denoted by the arrow.

RESULTS AND DISCUSSION

Direct RNA sequencing of human 28S rRNA, performed by reverse transcriptase extension of an oligonucleotide primer (30,21), has revealed a sequence dimorphism, with the presence of either a A or a G at position +60 (19,20), i.e., within a single-stranded, highly conserved, motif of the universal core of secondary structure of the molecule (Fig.1). As judged from the relative intensities of the two bands in sequencing patterns obtained with various human RNA samples, the relative abundance of the two variant forms seemed to exhibit substantial variations, depending upon the origin of the cells (results not shown). In order to obtain a more reliable estimate of these variations and to analyze potential differences among individuals in the abundance of the variant genes in genomic DNA, a titration assay has been developed (20), taking advantage of the isolation of rDNA plasmid clones encoding each form of human 28S rRNA. This assay involves the utilization of stringent hybridizations with a pair of synthetic oligonucleotides matching each form of variant sequence (Fig.1). Southern blot hybridizations confirm that these probes exclusively recognize their cognate rDNA fragment in genomic DNA. Thus, after a Eco RI digestion of human rDNA (Fig.2), the labeling is restricted to the 7.3 kb fragment which contains the 5'end of 28S rRNA sequence (20,28), this fragment being revealed by either the G-specific (Fig.2a) or the A-specific (Fig.2b) probe. However, marked differences are observed between human individuals regarding the relative levels of the signals obtained with each probe. Thus for HeLa cells (Fig.2, lanes b), the intensity of the signal with the G-specific probe is about 5 times higher than the signal obtained with the A-specific probe. However for another DNA sample (Fig.2, lanes d) the intensities of the two types of signal are almost identical. When chimpanzee (lane e) and mouse (lane f) genomic DNAs were analyzed in parallel, the presence of the G-form variant could not be detected, even for prolonged exposures (in conditions where a single copy per haploid genome would have been revealed).

In order to obtain a first insight on the range of individual variations in the relative abundance of the two variant forms in human genomes, 21 individuals (all of Caucasian origin except for one subsaharan African) were analyzed, using the dot-blot hybridization assay (20) on total cellular DNA, without prior digestion by restriction enzymes (see Material and Methods). We also studied three human cell lines, HeLa, MCF7 and HT-29. In all individuals and cell lines, the G-form appears as the major class of ribosomal genes (Fig.3) but the range of individual variation appears quite large (relative content in G-form: 53−96%, with 16 out of the 24 samples in the 70−90% range).

For all these individual samples, the relative content in the two variant forms was also examined at the level of cellular RNA, generally on leucocytes, using the same titration assay with the pair of oligonucleotides. In most cases, the G-form variant relative content in cellular 28S rRNA is closely similar to its relative content in genomic rDNA (Fig.3). However some individual samples represent marked and significant departures from this general rule. Thus in muscle cells of an individual for whom 55% of the ribosomal genes belong to the G-form, only 29% of cellular 28S rRNA corresponds to the G-form. However the two most outstanding cases of differential expression correspond to two human tumor cell lines, HeLa and HT-29 (a cell line isolated from a colon adenocarcinoma). In both cell lines, A-

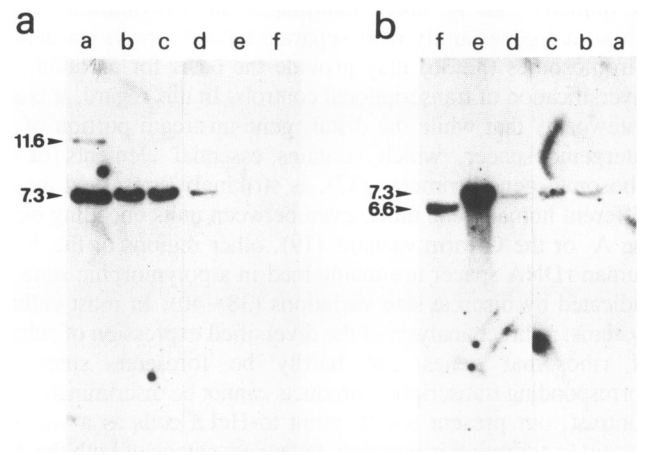

Fig. 2. Titration of both forms of 28S rRNA genes in genomic DNA. Southern blots prepared from Eco RI digests of human genomic DNA samples have been hybridized with either the G-specific (a) or the A-specific (b) labeled synthetic probes, in the conditions allowing an optimal discrimination (20). Eco RI-digested DNA samples have been loaded in duplicate on the two halves of the same 0.8% agarose gel. Lanes b, c and d correspond to human DNA samples (about 10 μg per lane) purified from HeLa cells, placenta and muscle (from two different individuals). DNAs isolated from chimpanzee brain and mouse liver have been analyzed in lanes e and f respectively. DNA of the plasmid recombinant pHrE7/5 (20), whose insert corresponds to a 7.3 kb Eco RI fragment of human rDNA encoding a G-form human 28S rRNA, has been digested by Eco RI and serves as a control in lanes a. Sizes of Eco RI fragments, deduced from the sequence analysis of human (15,28) and mouse (29) rRNA genes, are expressed in kb (the faint 11.6 kb band in lane 2 corresponds to residual amounts of linearized plasmid, as a result of an uncomplete Eco RI digestion).

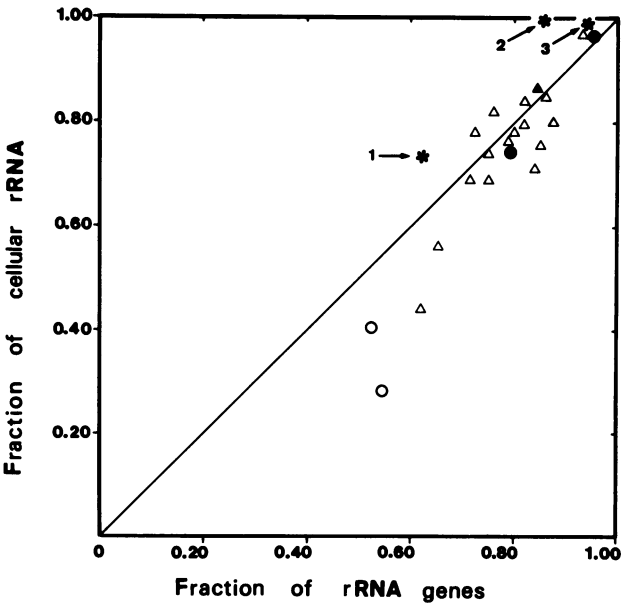

Fig. 3. Relative abundance of the G-form variant in cellular rRNA and in genomic rDNA of different human individuals and cell lines. For each cellular sample, titration of both variant forms was carried out on aliquots of total cellular RNA and DNA, by dot-blot hybridization with the pair of 22-mer oligonucleotide probes (20). Results are expressed as fraction of rRNA genes, or of cellular rRNA, corresponding to the G-form, with each symbol denoting a different individual. Triangles refer to leucocyte samples, either from African (filled triangle) or Caucasians (open triangles) individuals. Circles correspond to muscle (open circles), placenta (filled circle) and skin (crossed circle) samples from different Caucasians. Stars (designated by numbers) correspond to different transformed human cell lines: (1) MCF7 (ATCC HTB 22) ; (2) HeLa (ATCC CCL2); (3) HT-29 (ATCC HTB 38).

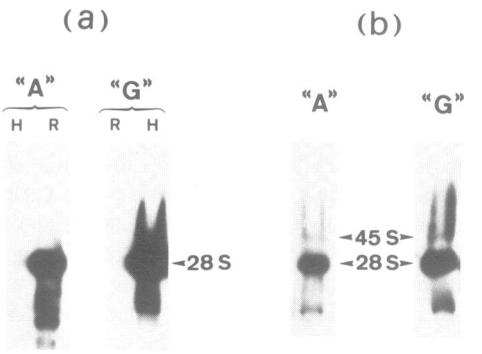

Fig. 4. Titration of both variant forms in cellular RNA. Purified total cellular RNAs (10 μg per lane) were analyzed on 0.8% agarose gels in denaturing conditions and transferred to nitrocellulose before hybridization with (^{32}P)-labeled synthetic oligonucleotides specific of each variant form. In (a), two sets of HeLa (H) and rat (R) samples were run on adjacent lanes of the same gels. Each set of blots was hybridized with each oligonucleotide probe, and the two sets were autoradiographed in parallel using the same film. In (b), two aliquots (10 μg) of HeLa cell RNA were analyzed, hybridized in parallel with each probe. In order to compare the 45S/28S signal ratio obtained with each probe, the two blots were autoradiographed separately (the exposure time was about 100 times longer for the blot hybridized with the A-specific probe in order to obtain similar intensities for the two 28S bands).

form variants represent less than 1% of cellular 28S rRNA, although amounting to 6 and 16% of the genomic content in ribosomal genes, for HT-29 and HeLa cells respectively. Since the differential expression is most dramatical in HeLa cells, we selected this cell line for a further analysis. The severe underexpression of the A-form genes in HeLa cells might result from impairments in the processing of rRNA precursor or from a markedly reduced life-time of mature rRNA. To test this possibility, we examined whether the relative abundance of the two variant forms was similar or not in rRNA precursors and in mature 28S rRNAs. Northern blot analysis of HeLa cell RNA (Fig.4b) reveals that the 45S/28S signal ratio remains roughly the same, using either the A-form or the G-form specific oligonucleotide probe: the A-form precursor also amounts to only about 1% of cellular 45S pre-rRNA. The parallel analysis of rat and HeLa RNA (Fig.4a) confirms the very high selectivity of the variant recognition: no labeled 28S band can be detected for rat with the G-probe, even after considerable overexposure of the HeLa RNA lane; conversely, with the A-probe, the 28S signal for HeLa RNA is about two orders of magnitude lower than for rat RNA. Our observation that the relative abundance of the two variant forms remains almost identical either in mature 28S rRNA or in the 45S rRNA precursor makes it likely that the underexpression of the A-form ribosomal genes in HeLa results from differences at the transcription level rather than at post-transcriptional stages.

DISCUSSION

The presence of a A/G dimorphism at position +60 of human 28S RNA constitutes an outstanding case of intraspecies polymorphism. Unlike the other examples reported so far in eukaryotes (9−16), it concerns a conserved, single-stranded, portion of the molecule, suggesting that this A to G change might significantly affect ribosome function. Moreover a preliminary survey shows large individual differences in the relative abundance of the two variant forms of ribosomal genes in a human genome. The presence of G variants could not be detected in any of the other mammals examined so far (21), including chimpanzee (Fig.2), strongly suggesting that the appearance and spreading of this nucleotide change is posterior to the human/chimpanzee split, about 5 millions years ago (31). Conversely this change, which has largely spread in populations, must involve several chromosomal loci, considering that the G variant is the major form of ribosomal genes which seem rather evenly distributed among five distinct chromosomes (32). Accordingly this mutation must have existed long enough to allow its transfer from one array of ribosomal genes in a single chromosome to the other chromosomal loci through relatively unfrequent non-homologous chromosomal exchanges.Further analyses of this particular sequence dimorphism in the populations may help to better evaluate the relative contribution and frequency of the different types of recombinational events involved in the concerted evolution of the ribosomal gene family (6−8). Since this nucleotide change is unlikely to be neutral in terms of ribosome function, this system may also prove useful for studying the complex interplay of selective constraints and internal dynamics of the genome on the homogenization of the multigene family.

Finally, our finding that in HeLa cells a sizable fraction of the ribosomal genes is dramatically underexpressed has interesting implications. Electron microscopy observation of transcriptionally active chromatin during development has shown that regulation of ribosomal gene transcription does not only involve a modulation of the level at which an average ribosomal gene is transcribed but also changes in the number of ribosomal genes that are active (33). Substantial evidence has also been reported that, within a tandem array, the transcription of the gene copies may not be independent of transcription of others (34,35) and a particular role has been proposed for the 5′ most copies in a transcriptional control of an entire array of repeats. Moreover, in humans like in other mammals, the distribution of the ribosomal gene family over separate tandem arrays on distinct chromosomes (32,36) may provide the basis for an additional diversification of transcriptional controls. In this regard, it is also noteworthy that while the distal, gene-upstream portion of the intergenic spacer, which contains essential elements of the ribosomal gene promoter (37), is strikingly conserved among different human gene units, even between units encoding either the A- or the G-form variants (19), other regions of the 30 kb human rDNA spacer are maintained in a polymorphic state, as indicated by discrete size variations (38−40). In most cellular systems, a direct analysis of the diversified expression of subsets of ribosomal genes can hardly be foreseen, since the corresponding transcription products cannot be discriminated. By contrast, our present results point to HeLa cells as a valuable system to address this question, taking advantage of both the A/G sequence dimorphism and the marked repression of the A-form subset of ribosomal genes. Obviously further progress in this direction may largely depend upon some knowledge of the relative organization of the two forms of variant genes along HeLa cell chromosomal rDNA. Given the large size (44 kb) of the repeat unit, this task still remains daunting and in fact no YAC clone containing more than one complete human ribosomal gene unit has been recovered so far (41). However, since A-form genes represent only 16% of the ribosomal genes in HeLa cells, the underexpressed variant genes could possibly be all clustered within a single chromosomal locus rather than being distributed among the five acrocentric chromosomes, thereby facilitating future analysis of transcriptional controls operating at the level of multigene arrays.

ACKNOWLEDGEMENTS

This work was supported by the Institut National de la Santé et de la Recherche Médicale (CRE 851001) and the Association pour la Recherche contre de Cancer (grant 6158). L.H.Q. benefited from a visiting scientist position from the Centre National de la Recherche Scientifique.

REFERENCES

1. Salim, M. and Maden, B.E.H. (1981) Nature 291, 205–208.
2. Veldman, G.M., Klootwijk, J., de Regt , V.C.H.F., Planta, R.J., Branlant, C., Krol, A. and Ebel, J.P. (1981) Nucleic Acids Res. 9, 6935–6952.
3. Ware, V.C., Tague, B.W., Clark, C.G., Gourse, R.L., Brand, R.C. and Gerbi, S.A. (1983) Nucleic Acids Res. 11, 7795–7817.
4. Hassouna, N., Michot, B. and Bachellerie, J.P. (1984) Nucleic Acids Res. 12, 3563–3583.
5. Gerbi, S.A. (1985), in Molecular Evolutionary Genetics (Mac Intyre R.J., ed.) Plenum Publishing Corp., New-York, pp. 419–517.
6. Dover, G. (1982) Nature 299, 111–117.
7. Arnheim, N. (1983) ,in Evolution of Genes and Proteins (Nei M. and Koehn R.K., eds) Sinauer, Sunderland, Mass., pp. 38–61.
8. Dover, G.A. (1986) Trends Genet. 2, 159–165.
9. Stewart, M.A., Hall, L.M.C. and Maden, B.E.H. (1983) Nucleic Acids Res. 11, 629–646.
10. Back, E., Müller, F. and Tobler, H. (1984) Nucleic Acids Res. 12, 1313–1332.
11. Kolosha, V.O., Kryukov, V.M. and Fodor, I. (1986) FEBS Lett. 197, 89–92.
12. Waters, A.P., Chiang Syin and Mc Cutchan, T.F. (1989) Nature 342, 438–440.
13. Bearson, S.R. and Kaufman, L.S. (1990) Mol. Cell. Biol. 10, 842–845.
14. Gonzalez, I.L., Gorski, J.L., Campen, T.J., Dorney, D.J., Erickson, J.M., Sylvester, J.E. and Schmickel, R.D. (1985) Proc. Natl. Acad. Sci. USA 82, 7666–7670.
15. Maden, B.E.H., Dent, C.L., Farrell, T.E., Garde, J., Mc Callum, F.S. and Wakeman, J.A. (1987) Biochem. J. 246, 519–527.
16. Gonzalez, I.L., Sylvester, J.E. and Schmickel, R.D. (1988) Nucleic Acids Res. 16, 10213–10224.
17. Hancock, J.M. and Dover, G.A. (1988) Mol. Biol. Evol. 5, 377–391.
18. Levinson, G. and Gutman, G.A. (1987) Mol. Biol. Evol. 4, 203–221.
19. Renalier, M.H., Joseph, N. and Bachellerie, J.P. (1989) FEBS Lett. 247, 298–302.
20. Nicoloso, M., Qu, L.H. and Bachellerie, J.P. (1989) Biochem. Biophys. Res. Comm. 159, 1233–1241.
21. Qu, L.H., Nicoloso, M. and Bachellerie, J.P. (1988) J. Mol. Evol. 28, 113–124.
22. Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107, 303–314.
23. Le Meur, M., Glanville, N., Mandel, J.L., Gerlinger, P., Palmiter, R. and Chambon, P. (1981) Cell 23, 561–571.
24. Lehrach, H., Diamond, D., Wozney, J.M. and Boedtker, H. (1977) Biochemistry 16, 4743–4751.
25. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
26. Blin, N. and Stafford, D.W. (1976) Nucleic Acids Res. 3, 2303–2308.
27. Southern, E.M. (1975) J. Mol. Biol. 98, 503–517.
28. Gonzalez, I.L., Chambers, C., Gorski, J.L., Stambolian, D., Schmickel, R.D. and Sylvester, J.E. (1990) J. Mol. Biol. 212, 27–35.
29. Michot, B., Bachellerie, J.P. and Raynal, F. (1983) Nucleic Acids Res. 11, 3375–3391.
30. Qu, L.H., Michot, B.and Bachellerie, J.P. (1983) Nucleic Acids Res. 11, 5903–5920.
31. Wilson, A.C., Ochman, H. and Prager, E.M. (1987) Trends Genet. 3, 241–247.
32. Wilson, G.N. (1982) in : The Cell Nucleus (Busch, H. and Rothblum, L. eds) vol. 10A, pp. 287–318, Academic Press, New-York.
33. Foe, V.E. (1977) Cold Spring Harbor Sympos. Quantit. Biol. 52, 723–740.
34. Baker, S.M. and Platt, T. (1986) Cell 47, 839–840.
35. Johnson, S.P. and Warner, J.R. (1989) Mol. Cell. Biol. 9, 4986–4993.
36. Worton, R.G., Sutherland, J., Sylvester, J.E., Willard, H.F., Bodrug, S., Dubé, I., Duff, C., Kean, V., Ray, P.N. and Schmickel, R.D. (1988) Science 239, 64–68.
37. Learned, R.M., Learned, T.K., Haltiner, M.M. and Tjian, R.T. (1986) Cell 45, 847–857.
38. Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O. and Zimmer, E. (1980) Proc. Natl. Acad. Sci. USA 77, 7323–7327.
39. Erikson, J.M. and Schmickel, R.D. (1985) Am. J. Hum. Genet. 37, 311–325.
40. La Volpe, A., Simeone, A., D'Esposito, M., Scotto, L., Fidanza, V., de Falco, A. and Boncinelli, E. (1985) J. Mol. Biol. 183, 213–223.
41. Labella, T. and Schlessinger, D. (1989) Genomics 5, 752–760.
42. Michot, B., Hassouna, N. and Bachellerie,J.P.(1984) Nucleic Acids Res. 12, 4259–4279.